INTEGRATING PREDATION RISK ACROSS SCALES: FROM NEURONS TO ECOSYSTEMS AND MILLISECONDS TO GENERATIONS

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INTEGRATING PREDATION RISK ACROSS SCALES: FROM NEURONS TO ECOSYSTEMS AND MILLISECONDS TO GENERATIONS

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EDITORIAL
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Editorial: Integrating Predation Risk Across Scales: from Neurons to Ecosystems and Milliseconds to Generations

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Keywords: predation risk, predator, prey, defense, fear

Editorial on the Research Topic

Integrating Predation Risk Across Scales: from Neurons to Ecosystems and Milliseconds to Generations

This editorial review of the Research Topic "Integrating predation risk across scales: from neurons to ecosystems and milliseconds to generations" explores prey responses to predation risk from an array of different perspectives. It includes work from neurobiologists, ecologists, and other disciplines interested in predator-prey interactions at varying spatial and temporal scales. Taken together, the 15 papers in this Research Topic represent an attempt to synthesize work across disciplines in search of intellectual synergies and new avenues of research collaboration.

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ECOLOGICAL RESPONSES TO PREDATION RISK: ORIGINAL RESEARCH

Papers by Madin et al. and Atwood et al. report on research addressing the phenomena of coral reef "halos," zones of unvegetated seafloor surrounding coral reef patches. These patches have long been thought to result from herbivorous fish seeking shelter from predators within reef cracks and crevices; they only feed on vegetation growing near the coral because foraging too far from shelter might expose them to predators. Madin et al. report that coral reef halos found in the Great Barrier Reef result from both prey fear and the action of bioturbators, sediment-dwelling organisms that churn up the seafloor and affect algal settlement. Atwood et al. conducted complementary research confirming the importance of predation risk in changing algal grazing rates and demonstrating that these changes decrease sedimentary carbon storage within the reef halos.

Both Chizzola et al. and Bleicher et al. describe work exploring the "landscape of fear" in terrestrial systems. Fear of lions has long been thought to shape the habitat choices and foraging decisions of many herbivorous mammals; Chizzola et al. combine behavioral, physiological, and isotopic information to reveal the highly dynamic and scale-dependent responses of wildebeest and impala to this fearsome threat. At a smaller spatial scale, Bleicher et al. explore how desert rodents on two continents respond to the common threat of owls and snakes, two dangerous but very different predators. They found that the two rodent communities differ in their assessment of which predator is the most threatening and discuss how local environments can profoundly shape prey responses.

The special feature also contains several articles exploring the impact of risk on individuals. First, Trakimas et al. discuss the connections between development rate and physiological stress in the cricket *Gryllus integer*. They found that slow-developing crickets had higher resting metabolic

rates than fast-developing ones, presumably reflecting the energetic costs associated with a higher resting metabolism. They also resumed activity faster following a stressor than did fast developers, a relationship that may translate into higher foraging rates. Second, Baranowski and Preisser report the results of work testing the impact of predation risk on the survival of luna moth (*Actias luna*) larvae. They found that caterpillars exposed to a threatening but non-lethal predator (a wasp with its jaws and stinger immobilized) would "freeze" in place, stop feeding, and die at a higher rate than larvae exposed to a similarly-sized but harmless fly. Finally, Eason et al. describe fascinating work addressing how threat type and refuge availability affect the trajectory of fleeing Eastern Gray Squirrels (*Sciurus carolinensis*). Briefly, they found that squirrels preferred not to run to refuges (trees) that were directly behind them and would actually run toward threats to reach a tree in front of them.

ECOLOGICAL RESPONSES TO PREDATION RISK: LITERATURE REVIEWS

The Research Topic includes two large-scale reviews of different ecological processes that affect predator-prey interactions. In the first, Weiss summarizes our current understanding of how *Daphnia*, a crustacean that is a model system for both ecology and evolutionary biology, detect and avoid predators. In addition to defending themselves against predators, Daphnia exposed to predator cues can produce offspring with spines and "helmets" that make them more difficult for predators to subdue and consume. The review traces response from the initial cues to the underlying neurophysiological mechanisms that structure prey responses, providing a concise overview of the molecular mechanisms underlying both short- and long-term phenotypic adaptation. In the second, Draper and Weissburg provide a review and synthesis of the impact of global warming and increased CO_2 levels on the sensory ecology of predator-prey interactions. Both factors affect multiple parts of the predator-prey encounter sequence, including the ability of predators to detect prey and the ability of prey to flee approaching predators. Although cue production, transmission, and reception will be globally affected by such large-scale environmental changes, they highlight the fact that aquatic predator-prey interactions may be disproportionately sensitive to these disruptions. Altered CO_2 levels are likely to have especially strong effects in marine systems due to ocean acidification, which can change Ph levels sufficiently to make the calcium-based shells of prey difficult or impossible to produce.

NEUROBIOLOGICAL RESPONSES TO PREDATION RISK: ORIGINAL RESEARCH

Moths and other night-flying prey are often targeted by bats that use ultrasonic calls to detect and help capture them. As a result, many moths possess tympanic organs capable of detecting bat sonar. Cinel and Taylor explored how chronic exposure to bat calls affected transcription in fall armyworms (*Spodoptera exigua*) that were either continuously exposed to foraging and attack calls or held in silence. The 290 transcripts they found either up- or down-regulated were involved in a broad range of cellular functions, information that lays the foundation for research addressing how these changes affect insect physiology, behavior, and demography. Working in a very different system, Tong et al. report on how testosterone affects both mouse fear and physiology. Exposing male mice to this chemical reduced their innate fear of predator cues (cat urine) when allowed to explore potentially risky habitats. In a complementary experiment, they also found that testosterone produced hypomethylation of the promoter region of the arginine vasopressin gene. The latter compound plays a key role in socio-sexual behavior; they suggest that testosterone-induced reductions in fear occur via manipulations of the trade-off between fear (reduced activity) and social/sexual interactions (increased activity).

NEUROBIOLOGICAL RESPONSES TO PREDATION RISK: LITERATURE REVIEWS

Recent technical advances in neurobiology have revolutionized our understanding of how predators and prey detect and respond to each other. This progress has heightened our appreciation for the role of the periaqueductal gray (PAG) in modulating such interactions; Franklin provides a cogent assessment of our current understanding. Work in rodent model systems demonstrates the role of the rostrolateral PAG in hunting behaviors and how the dorsal-ventral and rostral-caudal axes of the PAG differ in their impact on defensive behavior. The fact that brain imagining work in humans shows threat-induced PAG activity suggests that large portions of PAG function are likely conserved across an array of mammalian species.

The amygdala, and the basolateral neurons within it, also play a critical role in detecting and responding to predator cues; Mitra reviews the role of these neurons and how they change in response to predator odors. While neurons in the basolateral amygdala respond quickly to risk cues, continued exposure to stress-related hormones can change them in ways that also facilitate rapid endocrine responses to future stressors. Current evidence suggests that the basolateral amygdala plays an important role in risk-related activities across multiple taxa, and that it may be generally responsible for anticipatory defensive behaviors in threatening situations.

Fear generalization is an adaptive process that promotes survival in complex and dynamic environments. In their review, Asok et al. explore the behavioral, neural, genetic, and biochemical mechanisms involved in this process. As fear generalization is a hallmark of many anxiety and stress-related disorders (such as posttraumatic stress disorder, PTSD), Asok et al. highlights the importance of sex differences and remote timescales in rodent models to improve the dialogue between human and animal studies. Factors that contribute to PTSD were also considered in the review by Bhattacharya et al. Recent research in both humans and animals suggests that experience of the parent may influence its offspring. Bhattacharya et al. review studies in both humans with PTSD and laboratory predator stress models of PTSD that suggest changes to DNA methylation may underlie the generational effects of trauma transmission.

Both Asok et al., and Bhattacharya et al., argue that a deeper understanding of the mechanisms that promote stress-induced psychopathology will accelerate the development of effective therapeutics.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Predators Shape Sedimentary Organic Carbon Storage in a Coral Reef Ecosystem

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Trophic cascade theory predicts that predator effects should extend to influence carbon cycling in ecosystems. Yet, there has been little empirical evidence in natural ecosystems to support this hypothesis. Here, we use a naturally-occurring trophic cascade to provide evidence that predators help protect sedimentary organic carbon stocks in coral reef ecosystems. Our results show that predation risk altered the behavior of herbivorous fish, whereby it constrained grazing to areas close to the refuge of the patch reefs. Macroalgae growing in "riskier" areas further away from the reef were released from grazing pressure, which subsequently promoted carbon accumulation in the sediments underlying the macroalgal beds. Here we found that carbon stocks furthest away from the reef edge were ∼24% higher than stocks closest to the reef. Our results indicate that predators and herbivores play an important role in structuring carbon dynamics in a natural marine ecosystem, highlighting the need to conserve natural predator-prey dynamics to help maintain the crucial role of marine sediments in sequestering carbon.

Keywords: trophic cascades, blue carbon, trait-mediated effects, coral reefs, predators, herbivory, grazing halos, Great Barrier Reef

INTRODUCTION

There is growing evidence from a wealth of ecosystems that predators, via trophic cascades, play an important and potentially irreplaceable role in carbon (C) cycling (Wilmers et al., 2012; Atwood et al., 2014a,b; Schmitz et al., 2014), and that changes to predator populations can alter CO_2 concentrations and emissions from ecosystems (Schindler et al., 1997; Atwood et al., 2013; Hammill et al., 2015), C export from erosion (Coverdale et al., 2014), and C storage in plant biomass (Hawlena et al., 2012; Wilmers et al., 2012; Strickland et al., 2013; Heithaus et al., 2014). Yet, the influence of predators on one of our most important long-term C storage pools, marine sediments,

has rarely been explored (although, see Coverdale et al., 2014 on the influence of predators on C loss from salt marsh soils). Our study investigates how predators, through a naturally occurring trophic cascade, protect relatively young (0–40 years) and old (40–110 years) sedimentary organic carbon (OC) stocks in a coral reef ecosystem.

Marine ecosystems dominated by benthic macrophytes (seagrass, mangroves, salt marsh, and macroalgae) are important reservoirs in the global C cycle. Marine macrophytes represent ∼75% of marine autotrophic biomass (Smith, 1981; Gattuso et al., 1998), and coastal ecosystems dominated by macrophytes have the capacity to store large deposits of OC in their sediments (Fourqurean et al., 2012; Duarte et al., 2013; Krause-Jensen and Duarte, 2016; Atwood et al., 2017). The capacity of macrophyte systems to naturally sequester and store C in their sediments for millennia identifies these habitats as important C sinks (Nelleman et al., 2009; Duarte et al., 2013). Thus, it is crucial that we understand the important mechanisms that influence C accumulation and preservation in the sediments of these systems.

Herbivory is an important ecological process that can influence characteristics of the macrophyte community that underlie C deposition in marine systems. First and foremost, herbivores and grazers remove plant biomass that otherwise could be incorporated into the sediments (Rose et al., 1999; Atwood et al., 2015). A meta-analysis on the fate of macrophyte production suggests that 9–33% of macrophyte biomass returns to the biological pool through the process of herbivory, with the highest rates from macroalgal systems (Duarte and Cebrián, 1996). Second, grazers can alter the physical structure of the system by directly altering canopy height through consumption. For example, in Moreton Bay (Australia), grazing by dugongs reduced seagrass shoot density by 65–95% and above-ground seagrass biomass by 73–96% (Preen, 1995). The canopy and roots of macrophytes provide the necessary physical structure that allows allochthonous sediments and C being transported through the water column to be captured and accumulated in the macrophyte sediments. Clipping experiments have suggested that a 50% reduction in canopy height of seagrass can reduce sediment accumulation rates and increase sediment resuspension by 10-fold (Gacia et al., 1999). Despite evidence that herbivores influence the structure and composition of macrophyte communities, no study to date has investigated herbivory as a potential driver of spatial variation in marine sedimentary C deposits in a coral reef system.

Remotely sensed images of Earth's coral reef ecosystems reveal "halos" surrounding many patch reefs around the globe (**Figure 1A**). Patch reefs are series of spatially-isolated, lagoonal coral reefs that are often separated by seagrass or macroalgal meadows. The halos depicted in satellite images are caused by a disruption in the growth of seagrass or macroalgae that leaves a distinct band of unvegetated sediments surrounding a patch reef. Halo size (i.e., width of the halo) is variable, but can extend from only a couple of meters off the reef to >90 m from the reef's edge (Downie et al., 2013). It was originally thought that halos develop around patch reefs because wave surges and coarse sediments precluded seagrass or macroalgal growth near the reef. However, Ogden et al. (1973) found that when the herbivore *Diadema antillarum* was removed from heavily fished patch reefs in the West Indies, seagrass grew right to the reef edge and previously well-developed halos disappeared. These results have led many to argue that although physical processes may aid in the formation and maintenance of halos around patch reefs, the main mechanism driving halo formation is grazing patterns of herbivores (Randall, 1965; Sweatman and Robertson, 1994; Price et al., 2010; Madin et al., 2011; Gil et al., 2017). This has led to halos surrounding patch reefs being called "grazing halos."

It has been hypothesized that trophic cascades are responsible for concentrating herbivore abundance and grazing close to the refuge of the reef. A trophic cascade is the indirect and alternating effect of predators on lower trophic levels. Trophic cascades can be initiated as a result of predators consuming prey, which in turn reduces the preys' effects on their food source (density-mediated trophic cascade), or non-consumptive effects where the prey alters its foraging behavior in an attempt to avoid predation (behaviorally-mediated trophic cascade; Ripple et al., 2016). Although not well tested, it is thought that anti-predator shifts in herbivore foraging behavior are one of the underlying mechanisms behind the concentration of grazing close to patch reefs, which ultimately leads to halo formation (Madin et al., 2016; Gil et al., 2017). This risk of predation can create a "seascape of fear," whereby spatial variation in prey and primary producer biomass are inversely related as a function of predation risk levels across space (Brown and Kotler, 2004; Madin et al., 2010, 2011). If halos are indeed created by consumptive (lethal) and/or non-consumptive (risk) effects of predators, the poorly vegetated sediment surrounding patch reefs represents a zone of low predation risk, where herbivores can feed relatively close to a refuge. Conversely, the adjoining macroalgal or seagrass meadows represent areas in which it is generally too risky to forage.

Although the removal of vegetation due to grazing has obvious impacts on the distribution of plant biomass (Silliman and Bertness, 2002; Wilmers et al., 2012; Heithaus et al., 2014), and thus C cycling though photosynthesis (Wilmers et al., 2012), at present we do not understand whether these changes to vegetation have legacy effects on OC stocks in marine sediments. In this study, we investigate whether predators shape the distribution of sedimentary OC stocks by altering the abundance and foraging behavior of herbivores, which in turn alters benthic algal biomass and distribution (**Figure 1B**). We hypothesized that the occurrence of grazing halos in the Great Barrier Reef's Heron lagoon result from trophic cascades (**Figure 1B**). We predicted that, as on patch reefs elsewhere, the increased risk of predation with increasing distance from the refuge of the reef would result in a shift in herbivore behavior that would reduce grazing pressure and increase algal growth with increased distance from the reef edge. Furthermore, we predicted that the indirect effects of predators on the distribution of benthic algal biomass would have legacy effects on sedimentary OC stocks, with increased stocks at greater distances from refuges. This study helps us understand whether and how predator-prey interactions influence the distribution of sedimentary OC stocks in coral reef ecosystems. Such an understanding is becoming increasingly important as natural and anthropogenic disturbances continue

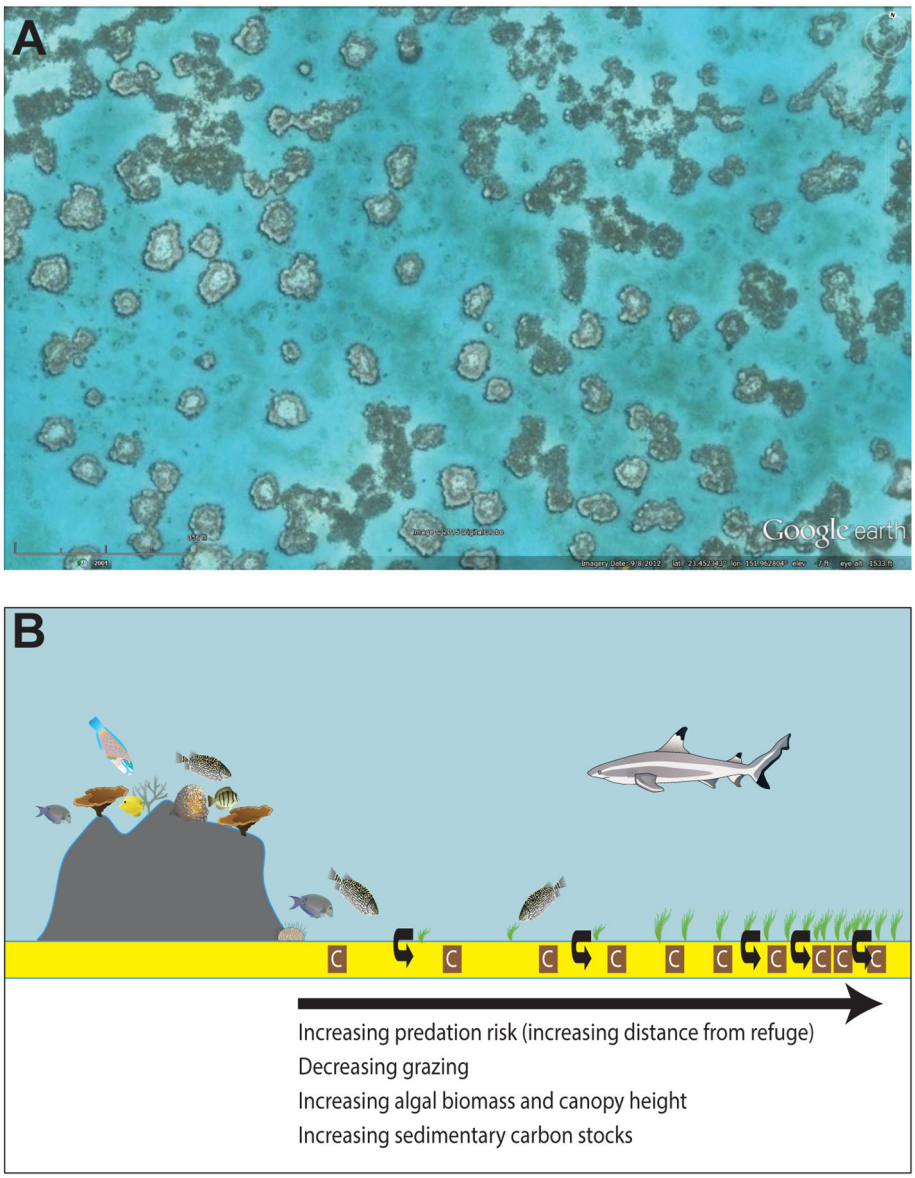

FIGURE 1 | Grazing halos. **(A)** Satellite image of macroalgal "grazing halos" in Australia's Heron lagoon. Image copyright Digital Globe and Google Earth. **(B)** Hypothesized non-consumptive effects of predators on grazing intensity, algal biomass, and sedimentary carbon stocks. The risk of predation constrains herbivore abundance to areas close to the reef, leading to reduced grazing, increased algal biomass, and increased sedimentary carbon stocks (brown boxes with "C") and sequestration (curved, black arrows) with increasing distance from reef refuges.

to alter marine communities in our greatest C sink (Sabine et al., 2004), the ocean.

MATERIALS AND METHODS

Study Area

This study was conducted on 22 patch reefs and their respective surrounding grazing halos within the shallow lagoon (total area 9.3 km^2) of Heron Reef in Australia's Great Barrier Reef Marine Park (23° 27′ S, 151° 55′ E). We focused our surveys on sheltered lagoonal reef habitat, which is typically shallow (<5 m) and features patch reefs isolated from one another by expanses of bare or algae-covered sand. Patch reefs that contained boulders, large coral fragments, or other hard substrates within the halos were not used in our study as these additional structures had the potential to influence fish behavior and algal growth. Furthermore, over the course of the surveys, selected patch reefs maintained a distinct separation between the edge of the reef and the sand flat. Heron's reef system represents the ideal location to study the effects of trophic cascades on sedimentary OC storage because its reefs have relatively intact predator and herbivore populations relative to other reefs that have been

more impacted by fishing. Furthermore, satellite imagery has documented the occurrence of grazing halos in the algal beds surrounding patch reefs in the Heron lagoon dating back to at least 1999 (**Figure 1A**), although the formation of the halos almost certainly pre-dates the availability of high-resolution satellite imagery for this area. This study was carried out in accordance with the Great Barrier Reef Marine Park Act (1975) and all protocols were approved by the Great Barrier Reef Marine Park Authority (Permit numbers: G14/37304.1 & G14/37182.1). No vertebrate fauna were taken or harmed, and no endangered or protected species were involved in this study.

Predator and Herbivore Abundance and Behavior

To quantify fish abundance and behavior, we deployed five GoPro underwater video cameras (www.gopro.com) in 2013. The cameras were anchored to the substrate and formed a line transect from the reef to the algal meadow. Cameras were placed at the center of the patch reef, and 2.5, 7.5, 15, and 22 m from the outer margin of the reef. A marker was positioned at a distance of 3 m in front of each camera to mark the end of the observation area. The camera's field of view at 3 m distance was 5.6 m wide. Recording times ranged from 119 to 285 min (mean ± SD: 215.49 ± 48.6 min) per camera. Remote underwater video surveys are becoming an important tool for assessing fish abundance and have several advantages over visual surveys. First, video surveys offer a permanent record that can be reviewed by others. Second, remote video surveys have minimal or no effect on fish behavior. This aspect was very important for our study, which aimed to examine changes in prey behavior with increasing predation risk.

In the laboratory, video footage was viewed on a computer screen, and all fishes passing through the defined field of view were recorded. We recorded the time, activity type (passing or foraging), and activity duration (in seconds) for each observation. In order to minimize overestimation of the number of roaming fish, we established a time threshold of 3 min between repeated occurrences of the same species to consider it as a new observation. We classified each fish to the lowest taxonomic level possible. For most observations, fish were identified to species level. Fish were further grouped into the following trophic categories: (i) herbivores: species that primarily feed on plant and detritus material (e.g., surgeonfishes, rabbitfishes, parrotfishes, and damselfishes other than planktivorous damselfishes), (ii) predators: species that feed mainly on fishes or invertebrates (including both top predators and meso-predators). For our analyses, however, we did not use the entire herbivore and predator data set. Rather, we only used data from herbivore species that we observed to actively forage on algae inside the halo (*Acanthurus* spp., *Zebrasoma veliferum*, *Naso unicornis*, and *Signaus canaliculatus*) and their predators (*Galeocerdo curvier, Carcharhinus melanopterus*, *Negaprion brevirostris* and jacks). We grouped meso-predators (e.g., jacks) and top predators (e.g., *G. curvier*) into a single group because we were interested in all the potential predators that could directly prey on our herbivore species. Furthermore, identifying a shark's trophic position without local diet data can be troublesome as a shark's trophic position can be influenced by not only the species of shark, but also its ontogeny, body size, behavior, and habitat (Heupel et al., 2014; Frisch et al., 2016; Roff et al., 2016). From our video records, we calculated the observed number of individuals per hour of video footage, and for foraging fish the number of bites per fish, per minute of video footage, as well as the time spent foraging. For the time spent foraging, the behavior ended when the fish began displaying a different behavior (i.e., resting) or when the fish left the camera frame. This means that fish may have foraged for longer periods than what was recorded by cameras. For each observation we identified the tidal stage (±3 h either side). Because this study was conducted over the course of several weeks and tides shift ∼1 h each day, tidal stage was not confounded with time of day. All videos were collected during daylight hours. Grazing assays using *Enteromorpha* spp. (previously identified as *Hincksia* sp.), were conducted by Madin et al. (2011); grazing assay data were reanalyzed for this study using the statistical methods described below.

Algal Growth

At each fish survey location, with the exception of on the reef, we calculated percent algal cover using benthic photo-quadrats (25 × 25 cm PVC pipe frame). We did not collect percent algal cover on the reef because we it is a vastly different substrate type compared to the sand flats and because *Enteromorpha* spp. was not found growing on the reef. Benthic quadrat images were analyzed with CoralNet online software (www.coralnet.ucsd.edu). One hundred dots were automatically laid over each quadrat and the substrate below each dot annotated. The substrate categories used in this study were sand and turf algae. In addition to percent cover, algal canopy height was also measured. Here, algal canopy height was measured with calipers to the nearest mm at distances of 0 m (directly off the reef), 2.5, 7.5, 15, 22, and 30 m from the outer reef edge. These distances were selected to match those from the carbon stock collections (see below). Because of weather constraints, which restricted access to the furthest and deepest patch reefs, only 15 of the original 22 patch reefs included in the fish and percent algal cover surveys were sampled for algal canopy height and carbon stocks.

Carbon Stocks, Sediment Nutrients, and Sediment Grain Size

In conjunction with algal canopy height (see above), two sediment cores from each distance at each reef were collected with a hand corer. Sediments from the first core were used to measure sedimentary OC stocks and total nitrogen concentrations. Sediments from the second core were analyzed for mean particle grain size using a Mastersizer particle size analyser (Malvern Instruments, Malvern United Kingdom). Cores were separated into surface (0–5 cm) and subsurface (5–14 cm) sediments. We wanted to provide evidence that the halo formations have had both short- and long-term effects on OC stocks. Based on Heron lagoon sedimentation rates (a method used to estimate the date of deposition) of

0.126 ± 0.14 cm yr^{-1} (Smith et al., 1998), surface sediments (0–5 cm) represent relatively young OC accumulation (over the past 40 years) that has occurred since the Great Barrier Reef Marine Park Act of 1975 was enacted, while subsurface sediments (5–14 cm) represent OC accumulation over the past century (40 to ∼111 years). Stable isotopic $\delta^{13}C$ signature of the surface and subsurface sediments were significantly different ($P < 0.001$), suggesting that there was little to no mixing between the two depth measurements during core extraction.

Sediment samples were dried at 60°C to a constant weight. Dry bulk density was calculated as the dry weight of the sediment sample divided by the volume of the core. Sediment samples were then finely homogenized and divided into two subsamples for OC stock assessment and stable isotopic $\delta^{13}C$ signature analysis. Sediment samples, regardless of depth or distance from patch reefs, were predominantly inorganic carbon (>90%). As a result we used the Campbell et al. (2014) burn method to estimate OC. Bulk density and percent OC were combined with core depth (either 5 cm for surface sediments or 9 cm for subsurface sediments) to obtain per-area sediment OC.

Sources of OC and Stable Isotope Analysis

We used stable isotopic $\delta^{13}C$ signature analysis to determine major sources of OC to the sediments and whether C sources varied with distance from refuges. Within the halos we collected abundant and common primary producers. *Enteromorpha* spp. was the most dominant algae and almost exclusively made up the grazing halos. In addition to *Enteromorpha* spp., we also collected *Hydroclathrus* sp., whose abundant detached fragments accumulated in depressions in the sediment. Surface sediment subsamples analyzed for stable isotopic $\delta^{13}C$ signature were acidified using 1M HCl to remove all inorganic C. Algal samples were thoroughly washed, but not acidified. Sediment and algal samples were analyzed for stable $\delta\ ^{13}C$ isotopic composition using an elemental analyzer coupled to an isotope ratio mass spectrometer at the University of Hawaii at Hilo. Stable isotope results are presented as deviations from Vienna Peedee Belemnite (VPDB) with a measurement precision of ± 0.2‰. A two-source mixing model was used to quantify the proportional contribution of OC by *Enteromorpha* spp. and *Hydroclathrus* sp. to halo sediments.

Statistical Analyses

Linear mixed effect (LME) models incorporating quadratic terms were used to quantify relationships between distance and each of algal canopy height, percent algal cover, $\delta^{13}C$ signature of the sediments, grazing assays, individual herbivore bite rates and time spent foraging. Time spent foraging was normalized using a square root function to account for the fact that the size of the residuals progressively increased with distance. We used generalized linear mixed effects models with a Poisson distribution, as the data were zero inflated, to investigate the independent and interactive effects of distance and tidal stage (high and low) on predator and herbivore fish distributions. Finally, we used LME models to investigate the effect of distance, sediment grain size and the total N concentration in the sediments on algal height. To investigate factors affecting sedimentary C stocks, we used a LME model with a quadratic term for distance and a linear term for algal canopy height. Quadratic terms were included to allow for curvilinear relationships between distance and each response variable. If the quadratic distance term was found to be non-significant, it was removed and the model was re-run (as was the case for surface sediments), to test if a linear relationship existed between distance and the response variable being tested. Within each model, "reef identity" was included as a random effect to account for unmeasured, between-reef differences and avoid issues relating to pseudo-replication. For all models we used step-wise removal of non-significant terms for model selection. For all graphical representations of our mixed effect models (**Figures 2–4**), we show the fixed effects, while accounting for the random effect, by approximating the means and 95% confidence intervals using the "predictSE.lme()" function in the "AICcmodavg" package in R. All analyses were conducted using the statistical programming package R (R Development Core Team, 2016).

RESULTS

Predator and Herbivore Abundance and Behavior

We found that the abundance of herbivores and large bodied predators differed with distance from the reef and between tides. Based on the species observed, predators were predominantly roving predators. Predator abundance was influenced by a significant interaction between distance and tide (distance × tide, $P = 0.002$; **Figure 2A**). Although for both high and low tide, predator abundance decreased with distance from the reef ($P < 0.001$), their abundance was about five times higher during low tide compared to high tide ($P = 0.036$). Herbivore abundance was greatest during high tide (tide: $P = 0.030$; **Figure 2B**) and at distances closest to the reef edge (distance $P < 0.001$). Furthermore, herbivores ventured further from the reef edge at high tide compared to low tide (distance × tide: $P = 0.016$; **Figure 2B**). Grazing assays using *Enteromorpha* spp. (the most common algae growing around the halos) showed that grazing decreased with distance from the reef ($P = 0.005$), with herbivores removing 68% more algae closest to the reef edge compared with the furthest point on the transect (**Figure 3A**). Herbivore individual bite rates were found to increase with increasing distance from the patch reef ($P < 0.001$; **Figure 3B**), while time spent foraging decreased with distance ($P < 0.001$; **Figure 3C**).

Algal Growth

We found that algal canopy height and percent algal cover significantly increased with distance from the reef edge (both $P < 0.001$; **Figures 3D,E**). Neither sediment grain size nor total nitrogen concentrations in the sediment showed a significant effect on algal height (all $P > 0.05$).

Sediment Carbon Stocks and Sources

OC stocks collected from surface sediments (0–5 cm) were positively affected by both algal canopy height ($P = 0.027$) and distance ($P = 0.005$) from the reef edge (**Figure 4A**). Because of the significant effect of distance on algal height, we investigated the variance inflation factor using the function "vif()"in the "CAR" package in R to determine the extent of co-linearity between the two factors. The variance of inflation factor was 1.491, suggesting only weak co-linearity. To further determine the validity of maintaining algal height in the model, we performed a likelihood ratio test. Here we found that the inclusion of algal height as a predictor variable marginally improved the model ($P = 0.057$). Subsurface OC stocks (5–14 cm) also increased with distance ($P = 0.046$), but did not significantly co-vary with algal canopy height (**Figure 4B**). Subsurface OC stocks had a curvilinear distribution with distance, where stocks remained relatively constant between 0 and 15 m from the reef edge, before rapidly increasing (**Figure 4B**).

We found that $\delta^{13}C$ signatures of the sediments did not differ with distance or algal canopy height for either surface or subsurface sediments (all $P > 0.05$). Two-source mixing models showed that 43 ± 3% of OC originated from *Enteromorpha* spp., the dominant algae growing in the lagoon, while the other 57 ± 2% originated from *Hydroclathrus* sp.

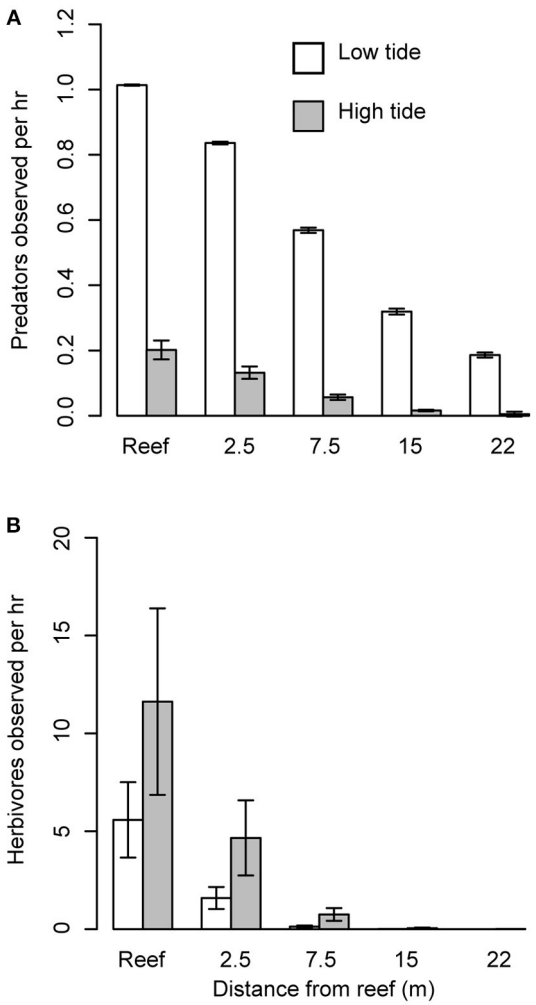

FIGURE 2 | Effect of predators on herbivore abundance. **(A)** Mean number (± s.e.) of predators observed per hour in Heron lagoon during high and low tide on the reef and with increasing distance from patch reef edge. **(B)** Mean number (± s.e.) of herbivores observed per hour in Heron lagoon during high and low tide and with increasing distance from patch reef (i.e., refuges).

DISCUSSION

Trophic cascade theory predicts that predator effects should extend to influence C cycling in ecosystems. Yet, there has been little empirical evidence in natural ecosystems to support this hypothesis. We demonstrate that marine predators can influence long-term OC storage in marine sediments via a behaviorally-mediated trophic cascade. Here we show that sedimentary OC stocks were greater in high-predation risk zones (i.e., further from the reef) compared to low ones (i.e., closer to the reef). Greater OC stocks further from the reef arose from behavioral responses by herbivores to the fear of predation, which provided a refuge for macroalgae growing further from the reef, which in turn aided C accumulation in these sediments. Our results indicate that predators may help protect the accumulation and preservation of OC in marine sediments, raising concerns regarding trophic downgrading of marine ecosystems.

We hypothesized that predators, via trophic cascades, were a major driver behind the development of grazing halos in Heron lagoon. One way in which predators can induce a trophic cascade is through direct consumption (Paine, 1980), which reduces prey abundance in locations where predation pressure is high (i.e., density-mediated trophic cascade). It is possible that a density-mediated cascade may be occurring in this system, i.e., predators may reduce herbivore density via consumption as herbivores venture away from the reef into unsheltered areas. In 311.5 h of remote daylight observation, we observed no successful attacks of predators on herbivores in the halo. Although these results could suggest that density-mediated effects are relatively weak in this system, it is important to note that our cameras captured only a limited field of view and were constrained to filming during daylight hours. Further studies investigating predation events, especially nocturnal predation events, are needed to determine the contribution of density-mediated trophic cascades on the presence and maintenance of grazing halos in our study system.

Trophic cascades, however, are not always manifested through direct consumption. Trophic cascades can also arise from adaptive shifts in prey behavior that aim to minimize predation risk (Schmitz et al., 2004). Specifically, prey can allocate time differently to activities that vary in predation risk and feeding opportunity, or use vigilance to trade-off feeding rate and predation risk (Brown, 1999). These behaviorally-mediated trophic cascades can be as strong, or even stronger than, consumptive effects (Preisser et al., 2005; Hammill et al., 2015). To determine whether a behaviorally-mediated trophic cascade was occurring in Heron lagoon, we first investigated

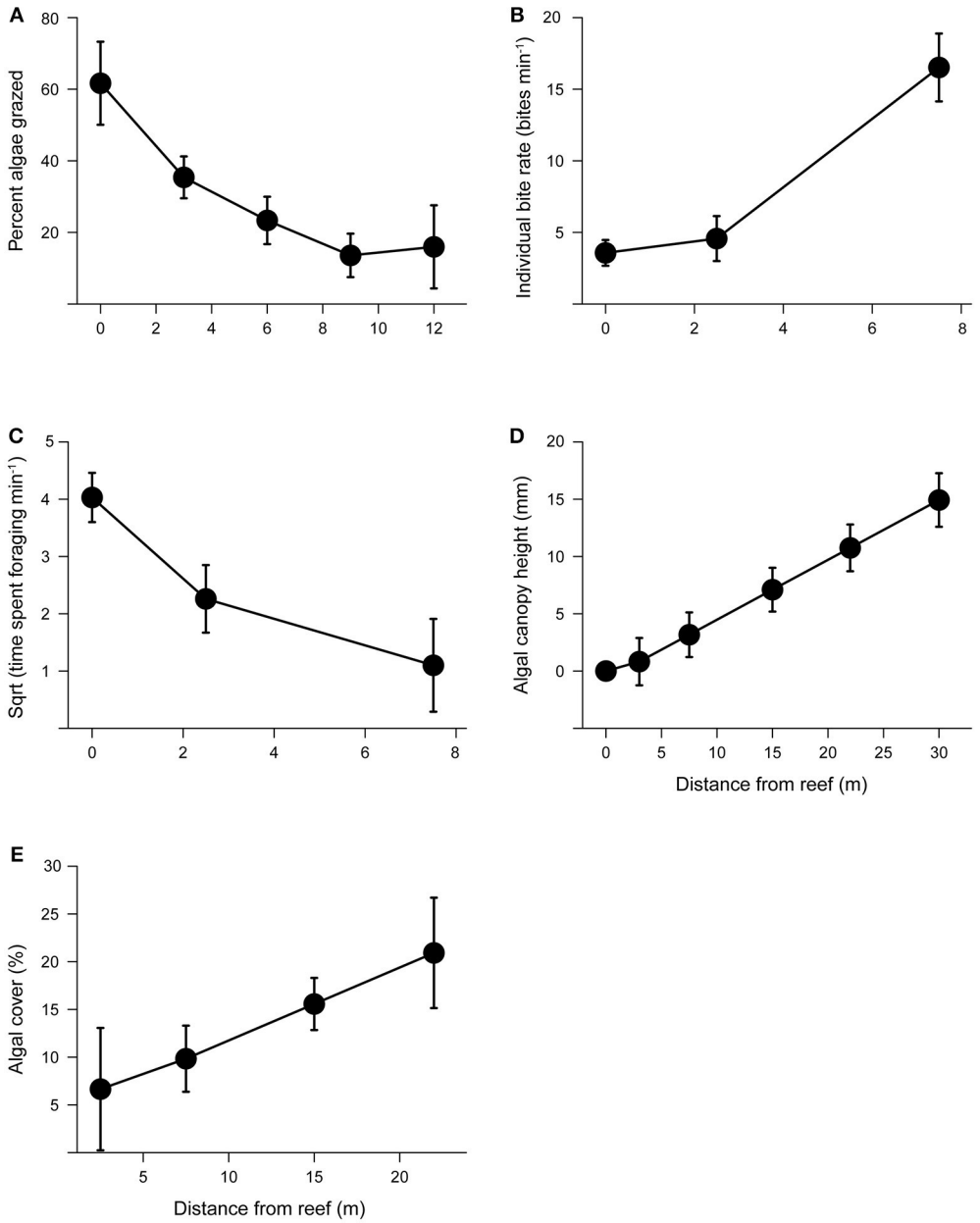

FIGURE 3 | Grazing intensity and its effects on algal growth with increasing distance from patch reefs. **(A)** Mean percent algae removed (± s.e.) with increasing distance from patch reef following 72 h grazing assays. **(B)** Mean individual bite rates (± s.e.) by herbivores with increasing distance from patch reefs. We did not observe any herbivorous fish foraging at distance >7.5 m from the reef edge. **(C)** Mean herbivore foraging time (± s.e.) with increasing distance from patch reefs. **(D)** Mean algal canopy height (± s.e.) with increasing distance from the patch reef. **(E)** Mean percent algal cover (± s.e.) with increasing distance from the patch reef.

the abundance and behavior of predators (sharks and jacks) and herbivores in our system. We then determined whether herbivores were responding behaviorally to predator patterns in ways that are consistent with a top-down effect.

According to Ideal Free Distribution theory, consumers with no perceived risk should allocate foraging time in proportion to food availability (Wirsing et al., 2007). However, where predation risk occurs, foraging theory predicts that animals should harvest fewer resources in habitats that are more exposed to predation risk (Brown and Kotler, 2004). We found that herbivore abundance (**Figure 2B**) and the amount of algae removed from grazing assays (**Figure 3A**) declined with distance from the reef edge. This pattern occurred despite the fact that highly-palatable benthic macroalgae was far more abundant at distances greater from patch reefs (**Figures 3D,E**). The fact that predator abundance, which was largely composed of roving predators, was also greatest near the reef (**Figure 2A**) highlights the complexity of predator-prey interactions. Naturally, predator abundance should be highest where their food source is greatest. Thus, prey must not only monitor the presence of predators

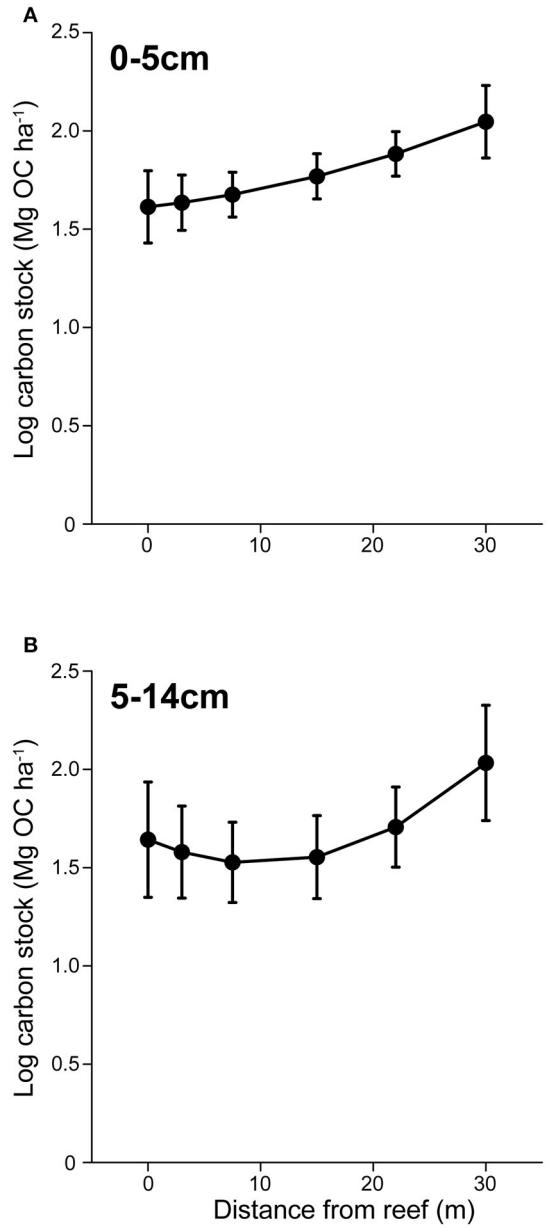

FIGURE 4 | Sedimentary carbon storage in a seascape of fear. **(A)** Mean (± s.e.) sedimentary organic carbon (OC) in surface (0–5 cm) sediments with increasing distance from herbivore refuges. **(B)** Mean (± s.e.) sedimentary OC stocks in subsurface (5–14 cm) sediments with increasing distance from herbivore refuges.

to reduce predation risk, but also make decisions about when to flee based on a margin of safety. Theory on flight initiation distance (FID; the distance at which prey flee an approaching predator) shows that the margin of safety for a prey is based on its distance from a refuge, the predators distance from the refuge, and the prey's speed relative to the predator's (Cooper, 2016). As the prey's distance to the refuge increases, so does the FID. Thus, herbivores foraging further from the reef are at a greater risk of predation by roving predators than those foraging close to the reef. We did not observe any herbivores at distances >7.5 m from the reef edge, suggesting that this distance may be the FID threshold for our predator-prey species.

To further support our hypothesis that herbivores in our system are responding to predation risk, we found that herbivores in Heron lagoon appeared to respond to temporal patterns in predator abundance by altering their abundances in an opposing way. We found that the abundance of large bodied predators on and around patch reefs were not consistent across high and low tides. Instead, predators appear to patrol patch reefs in the lagoon primarily at low tide when water depth is lowest, and all but vanish during high tide, when water depth is at its max (**Figure 2A**). This hunting behavior may be used to reduce the three-dimensional space in which prey can move, a known strategy employed by marine predators to increase the likelihood of capturing their prey (Heithaus et al., 2009). Conversely, herbivores were more abundant and ventured further from the reef edge at high tide when predator abundance was lowest (**Figure 2B**). Since net population sizes of predator and prey populations are not significantly increasing and decreasing due to births and deaths over the time scale of tidal cycles, predators and herbivores must be either migrating into and out of the lagoon with the tides or restricting their movements such that they are observed less frequently.

Finally, we found that herbivore individual bite rates increased with distance from the reef, but time spent foraging decreased (**Figures 3B,C**). There are two potential reason why herbivores would spend less time foraging further from the reef. First, the cost of energy spent foraging further from the reef is greater than the net energy gained from foraging in these food patches. Second, the risk of predation at food patches further from the reef is higher than those closer to the reef; thus herbivores are trading foraging time for safety. Although we do not have giving-up densities for our system, a study conducted on similar herbivorous species found that over a distance of 30 m from a refuge, harvest rates by herbivores were dependent on resource availability (Gil et al., 2017). Thus, it is unlikely that the energetic costs of foraging further from the reef is driving the herbivore abundance patterns seen in Heron lagoon. Furthermore, the fact that herbivores increased their bite rates with increased distance from the reef suggests that herbivores are trying to maximize their food intake when foraging in these riskier areas. Overall, our results show that herbivores in Heron lagoon reduce predation risk through time allocation. Specifically, herbivores altered where they foraged, when they foraged, and for how long they foraged in different microhabitats. Consistent with a trophic cascade, these effects on herbivore foraging behavior resulted in an increase in algae with increasing distance from the reef.

The observed changes in herbivore foraging intensity and its effects on the distribution of algae, subsequently had legacy effects on the spatial distribution of OC stocks in coastal marine sediments. OC stocks collected from surface sediments (0–5 cm), which represent OC storage over the past ∼40 years, were positively affected by both algal canopy height and distance from the reef edge (**Figure 4A**). This resulted in surface OC stocks being up to 24% greater at 30 m from the reef's edge compared

to adjacent to the reef. Subsurface OC stocks, which represent OC storage between ∼40 and 110 years, had a curvilinear distribution with distance, where stocks remained relatively constant between 0 and 15 m from the reef edge, before increasing. Although we were unable to collect sediment cores from some of the deepest reefs (see section Materials and Methods above), consistent patterns in herbivore and predator behavior and abundance, as well as percent algal cover across the different patch reefs suggest that patterns in OC stocks should be similar in these areas. It is important to note that although we saw a positive effect of predators on sedimentary OC stocks in Heron lagoon, the direction of predator effects on OC storage is dependent on food chain length. The mechanisms behind halo formation in Heron lagoon appeared to be driven by interactions between species in an odd-numbered food chain (predator > herbivore > primary producer); thus we would predict that predators should have a net positive effect on primary producers and C storage. However, in even-numbered food chains, predators are predicted to have a net negative effect on primary producer, subsequently reducing OC storage (Schindler et al., 1997; Hammill et al., 2015).

We found that $\delta^{13}C$ signatures of the sediments did not differ with distance or algal canopy height for either surface or subsurface sediments. This homogeneity in $\delta^{13}C$ signatures suggests that sources of OC to the sediments arise from the same basal resource regardless of distance from the reef or sediment age. Two-source mixing models using $\delta^{13}C$ stable isotopic signatures for surface sediments showed that 43 ± 3% of OC originated from *Enteromorpha* spp., the dominant algae growing in the lagoon, while the other 57 ± 2% originated from *Hydroclathrus* sp. Although live *Hydroclathrus* sp. was not seen growing in the algal halos at the time of this study, large deposits of detached *Hydroclathrus* sp. are found throughout the algal beds in sand depressions. The contribution of autochthonous C to OC sediment pools can be highly variable in macrophyte systems, with past results ranging from >80% to <50% (Kennedy et al., 2010; Reef et al., 2017). These results suggest that herbivores in Heron lagoon not only influence C storage by consuming autochthonous sources of OC to sediments (e.g., *Enteromorpha* spp.), they also remove the structure (canopy height and density) by which allochthonous OC is captured. These findings shed light on some of the biotic processes that influence OC accumulation and retention in marine sediments, as well as provide important insights into the potential community- and ecosystem-level consequences of marine predator declines.

We investigated several alternative hypothesis (sediment grain size, nutrient availability in the sediment, bioturbation, or water movement) for halo formation in Heron lagoon. However, we found no evidence to support these other hypotheses. First, although average algal cover immediately adjacent to patch reefs was far lower than algal cover farther from the reef, some individual patch reefs had an algal cover as high as 43% at the closest distance to the reef (2.5 m). This suggests that although algal growth was stunted near the reef, physiochemical conditions (e.g., grain size or wave action) close to the reef do not completely prevent growth. Second, neither sediment grain size nor total nitrogen concentration in the sediment showed a statistically significant effect on algal height. If nutrient levels in the water column were driving algal growth, we would expect more algae closer to the reef because of the greater availability of nutrients being transferred off the reef. Third, a study conducted concurrently with ours found no indication that benthic meiofauna were contributing to the patterns in algae (Ollivier et al., 2018). Finally, if water movement around the reef was responsible for the halo-type pattern in algal growth, we would expect asymmetrical radii of halos to be in accordance with the dominant direction of water flow. However, Madin et al. (2011) did not find any evidence of this for Heron lagoon halos. Although it is possible that other untested physical or biological factors are influencing the growth of algae close to the reef, it is clear that spatial differences in herbivore foraging intensity plays a major role in the formation and maintenance of grazing halos in Heron lagoon.

Our ability to link trophic cascades in Heron lagoon with patterns in ancient OC stocks (e.g., subsurface stocks) is somewhat limited considering that the lagoon landscape could have been vastly different ∼110 year ago when this deeper layer of OC was first accumulated. If trophic cascades 110 years ago were operating similar to current day cascades in this system, then the observed rapid increase in subsurface OC stocks beyond 15 m from the reef edge could suggest that this distance represents a long-term threshold for algal growth and OC accumulation. However, this would only be the case if our patch reefs maintained a similar size over the past ∼110 years. In the GBR live coral colonies can expand linearly by ∼6 cm yr^{-1} and dead corals can erode by as much as ∼30 cm yr^{-1} (Ferrari et al., 2017). Thus, if our patch reefs grew or eroded over the past 110 years, our distances from the reef edge would not accurately represent historical distances. Furthermore, because we do not know whether historical halos existed at these patch reefs 110 years ago, it is unclear whether our results represent the effects of historical grazing on then OC stocks, or whether current grazing patterns are liberating ancient OC stocks close to the reef. Studies have shown that disturbances that decrease macrophyte density can lead to the rapid loss of sediment OC down to one meter deep in the sediment (Pendleton et al., 2012) by increasing microbial demineralization rates or by enhancing sediment resuspension and transportation (Lovelock et al., 2017). Regardless of the overall mechanism, our results suggest that current day predators protect OC stocks in Heron lagoon by creating high-risk predation zones that offer a refuge for algal growth and OC accumulation and retention in sediments.

It is now widely recognized that human activities are responsible for rapid and large declines in aquatic and terrestrial predator populations (Pauly et al., 1998; Myers and Worm, 2003; Darimont et al., 2015; McCauley et al., 2015). However, despite the strong suggestion that the extirpation of predators can have far reaching effects on C cycling (Estes et al., 2011; Schmitz et al., 2014; Atwood et al., 2015), very few studies have explicitly documented this in natural ecosystems. Our study demonstrates that trophic cascades can indeed lead to dramatic legacy effects on relatively young and old OC stocks in ocean sediments. Unlike animals which offer only short-term (days to years) C storage potential and corals which

actually act as net sources of CO_2, marine sediments can "lockup" OC for thousands of years (Howard et al., 2017). Thus, our results suggest that changes to both marine predator and herbivore populations can have consequences for ocean C cycling. Furthermore, marine predators often have slow population growth rates, and recovery can take decades or longer (Hutchings and Reynolds, 2004). As a result, the consequences of marine predator declines on C cycles may be significant and long-lasting. Our empirical demonstration of the important role of predators and herbivores in structuring C processes in a natural marine ecosystem highlights that conservation of predators is critical and urgent, not just for their intrinsic value, but due to their role in regulating fundamental ecosystem services (Estes et al., 2011; Ritchie et al., 2012; Atwood et al., 2014a).

AUTHOR CONTRIBUTIONS

TA, EM, AH, PM, and CL designed the study. TA, EM, AH, EH, and QO collected data. EM, OL, EH, and CR analyzed data. TA wrote the first draft of the manuscript. All authors contributed to the writing and editing of the manuscript.

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Landscape Level Effects of Lion Presence (*Panthera leo*) on Two Contrasting Prey Species

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Due to the strong individual cost of being predated, potential prey species alter their behavior and physiology in response to predation risk. Such alterations may cause major indirect consequences on prey populations that are additive to the direct demographic effects caused by prey being killed. However, although earlier studies showed strong general effects of the presence of apex predators, recent data suggest that indirect effects may be highly context dependent and not consistently present. We combined behavioral data with data on endocrine stress and stable isotopes to assess landscape level effects of lion (*Panthera leo*) presence on two prey species in South Africa, impala (*Aepyceros melampus*) and blue wildebeest (*Connochaetes taurinus*). We also evaluated if there was any seasonal variation in such effects. In addition, we provide results from a physiological validation for an enzyme-linked immunoassay (EIA) that can be used for non-invasive monitoring of glucocorticoid stress metabolite concentrations in impala from fecal pellets. We did not find any significant differences in vigilance behavior, fecal glucocorticoid metabolite concentrations, $\delta^{13}C$ values or isotope niche breadth between animals living with and without lions for either species. However, wildebeest living in a reserve with lions spent more time foraging compared to wildebeest in a lion-free environment, but only during the wet season. Values of fecal $\delta^{15}N$ suggest a shift in habitat use, with impala and wildebeest living with lions potentially feeding in less productive areas compared to animals living without lions. For both species, characteristics of the social groups appeared to be more important than individual characteristics for both foraging and vigilance behavior. Our results highlight that antipredator responses may be highly dynamic and scale-dependent. We urge for further studies that quantify at what temporal and spatial scales predation risk is causing indirect effects on prey populations.

Keywords: anti-predator response, predation, lion, Africa, behavioral observation, stable isotopes, fecal glucocorticoid metabolites, validation

INTRODUCTION

Predation has major impacts on ecosystem processes by influencing the dynamics of prey populations (Lima, 1998). The effects of predation on prey demography are partly caused by prey being killed, and partly by indirect effects caused by anti-predatory responses (Lima, 1998; Creel and Christianson, 2008). These so called "risk effects" can be responsible for considerable portion of the total role of predation on prey demography (Creel and Christianson, 2008; Valeix et al., 2009; Creel et al., 2014). Behavioral responses to predation risk are diverse, but may include shifts in habitat use (Creel et al., 2005), changes in movement patterns (Sih and McCarthy, 2002), adjustment of the relative time spent vigilant and foraging (Lima and Bednekoff, 1999; Abramsky et al., 2002), and social aggregation (Lima, 1995). It can also trigger physiological stress responses (Clinchy et al., 2004), which may have negative effects on prey populations (Hawlena and Schmitz, 2010; Boonstra, 2013).

A number of studies from a wide range of taxa show that antipredator responses often lead to a decreased foraging success, caused by a shift in the trade-off between vigilance and feeding habits (Werner et al., 1983; Creel et al., 2014). Lima and Bednekoff (1999) suggested that under temporal fluctuations of predation risk, prey should balance their anti-predator behavior and energetic intake requirements ("the risk allocation hypothesis"). Animals will spend more effort in antipredator behaviors when predation risk is high and prioritize feeding efficiency when predation risk is low and prolonged. In this situation, prey need to reduce anti-predator behavior in favor of food intake (Lima and Bednekoff, 1999). This trade-off should become more pronounced in the presence of food limitation (Dias et al., 2011). In general, prey demography is affected by the reduction of foraging success to favor anti-predator vigilance (Lima and Dill, 1990). Animals can also alter the amount of time spent vigilant or the frequency of scanning bouts, influencing their foraging behavior differentially (Roberts, 1996). The tendency of prey to forage gregariously promotes risk dilution and confusion effects, but also enhances the possibility to detect predation risk reducing an individual's time spent vigilant while favoring food intake (Elgar, 1989; Dalerum et al., 2008a). Several studies have demonstrated the cost of vigilance for foraging. For example, Wolff and Van Horn (2003) found that female elk (*Cervus elaphus*) in Yellowstone National Park, a predator-rich environment, spent more time being vigilant and less time foraging than female elk in Rocky Mountain National Park, a predator-free system.

Predation risk can also cause physiological stress responses, which include an increased activity of the hypothalamic-pituitary-adrenal (HPA) axis (Hawlena and Schmitz, 2010). Increased levels of adrenocorticotrophic hormone (ACTH) promote the adrenal cortex to release glucocorticoids (GCs, i.e., cortisol or corticosterone), which induce elevation of blood pressure, cardiovascular function and respiration (Sapolsky et al., 2000). Elevated GC levels also directly inhibit the immune and digestive systems, and suppress growth and reproduction (Dobson and Smith, 2000). Therefore, a prolonged stress response may cause pathologies, such as inhibition of fundamental non-emergency body functions and drops in reproductive output, which may have far-reaching consequences for predator-prey dynamics (Boonstra et al., 1998; Creel et al., 2009; Hawlena and Schmitz, 2010). However, although most vertebrates show a physiological stress response to immediate predation risk, the occurrence of chronic stress as a response to anticipated predation may differ between prey species depending on life history characteristics (Boonstra, 2013).

Predation may also cause shifts in foraging patterns and habitat utilization, if animals are forced to feed in lower quality habitats to limit risk (Creel and Christianson, 2008). Prey tend can avoid areas with high predation risk by selecting habitats with better opportunities for detection of predators and better opportunities to escape (Valeix et al., 2009). However, the decisions associated with habitat use may involve trade-offs between nutritional needs to optimizing energy intake while simultaneously reducing the risk of predator exposure. For instance, a study in the Yellowstone National Park showed that the presence of wolf (*Canis lupus*) led elk to shift their habitat use from favored grassland to wooded areas, and to shift their feeding habits from grazing to browsing (Creel et al., 2005; Creel and Christianson, 2009). Similarly, Thaker et al. (2011) found that in Africa, smaller ungulates, such as impala (*Aepyceros melampus*), warthog (*Phacochoerus africanus*) and kudu (*Tragelaphus strepsiceros*) avoided the areas used by predators living nearby. However, larger ungulates, such as blue wildebeest (*Connochaetes taurinus,* hereafter referred to as wildebeest), plains zebra (*Equus quagga*) and giraffe (*Giraffa camelopardalis*) avoided areas utilized by larger predators, such as lions and leopards. Due to of such trade-offs, seasonal variation in food availability and quality can influence habitat choice (Jarman, 1974; Périquet et al., 2012).

African ecosystems support some of the most diverse groups of large carnivores and associated assemblages of ungulate prey on Earth (Sinclair et al., 2003; Dalerum et al., 2009; Dobson, 2009). Therefore, they offer excellent environments in which to evaluate the indirect effects of predation risk. The African savanna hosts diverse herbivore assemblages with species of contrasting morphology, physiology and behavior due to high plant diversity and spatiotemporal heterogeneity (du Toit et al., 2004). In the case of large herbivores, local extinction of predator populations may not necessarily cause a loss in the ability of prey species to respond to predators (Hettena et al., 2014), and ecologically naive prey in Africa have showed similar responses to lion exposure as populations facing lion predation (Dalerum and Belton, 2015). This finding agrees with evolutionary arguments for retention of anti-predatory behavior in multi-predatory environments (Blumstein, 2006), although it stands in contrast to some studies from the northern hemisphere (Sand et al., 2006; Berger, 2007). Hence, the repatriation of locally extinct African predators has the potential to rapidly reconstruct ecological effects of predation that may have been lost (Dalerum and Belton, 2015).

Stable carbon and nitrogen isotope measurements can be good indicators of the foraging ecology and habitat utilization of African ungulates (Codron et al., 2007; Miranda et al., 2014). Three main foraging strategies can be identified among African herbivores; grazers, browsers and mixed-feeders (Hofmann and Stewart, 1972). The majority of African grasses follow the C4

photosynthetic pathway, whereas forbs, shrubs and trees utilize the C3 photosynthetic pathway. Because these pathways cause different rates of incorporation of ^{13}C, stable carbon isotope ratios can be used to distinguish grasses from trees, shrubs and forbs (Farquhar et al., 1989). Subsequently, $^{13}C/^{12}C$ isotope ratios allow for the identification of feeding strategies in African ungulates (Codron J. et al., 2005). In contrast, $^{15}N/^{14}N$ ratios in plants reflect plant physiology and environmental conditions, such as aridity, soil type, climate and nitrogen sources (Handley and Raven, 1992). Stable nitrogen isotope ratios can be good indicators of habitat utilization (Ambrose and De Niro, 1986), although they may also reflect physiological mechanisms of rumination (Ambrose, 1991). Combining carbon and nitrogen isotope data may therefore offer complementary information regarding the ecology of African ungulates (Miranda et al., 2014).

In this study, we evaluated how the presence of lions (*Panthera leo*) influenced foraging and vigilance behavior, stress hormone levels, and stable isotope markers of foraging ecology in two African ungulates, impala and wildebeest. We compared data from two reserves in northern South Africa, of which one supports a re-introduced lion population, while the other has been without lions since they went regionally extinct over 100 years ago (Dalerum and Belton, 2015). We also provide a physiological validation for an enzyme-linked immunoassays (EIAs) to ensure its suitability for a non-invasive monitoring of a physiological stress response in impala measuring fecal glucocorticoid metabolite (fGCM) concentrations (Ganswindt et al., 2012). Wildebeest are one of the most preferred prey species for lions (Hayward and Kerley, 2005), whereas impala are one of the least preferred (Owen-Smith, 2008). Both species are gregarious ungulates (Périquet et al., 2012; Sagamiko et al., 2015), impala are mixed feeders utilizing both grassland and bushland habitats according to seasonal change in food resources (Miranda et al., 2014), whilst wildebeest are regarded as grazers usually confined to grassland areas (Codron et al., 2007; Valeix et al., 2009; Périquet et al., 2012).

We hypothesized that the presence of lions would affect behavioral time budgets, stress-related hormone concentrations and foraging ecology of both species, and made the following predictions: (1) ungulates living in the presence of lions will forage less and be more vigilant than ungulates living in a lion-free environment; (2) ungulates living in an area with lions will have higher stress hormone concentrations than ungulates living in a lion-free environment; (3) lions will influence the foraging patterns in the two ungulates which will be reflected in the stable isotope ratios; (4) the effects of lions on time budgets will be most pronounced during the dry season, which has limited resources; and (5) the presence of lions will have an overall greater effect on the behavior of the most preferred prey, wildebeest, than on impala.

METHODS

Study Area

We conducted the study in two reserves in the Waterberg region of the Limpopo province, South Africa (**Figure 1A**). The Waterberg region was declared a biosphere reserve (the Waterberg Biosphere Reserve) by United Nations Educational, Scientific and Cultural Organization (UNESCO) in 2001 (Pool-Stanvliet, 2013). The region is dominated by highland plateaus and steep rocky hills and the altitude ranges from 1,100 m in the north-west to 2,100 m in the south-western part of the area (Wellington, 1955; Dalerum and Belton, 2015). The area is characterized by numerous small and large rivers, streams and gullies, but other water support systems, such as wetlands, marshes and artificial waterholes are present (Swanepoel, 2008). The vegetation is classified as Waterberg Mountain Bushveld, where the flora ranges from short closed woodland to low brake (Mucina and Rutherford, 2006). The area is located in a summer rainfall region with distinct dry (April–September) and wet (October–March) seasons (Codron D. et al., 2005). The mean annual rainfall ranges from 650 to 900 mm with more intense precipitation during the wet season with a maximum monthly rainfall of 302.6 mm (Low and Rebelo, 1996). The mean annual maximum temperature is 26.5°C and the mean annual minimum temperature is 11°C (Kilian, 2003). The average maximum summer temperature is 32°C and the minimum is 18°C. In winter, the mean maximum temperature is 22°C and the mean minimum is 4°C, with the possibility of frost to occur (Ben-Shahar, 1987).

The study was conducted in two private reserves, Lapalala Wilderness (hereafter Lapalala, 23°51S, 28°16E) and Welgevonden Game Reserve (hereafter Welgevonden, 24°18S, 27°80E). Both study areas were previously agricultural lands which were transformed into wilderness areas in 1993 and 1981, respectively. The two reserves are 50 km apart and similar in size, topography, geology, hydrology, vegetation and fauna (**Figure 1B**, Dalerum and Belton, 2015). In both reserves, upland areas are based on quartzite soils and low productivity semi-open woodlands, whereas lower elevations are characterized by a mixture of sand and clay based soils of higher productivity, with more open grassland patches. These areas of contrasting productivity are approximately equally distributed in the two areas. Both properties are privately owned and heavily fenced. Lapalala covers about 36,000 h and was at the time of data collection closed to the public. However, Lapalala hosted a wilderness school and a limited number of guided trophy hunts were permitted (Dalerum and Belton, 2015). Welgevonden, which encompasses 37,500 ha, contains 15 commercial game lodges and several private ones. Over 50 land-owners have access rights over Welgevonden. Hunting is prohibited, but game-viewing is allowed (Dalerum and Belton, 2015). Our observations suggest that most animals have become habituated to game viewing vehicles, as has been suggested elsewhere (Bateman and Fleming, 2017). In both reserves, management-related activities, such as burning and bush-clearing occurred during the study (Isaacs et al., 2013).

Both study areas support abundant and diverse populations of herbivores, from large-sized ungulates, such as giraffe, white (*Ceratotherium simum*) and black (*Diceros bicornis*) rhinos, plains zebra, blue wildebeest and greater kudu to medium-sized ungulates like impala and warthog (Isaacs et al., 2013; Dalerum and Belton, 2015). Similarly, both reserves have similar resident carnivore communities including brown hyena (*Parahyaena*

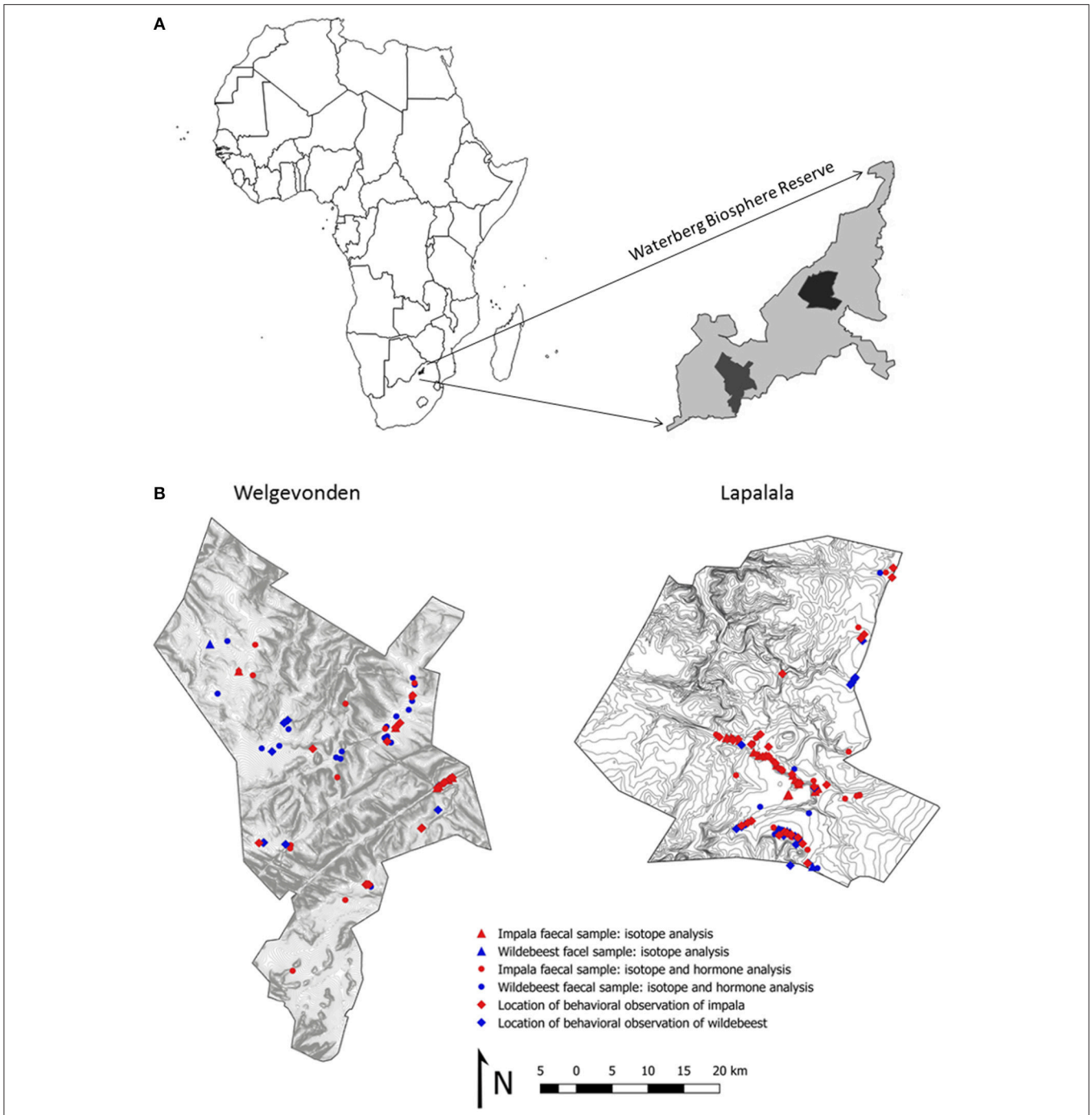

FIGURE 1 | Location of the two study sites within the Waterberg Biosphere Reserve **(A)** as well as detailed maps of each reserve including locations for behavioral observations and collected fecal samples **(B)**.

brunnea), black-backed jackal (*Canis mesomelas*) and leopard (*Panther pardus*) (Swanepoel et al., 2015). Individual cheetahs (*Acinonyx jubatus*) are transitory in Lapalala and occasionally present in Welgevonden (Dalerum and Belton, 2015), while wild dog (*Lycaon pictus*) occasionally occur in Lapalala (Ramnanan et al., 2013). Lapalala is characterized by the absence of lions, which were eradicated in the north-central part of South-Africa at the beginning of the twentieth century (Skead, 2011). Conversely, to develop Welgevonden as a Big Five wildlife reserve, lions were reintroduced in 1998 together with elephant (*Loxodonta ajricana),* white rhinocero and buffalo (*Syncerus caffer*) (Kilian, 2003). Apart from these differences, densities of ungulates and

carnivores were comparable between the reserves, although temporal fluctuations occurred (Dalerum and Belton, 2015; Swanepoel et al., 2015). At the time of data collection (2008–2012), Welgevonden hosted a lion population ranging from 8 to 14 animals, distributed across two prides and one male coalition (Dalerum and Belton, 2015). The two reserves are separated by ∼50 km of farmland, so there is very little likelihood of the Welgevonden lions moving into the Lapalala area and it is unlikely that prey species have dispersed between them (Dalerum and Belton, 2015).

Collection of Behavioral Data

Behavioral observation periods of 1–2 weeks took place from July 2008 to June 2012, during both the dry and wet seasons. We opportunistically encountered groups of herbivores while driving in the reserve at dawn and dusk, and recorded data from groups where at least half of the animals were actively foraging to avoid biases associated with animals experiencing different motivational drivers for various behaviors (Dalerum et al., 2008a, see also Clutton-Brock et al., 1999). The observations were conducted in open landscape or open patches in the woodlands throughout both reserves (**Figure 1B**). Observations of the same group were prevented as far as possible by avoiding repeated sampling at the same location during each field period. Although this may have prevented pseudo replication within each season, we cannot rule out that the same groups were observed during different years and seasons. Groups were observed from within a vehicle at distances ranging from 25 to 200 m from the animals. For each group, the group size and the group composition based on a crude age class distinction (adult, sub-adult and juvenile) and sex was noted, as well as distance of the center of the group to nearest available cover. These distances ranged from 0 to 300 meters for both impala and wildebeest. While distance to cover did not differ between the reserves for impala (Lapalala [mean ± sd]: 54 ± 76 m, Welgevonden: 44 ± 46 m, $\beta = -11.4$, $t_{56} = 0.60$, $p = 0.553$), wildebeest had longer distance to cover in Welgevonden (110 ± 88 m) than in Lapalala (48 ± 50 m) ($\beta = 66.8$, $t_{41} = 2.80$, $p = 0.008$). We recorded the whole group, or as large part of the group as possible, for 5 min or until the group was out of view using a small handheld digital video camera. Recordings were not initiated until the group showed no signs of disturbance from the observation vehicle (Dalerum and Belton, 2015). We also conducted scan observations of the groups while they were being observed in the field.

Time spent foraging and being vigilant was quantified using focal sampling techniques from the recorded videos (Altmann, 1974; Dalerum and Belton, 2015). The duration of all behaviors was timed to the closest second by recording them on a PDA device or onto a laptop computer (Dalerum and Belton, 2015). We also counted the number of vigilance scan bouts for each focal individual. The focal observations on each adult or sub-adult group member was done for 5 min or until the individual was out of sight. Animals with <90 s of observation were excluded from analyses. An animal was defined as foraging if it was standing up with its head distinctly below its shoulders or if it was standing up and feeding from a bush. Animals were defined as vigilant if they were standing up with their head clearly above their shoulders and scanning the surroundings. We attempted to distinguish social from anti-predatory vigilance, and discarded all vigilance bouts where vigilance clearly was directed towards group members.

The scan observations were conducted by simultaneously recording the activity of each individual at specific time points and summarizing the number of animals engaged in each behavior. Animals for which we could not define the behavior because they were obscured or out of sight were omitted from that individual scan. In contrast to the focal observations, which give behavioral time budgets, the scan observations provide an indication of how large a proportion of each group are engaged in the respective behaviors at a given time. Scan observations were scored at 1-min intervals for 5 min. For each scan event, we calculated the proportions of the total number of observable adult and sub-adult individuals that were engaged in foraging and vigilance for subsequent analyses (Dalerum and Belton, 2015).

Throughout the two seasons we scored behavior from a total of 171 impala (95 in dry and 76 in wet) and 106 wildebeest individuals (64 in dry and 42 in wet) belonging respectively to 42 and 29 groups in Lapalala for focal recordings, and 75 impala (29 in dry and 46 in wet) and 61 wildebeest (46 in dry and 15 in wet season), respectively distributed in 16 and 14 groups in Welgevonden. Number of individuals per group ranged from 1 to 7 for impala and from 1 to 5 for wildebeest. For scan observations we recorded a total of 33 groups of impala (21 in dry and 12 in wet season) and 22 groups of wildebeest (13 in dry and 9 in wet season) in Lapalala, and 15 groups of impala (6 in dry and 9 in wet season) and 11 groups of wildebeest (8 in dry and 3 in wet season) in Welgevonden.

Collection of Fecal Samples and Plant Reference Samples

Fecal samples from the target species were collected from the two reserves during the same periods as the behavioral recordings, i.e., from July 2008 to June 2012. Fecal samples were used for both glucocorticoid metabolite and isotope analyses. Groups or single study animals were spotted from a vehicle and followed until they defecated. Feces were collected within 15 min after defecation, put into labeled zip-lock bags, and stored in a cooling box containing ice to avoid bacterial and microbial degradation of fGCMs (Hulsman et al., 2011). Back at the research base, the samples were stored in a freezer at −20°C. Samples were collected only from adult or sub-adult individuals and we noted age, sex as well as group size and composition. Attempts were made to collect a unique sample per group in order to decrease interdependency between samples (Miranda et al., 2014). A total of 85 impala samples were collected for C and N isotope analysis, from 59 individuals (24 in dry and 35 in wet season) that belonged to 46 groups in Lapalala and 26 individuals (5 in dry and 21 in wet season) that belonged to 17 groups in Welgevonden. A total of 56 wildebeest samples were collected, of which 29 individuals (14 in dry and 15 in wet season) belonged to 24 groups in Lapalala, and 27 animals (9 in dry and 18 in wet season) belonged to 23 groups in Welgevonden.

In order to provide a baseline isotopic dataset for interpreting the stable isotope values from the fecal samples, 34 reference plant samples from 10 different species in Lapalala were collected. These were either shrubs or grasses and were collected in diverse topographical sites to reflect a wide range of environmental conditions. These reference samples were collected during the dry season in 2017.

Hormone Analysis

The evaluation of physiological stress was determined non-invasively by measuring fGCM concentrations using a random sub-set of the collected fecal samples. The analysis were performed on 43 fecal samples of impala (18 in dry and 25 in wet season) from 31 groups in Lapalala and on 19 impala samples (7 in dry and 12 in wet) from 14 groups in Welgevonden. We analyzed 27 fecal samples of wildebeest (10 in dry and 17 in wet season) from 22 groups in Lapalala, and 27 samples (9 in dry and 18 in wet season) from 23 groups in Welgevonden for a total of 54 samples for this species.

We lyophilized the fecal samples for 72 h and pulverized them using a pestle and mortar in order to remove solid inert matter, such as seeds and fibrous dietary material (Heistermann et al., 1993). A sample of about 0.010–0.011 g of dry powder was extracted by vortexing for 15 min with 80% ethanol in water (3 ml). Afterwards, the suspension was centrifuged at 1,500 g for 10 min and the supernatant decanted into Eppendorf tubes, which were stored at −20°C until hormone analysis. Immunoreactive fecal glucocorticoid metabolite (fGCM) concentrations were measured from the fecal extracts of Impala and Wildebeest samples using enzyme immunoassays (EIAs). For impala we used an antibody detecting fGCMs with a 5β-3α-ol-11-one structure (3α,11oxo-CM) previously described by Möstl and Palme (2002), and for wildebeest an antibody detecting 11,17 dioxoandrostanes (11,17-DOA) previously described by Palme and Möstl (1997). The sensitivity of the 11,17-DOA and 3α,11oxo-CM EIAs was 0.6 ng/g dry weight, respectively. Intra-assay coefficient of variation of high- and low-value quality controls were 3.05 and 5.71% for the 11,17-DOA EIA, and 5.27 and 5.76% for the 3α,11oxo-CM EIA, respectively. Inter-assay coefficient of variation of high- and low-value quality controls were 3.84 and 6.59% for the 11,17-DOA EIA, and 10.39 and 12.15% for the 3α,11oxo-CM EIA. These antibodies were selected because they provided the strongest species-specific response in fGCM concentrations during physiological validation using ACTH challenge tests. Respective validation experiments have been described elsewhere for wildebeest (de Haast, 2016), whereas the ACTH challenge test for impala is described below. We expressed fGCM concentrations as μg/g dry of weight extracted fecal material. All fecal samples were analyzed at the Endocrine Research Laboratory, University of Pretoria, South Africa.

ACTH Challenge Test for Impala

We physiologically validated an assay for the measurement of fGCMs in impala by conducting an adrenocorticotrophic hormone (ACTH) challenge test (Touma and Palme, 2005). The tests were done on three adult impala males located in standard hospital enclosures at the National Zoological Gardens (NZG) of South Africa, Pretoria. They were housed singly but adjacent to conspecifics. The animals were injected with 0.5–1.0 IU/kg of Synacthen depot (Novartis, Australia) intramuscularly via remote injection (Daninject 1.5 or 3 ml dart). A 20 × 1.5 mm barbless needle was attached to the darts, which fell out within 1–2 h. Fecal samples were collected daily from each individual for 5–7 days prior to ACTH administration and 72 h post-injection and 1–2 samples per day during the following 3–4 days. We collected respective sample material from the center of a dropping to avoid cross-contamination with urine or contamination with other nearby scats. A portion of 5–10 g was homogenized and stored in individual collection vials and frozen immediately upon collection at −20°C. We used the same extraction protocol as described above (2.4). Using partly a reduced sample set, we evaluated EIAs detecting (i) 11,17-dioxoandrostane (11,17-DOA), (ii) fGCMs with a 5β-3α-ol-11-one structure (3α,11oxo-CM), as well as (iii) a cortisol and ivcorticosterone EIA. For more details on these EIAs, see Palme and Möstl (1997) for the corticosterone, cortisol, and 11,17-DOA assays, and Möstl and Palme (2002) for the 3α,11oxo-CM assay. Following the experiment, the animals were given access to join each other for the acclimatization period before being loaded and returned to their standard enclosures.

Of the four different assays tested, the 3α,11oxo-CM assay detected the strongest response and was regarded as the most appropriate assay for impala. This assay showed post-injection peak concentrations of 232% (animal 1), 936% (animal 2), and 936% (animal 3) above baseline (**Figure S1A**). In terms of performance, this assay was followed by the 11,17-DOA EIA (animal 1: 142%; animal 2: 597%; animal 3: 338%; **Figure S1B**). Peak values for both of these assays occurred about 20 h post-injection. The corticosterone EIA showed a less pronounced increase (animal 1: 104%; animal 2: 138%; animal 3: 214%) with no consistent peak in time (**Figure S1C**), whereas the cortisol EIA showed inconsistent results with no increase post-injection detected in samples from animal 1 (96%) and only low increases in the other two individuals (animal 2: 163%; animal 3: 119%, **Figure S1D**).

Isotope Analyses

For the isotope analyses, fecal material was oven dried at 37°C for at least 24 h, all traces of surface contaminants removed and then pulverized with a mortar and pestle (Miranda et al., 2014). All equipment was cleaned with 70% ethanol between samples. Aliquots of 1.0–1.1 mg of fecal powder were weighed into tin capsules that had been pre-cleaned with toluene. Isotope analysis was done on a Flash EA 1112 Series coupled to a Delta V Plus stable light isotope ratio mass spectrometer via a ConFlo IV system (all equipment supplied by Thermo Fischer, Bremen, Germany). Fecal stable isotope analyses were conducted at the UP Stable Isotope Laboratory at the Mammal Research Institute, University of Pretoria, South Africa. Carbon isotope ratios are referenced to Vienna Pee-Dee Belemnite, whereas nitrogen isotope values reference to atmospheric N_2. Results are expressed in delta notation with a per mill (‰) scale using the

standard equation:

$$\delta X(‰) = [(R_{sample}/R_{standard}) - 1] \times 1,000 \quad (1)$$

where X = ^{13}C or ^{15}N and R represents ^{13}C/^{12}C or ^{15}N/^{14}N, respectively.

Two laboratory running standards (Merck Gel: $\delta^{13}C = -20.26‰$, $\delta^{15}N = 7.89‰$, C% = 41.28, N% = 15.29) & (DL-Valine: $\delta^{13}C = -10.57‰$, $\delta^{15}N = -6.15‰$, C% = 55.50, N% = 11.86) and a blank sample were run after every 11 unknown samples. Data corrections were performed using results for the Merck Gel standard for each run. The results for the DL-Valine standard provided the precision for each run, which was <0.08‰ for both carbon and nitrogen, respectively.

Data Analysis

For all analyses described below, separate models were run for each species. These models included the presence or absence of lions (i.e., reserve), season, their interaction effect as well as the main effects of group size, the number of juveniles, age and sex as fixed terms, except for models on scan data where we did not fit effects of sex and age. They also included group identity and year as random terms. We used generalized linear mixed-effects models with a binomial error and a logit link to evaluate the effect of the presence of lions and season on the proportion of time animals were engaged in foraging and vigilance as well as on the proportion of animals engaged in foraging and vigilance. For the models on the focal data, we regarded each second as a binomial variable that could be spent doing the behavior in question (i.e., foraging or being vigilant) or not (Dalerum and Belton, 2015; Périquet et al., 2017). We therefore added the number of seconds an animal spent foraging and vigilant in relation to the number of second the animals were not foraging and vigilant as response variables for each individual, and the number of foraging and vigilant animals in relation to the total number of observed animals estimated from the scan data as response variables for each group. We used generalized linear mixed-effects models with a Poisson error and a log link to evaluate the effect of the presence of lions and season on the number of vigilance bouts. These models had the number of vigilance bouts recorded for each focal observation as response observation length as an offset to correct for unequal length of the different observation periods. For all models on behavior, we initially used distance to cover as an additional fixed co-variate. However, since this did not approach significance for any model ($p > 0.18$ for all models), we did not retain distance to cover in the final set of analyses. We used mixed linear models to evaluate the effects of presence of lions and season on fGCM concentrations. In these models, the fGCM concentrations were log-transformed to account for heteroscedasticity before being used as a response variable. Similarly, we evaluated the effects of presence of lions and season on isotope values and isotope niche breadth using mixed linear models. Raw values were used a response variable for the models on δ^{13}C, whereas log transformed values were used as response variables for the model on δ^{15}N for wildebeest and the model on estimates of isotope niche breadth (see below) for impala. Isotope niche breadth was quantified for each species and season as the mean Euclidian distance from each sample to the group centroid of each reserve and season in a two-dimensional isotope space formed by δ^{13}C and δ^{15}N values (Dalerum et al., 2012). For all analyses, we treated an alpha error of 0.05 as a threshold of statistical significance. Statistical analysis was conducted in R version 3.4.4 for Linux (http://www.r-project.org) using the nlme (Pinheiro et al., 2018) and MASS packages (Venables and Ripley, 2002).

Animal Ethics and Institutional Approval

The study was performed with approval of the Animal Use and Care Committee of University of Pretoria (AUCC, Reference EC17-12) the Ethics and Scientific Committee of the National Zoological Gardens of South Africa, Pretoria (Reference # PlO/33).

RESULTS

Foraging Behavior

Based on our focal observations, the presence of lions did not seem to influence the proportion of time that either impalas ($\beta = 0.03$, $t_{49} = 0.39$, $p = 0.700$) or wildebeests ($\beta = 0.07$, $t_{23} = 0.67$, $p = 0.508$) spent foraging (**Figure 2A**), nor did effects of lions differ between seasons (impala: $\beta = 0.09$, $t_{49} = 0.79$, $p = 0.436$; wildebeest: $\beta = 0.21$, $t_{23} = 1.07$, $p = 0.256$). Similarly, there were no differences between the two seasons in the proportion of time spent foraging for either impala ($\beta = 0.05$, $t_{49} = 0.91$, $p = 0.367$) or wildebeest ($\beta = 0.03$, $t_{23} = 28$, $p = 0.780$). Group size was positively associated to the amount of time spent foraging in impala, but not the number of juveniles, whereas neither group size nor sex influenced the proportion of time foraging in wildebeest (**Table 1**). Sub-adult animals spent less time foraging compared to adult individuals for both impala and wildebeest, but there were no effects of sex for either species (**Table 1**).

Based on our scan observations, the presence of lions did not seem to influence the proportion of impalas that were engaged in foraging ($\beta = 0.00$, $t_{39} = 0.05$, $p = 0.962$), nor did any such effects differ between seasons ($\beta = 0.09$, $t_{39} = 0.69$, $p = 0.49$). For wildebeest, however, there was a seasonal difference in the effects of lions ($\beta = 0.25$, $t_{24} = 2.08$, $p = 0.048$), with more animals being engaged in foraging in the presence of lions during the wet, but not the dry season (**Figure 3A**). Neither group size nor the number of juveniles influenced the proportion of animals engaged in foraging for either species (**Table 1**).

Vigilance Behavior

Based on our focal observations, the presence of lions did not seem to influence the proportion of time that impalas ($\beta = 0.29$, $t_{49} = 0.92$, $p = 0.364$) or wildebeests ($\beta = 0.74$, $t_{23} = 1.50$, $p = 0.146$) spent being vigilant (**Figure 2B**), nor were there any seasonal differences (impala: $\beta = 0.04$, $t_{49} = 0.10$, $p = 0.921$; wildebeest: $\beta = 1.78$, $t_{23} = 1.80$, $p = 0.084$). However, impala spent less time being vigilant in the wet than in the dry season ($\beta = 0.46$, $t_{49} = 2.18$, $p = 0.034$), but there were no seasonal differences for wildebeest ($\beta = 0.32$, $t_{23} = 0.72$, $p = 0.476$, **Figure 2B**). The proportion of time spent vigilant was negatively

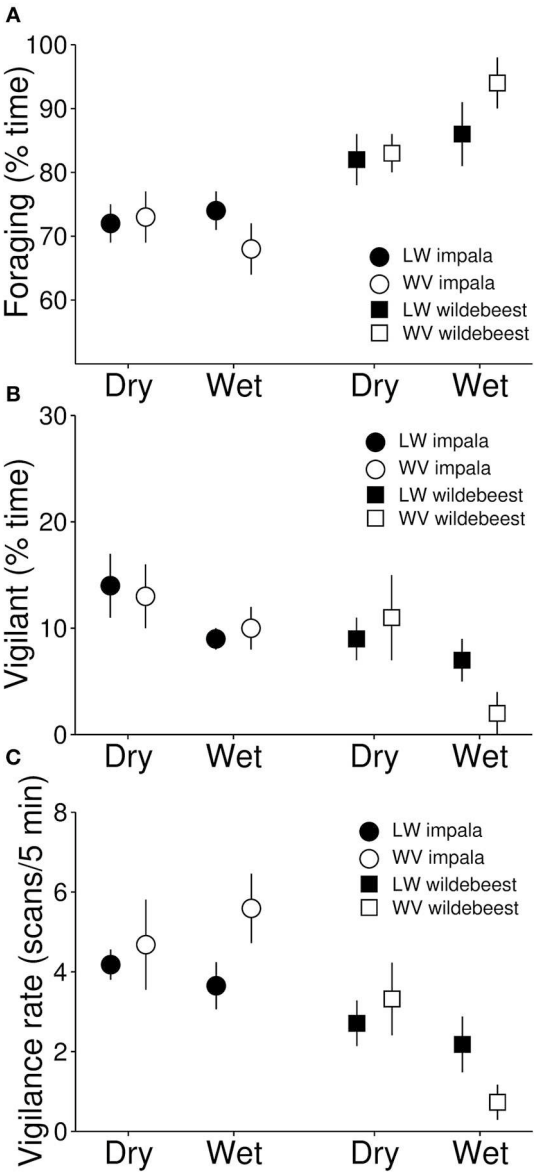

FIGURE 2 | Proportion of time spent foraging **(A)**, proportion of time spent vigilant **(B)** and vigilance scan rates (scans/5 min observation period; **C**) calculated from a total of 413 individuals during 5 min focal observations conduced on 42 groups of impala and 29 groups of wildebeest in Lapalala and 16 groups of impala and 14 groups of wildedebeest in Welgevonden. The figure represents means of group means ± 1 SE.

associated to group size in impala, but not in wildebeest and there was no effect of the number of juveniles for either species (**Table 1**). Sub-adult impalas spent more time being vigilant than adults, while age did not affect the proportion of time being vigilant in wildebeest. Similarly, there were no effects of sex on the proportion of time spent vigilant for either species (**Table 1**).

Similarly based on the focal observations, the presence of lions did not seem to influence vigilance scan rate in either impala ($\beta = 0.09$, $t_{49} = 0.41$, $p = 0.68$) or in wildebeest ($\beta = 0.58$, $t_{23} = 1.48$, $p = 0.152$), nor did season influence any such effects (impala: $\beta = 0.43$, $t_{49} = 1.56$, $p = 0.125$; wildebeest: $\beta = 1.44$, $t_{23} = 1.76$, $p = 0.092$, **Figure 2C**). Similarly, there were no differences between seasons for either impala ($\beta = 0.23$, $t_{49} = 1.49$, $p = 0.144$) or wildebeest ($\beta = 0.04$, $t_{23} = 1.12$, $p = 0.909$). Group size was negatively associated while the number of juveniles was positively associated with vigilance scan rate in impala but not in wildebeest, whereas neither age nor sex was associated with vigilance scan rate of either species (**Table 1**).

Based on the scan observations, the presence of lions did not seem to influence the proportion of either impala ($\beta = 0.25$, $t_{39} = 0.71$, $p = 0.479$) or wildebeest ($\beta = 0.16$, $t_{24} = 0.48$, $p = 0.632$) that were vigilant, nor did seasons influence any such effects (impala: $\beta = 0.16$, $t_{39} = 0.35$, $p = 0.782$; wildebeest: $\beta = 27.96$, $t_{24} = 0.00$, $p = 0.999$, **Figure 3B**). Group size was negatively associated with proportion of animals being vigilant for impala but not for wildebeest, whereas the number of juveniles was negatively associated to the proportion of animals being vigilant for wildebeest but not for impala (**Table 1**).

Stress Hormone Levels

The presence of lions did not influence fGCM concentrations in samples from either impalas ($\beta = 0.27$, $t_{35} = 0.93$, $p = 0.358$, **Figure 4A**) or wildebeests ($\beta = 0.14$, $t_{35} = 0.46$, $p = 0.650$, **Figure 4B**), nor did season influence any such effects (impala: $\beta = 0.20$, $t_{35} = 0.58$, $p = 0.563$; wildebeest: $\beta = 0.16$, $t_{35} = 0.42$, $p = 0.679$). Similarly, there were no differences between seasons for either impala ($\beta = 0.26$, $t_{35} = 1.03$, $p = 0.310$) or wildebeest ($\beta = 0.31$, $t_{35} = 1.14$, $p = 0.262$). Group size did not influence fGCM concentrations in either species, but the number of juveniles was positively associated with fGCM concentrations in impala but not in wildebeest (**Table 2**). Neither age nor sex influenced fGCM concentrations.

Stable Isotope Values

Reference Plant Values

Grasses were enriched in ^{13}C compared to shrubs, which correspond to plants following a C4 pathway. Conversely, we observed lower $\delta^{13}C$ values in shrubs, which is characteristic of a C3 photosynthesis pathway. Plant $\delta^{15}N$ values had lower inter specific variation than what was observed for $\delta^{13}C$, but grass had slightly lower values compared to shrubs (**Table S1**).

Carbon Isotope Values in Ungulate Feces

The presence of lions did not influence $\delta^{13}C$ values in feces from either species (impala: $\beta = 1.24$, $t_{53} = 1.15$, $p = 0.255$; wildebeest: $\beta = 0.01$, $t_{35} = 0.60$, $p = 0.553$), nor was there a seasonal variation of any such effects (impala: $\beta = 0.83$, $t_{53} = 0.66$, $p = 0.51$; wildebeest: $\beta = 0.01$, $t_{35} = 0.30$, $p = 0.766$). However, samples from both impala ($\beta = 4.28$, $t_{53} = 6.42$, $p < 0.001$) and wildebeest feces ($\beta = -0.06$, $t_{35} = 3.29$, $p = 0.002$) were enriched in ^{13}C in the wet compared to the dry season. Values for impala reflected a mixed-strategy during the dry and a grazing feeding strategy during the wet season whereas wildebeest values for both seasons conformed to a strict grazing feeding strategy (**Figure 5A**). Neither group size, the number of juveniles nor sex influenced $\delta^{13}C$ values, but sub-adult wildebeest had lower $\delta^{13}C$ values than adults (**Table 2**).

TABLE 1 | Estimated effects of covariates related to group (group size, number of juveniles) and individual (age, sex) characteristics on proportion of time spent foraging and being vigilant, vigilance scan rate, proportion of animals engaged in foraging and proportion of animals engaged in vigilance.

	Time spent foraging				Time spent vigilant				Vigilance scan rate				Proportion of animals foraging				Proportion of animals being vigilant			
Covariate	β	*t*	df	*p*	β	*t*	df	*p*	β	*t*	df	*p*	β	*t*	df	*p*	β	*t*	df	*p*
IMPALA																				
Group size	0.01	2.10	184	0.037	−0.04	3.15	184	0.002	−0.02	3.15	184	0.002	0.00	0.96	221	0.340	−0.03	2.45	221	0.015
Number of juveniles	0.00	0.31	49	0.756	0.07	1.35	49	0.183	0.08	2.59	49	0.013	−0.03	0.85	39	0.398	0.10	0.73	39	0.470
Age class[a]	−0.31	4.28	184	0.001	0.61	3.51	184	>0.001	0.19	1.32	184	0.187								
Sex[b]	0.00	0.08	184	0.939	−0.05	0.33	184	0.740	−0.11	0.93	184	0.356								
WILDEBEEST																				
Group size	0.01	1.54	23	0.137	−0.04	1.95	23	0.064	−0.03	1.86	23	0.080	0.00	0.18	24	0.860	−0.01	1.06	24	0.301
Number of juveniles	0.01	0.94	23	0.358	−0.09	1.23	23	0.231	−0.06	0.96	23	0.347	0.01	1.37	24	0.181	−0.09	2.39	24	0.020
Age class[a]	−0.53	2.08	23	0.049	1.76	1.86	23	0.070	0.93	1.15	23	0.263								
Sex[b]	0.05	0.55	84	0.584	−0.24	0.60	84	0.548	−0.13	0.37	84	0.712								

Estimated coefficients are from generalized mixed linear models, and reflect main effects that are estimated across animals living both with and without lions.

[a] *Nominate level was "adult."*

[b] *Nominate level was "female."*

Nitrogen Isotope Values in Ungulate Feces

Feces from both impala ($\beta = 1.32$, $t_{53} = 2.18$, $p = 0.034$) and wildebeest ($\beta = 0.94$, $t_{35} = 3.86$, $p < 0.001$) that were living with lions had higher δ^{15}N values than feces from animals living in a lion free environment (**Figure 5B**). However, there were no seasonal differences in these effects for either species (impala: $\beta = 0.64$, $t_{53} = 0.90$, $p = 0.370$; wildebeest: $\beta = 0.22$, $t_{35} = 0.46$, $p = 0.651$), although feces from the wet season had higher δ^{15}N values than feces from the dry season for animals from both environments (impala: $\beta = 0.88$, $t_{53} = 2.32$, $p = 0.024$; wildebeest: $\beta = 1.35$, $t_{35} = 3.86$, $p < 0.001$). Neither group size, the number of juveniles, age nor sex influenced δ^{15}N values (**Table 2**).

Isotope Niche Breadth Estimated From Isotope Values in Ungulate Feces

The presence of lions did not influence isotope niche breadth for either species (impala: $\beta = 0.36$, $t_{53} = 1.22$, $p = 0.23$; wildebeest: $\beta = 0.42$, $t_{35} = 1.20$, $p = 0.24$), nor was there a seasonal variation of any such effects (impala: $\beta = 0.48$, $t_{53} = 1.39$, $p = 0.169$; wildebeest: $\beta = 0.33$, $t_{35} = 0.73$, $p = 0.469$, **Figure 5C**). Similarly, there were no differences between seasons in isotope niche breadth (impala: $\beta = 0.00$, $t_{53} = 0.01$, $p = 0.989$; wildebeest: $\beta = 0.52$, $t_{35} = 1.66$, $p = 0.107$). Neither group size, the number of juveniles, age nor sex influenced isotope niche breadth (**Table 2**).

DISCUSSION

Despite strong claims of the ecological consequences of indirect predation effects (e.g., Preisser et al., 2005; Creel and Christianson, 2008), our observations did not suggest that impala and wildebeest living in the presence of lions foraged less or were more vigilant than impala and wildebeest living in a lion free environment. Similarly, there were no differences between the two species with respect to the influence of lions on their fGCM concentrations. We interpret these results as support for recent suggestions that the consequences of predation risk may be highly context dependent or not necessarily present at the landscape level (e.g., Périquet et al., 2010; Middleton et al., 2013). For instance, a parallel study in our study system did not find uniform effects of lion presence on zebras, but rather that that seasonal variation in food supply and vegetation dictated how lions influenced zebra's vigilance behavior and stress physiology (Périquet et al., 2017). We suggest that anti-predatory responses in many, if not most, predator-prey systems may be influenced by spatial and temporal variation in immediate predation risk, resource availability, and life history characteristics (Bolnick and Preisser, 2005; Boonstra, 2013; Courbin et al., 2016).

Despite a general lack of influence of lion presence on the behavior and stress physiology of impala and wildebeest, we found that the feces from animals in Welgevonden (where lions were present) had lower δ^{15}N values than feces from animals in Lapapala (which was lion free). Since soil ^{15}N often is associated with higher productivity, and this is expected to be reflected in plant and also herbivore fecal nitrogen isotope values (Miranda et al., 2014), this finding may indicate that both impala and

TABLE 2 | Estimated effects of covariates related to group (group size, number of juveniles) and individual (age, sex) characteristics on fecal glucocorticoid metabolite (fGCM) concentrations, $\delta^{13}C$, $\delta^{15}N$, and isotope niche breadth in impala and wildebeest.

	fGCM				$\delta^{13}C$ (‰)				$\delta^{15}N$ (‰)				Isotope niche breadth			
Covariate	**β**	***t***	**df**	***p***	**β**	***t***	**df**	***p***	**β**	***t***	**df**	***p***	**β**	***t***	**df**	***p***
IMPALA																
Group size	−0.01	0.18	35	0.860	0.02	0.03	53	0.561	0	0.15	53	0.880	0.01	0.38	53	0.704
Number of juveniles	0.04	2.41	35	0.022	−0.06	0.88	19	0.392	−0.06	1.67	19	0.110	−0.02	1.05	19	0.307
Age class[a]	0.32	0.89	35	0.389	0.25	0.35	19	0.728	−0.38	0.14	19	0.270	0.12	0.46	19	0.625
Sex[b]	0.13	0.78	35	0.448	0.32	0.64	19	0.526	0.24	0.97	19	0.343	−0.14	0.89	19	0.385
WILDEBEEST																
Group size	−0.01	0.87	35	0.391	−0.01	0.03	35	0.977	−0.01	0.56	35	0.581	0.01	0.81	35	0.435
Number of juveniles	−0.01	0.06	35	0.950	−0.01	1.17	3	0.327	0.01	0.24	3	0.823	−0.01	0.28	3	0.792
Age class[a]	0.48	1.36	4	0.246	−0.03	3.04	3	0.056	0.22	0.50	3	0.651	−1.04	2.71	3	0.073
Sex[b]	0.44	2.30	4	0.083	−0.01	0.95	3	0.412	−0.19	0.76	3	0.502	−0.11	0.510	3	0.64

Estimated coefficients are from mixed linear models, and reflect main effects that are estimated across animals living both with and without lions.

[a] *Nominate level was "adult."*

[b] *Nominate level was "female."*

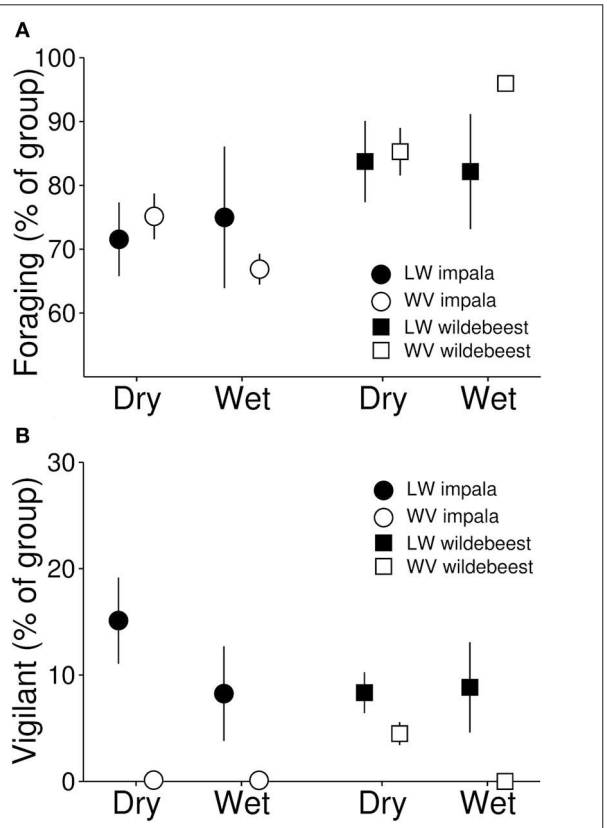

FIGURE 3 | Proportion of individuals engaged in foraging **(A)** and vigilance behavior **(B)** recorded during 335 scan observations on 33 groups of impala and 22 groups of wildebeest in Lapalala and 15 groups of impala and 11 groups of wildebeest in Welgevonden. Scan recordings were performed with 1-min intervals for 5 min. The figure represents means of group means ± 1 SE.

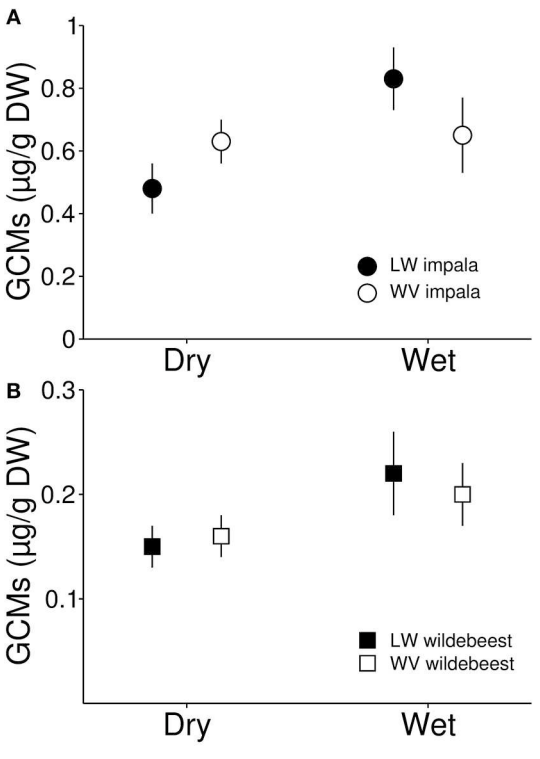

FIGURE 4 | Fecal glucocorticoid metabolite concentrations in impala **(A)** and wildebeest **(B)** living in Lapalala and Welgevonden during the dry and wet season. Data comes from 119 individual samples collected from 31 groups of impala and 24 groups of wildebeest in Lapalala and from 14 groups of impala and 26 groups of wildebeest in Welgevonden. The figure represents means of group means ± 1 SE.

wildebeest may have been utilizing habitats with lower primary productivity and less grazing pressure in Welgevonden compared to Lapalala. This could reflect a habitat choice were both species preferred areas that are less productive, but potentially safer when lions are present, although we cannot rule out that regional differences in plant $\delta^{15}N$ could partly explain the observed

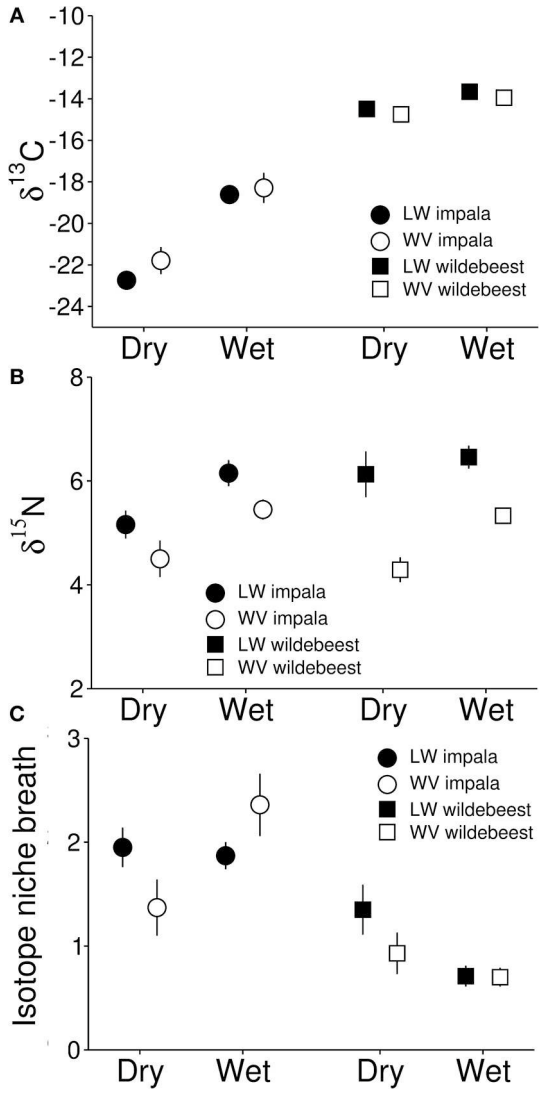

FIGURE 5 | Values of $\delta^{13}C$ **(A)**, $\delta^{15}N$ **(B)**, and isotope niche breadth **(C)** from fecal samples of impala and wildebeest in the two reserves during dry and wet seasons. Isotope niche breath was quantified as the Euclidean distance of each sample to group centroids in a two-dimensional isotope space created by $\delta^{13}C$ and $\delta^{15}N$ values. Figures present the mean of 46 groups of impala (containing a total of 59 individuals) and 25 groups of wildebeest (containing a total of 29 individuals) collected in Lapalala, and 17 groups of impala (containing a total of 26 individuals) and 23 groups of wildebeest (containing a total of 27 individuals) collected in Welgevonden. The figure represents means of group means ± 1 SE.

differences as well. In both Welgevonden and Lapalala the most productive habitat tends to be associated with restricted patches in the valley bottoms, and the utilization of these areas in the presence of lions could incur elevated predation risks (e.g., Thaker et al., 2011). Such predation-induced habitat shifts have been previously observed (Caldwell, 1986; Creel et al., 2005), and recent studies have shown that ungulates may use immediate cues about predation risk when selecting where to forage (Valeix et al., 2009; Creel et al., 2014). Although the $\delta^{15}N$ data suggests that the presence of lions may have influenced the habitat use of impala and wildebeest, the $\delta^{13}C$ values indicate that they did not alter their broad foraging strategies. Combined, these results points to complex and inconsistent influences of predation risk on the foraging ecology of potential prey, where some aspects of their foraging strategies may be influenced by predation risk. Such inconsistencies have ramifications for evaluating the demographic and evolutionary consequences of living under the risk of predation that warrants further attention.

Our results suggest that group characteristics (i.e., group size and composition) may have been more influential than individual characteristics (age and sex) on foraging and vigilance behavior. We argue that these results point to context dependent anti-predatory responses, where the immediate social environment combined with the intensity of predation risk and other environmental factors determine individual anti-predatory behavior. This is not a novel suggestion (Elgar, 1989; Lima and Dill, 1990), and several mechanisms have been put forward to explain how the social context may influence individual responses to predation risk (Welty, 1934; e.g., dilution and confusion effects: Hamilton, 1971; the "many eyes" hypothesis: Pulliam, 1973). However, African predators have both age and sex preferences when targeting prey (Kruuk, 1972; Schaller, 1976; FitzGibbon, 1990; Creel and Creel, 2002). The lack of strong general effects of age class and sex is therefore puzzling, but subtle adjustments of individual predation risk within the social groups, for instance by shifting to more central positions within the herds (Morrell et al., 2010) or by fine tuning anti-predatory responses to immediate risk (Lima and Bednekoff, 1999) may have been contributing factors.

We acknowledge several caveats to this study. First we appreciate the potential for a lack of spatial replication and the potential for temporal pseudoreplication. We did not have replicates of reserves with and without lions, which obviously limits the statistical sample size to one at a landscape level (Hurlbert, 1984). However, we still suggest that our results can provide useful insights into prey ecology and their proximity to an apex predator, although we acknowledge that more data are needed before our interpretations can be applied to different geographic areas and ecological systems. In addition, we appreciate that the lions had only been present for 10–14 ears in Welgevonden at the time of our study. Although observations from the northern hemisphere suggest a low retention of anti-predatory behavior (Berger, 2007), such lack of retention appears not to be uniform (Hettena et al., 2014). This agrees with evolutionary arguments for the retention of anti-predatory behaviors in multi-predator environments (Blumstein, 2006), such as the one in South Africa (Dalerum et al., 2008b). We therefore regard it as likely that the animals in Welgevonden were exhibiting the same range of anti-predatory behavior to that of populations that had not experienced an ecological period without lions. We also did not work with marked groups and individuals, so that we cannot rule out that we sampled the same individuals in different years or seasons. However, we sampled a very small portion of the total populations, and we therefore regard such repeated sampling relatively unlikely. We did not distinguish routine vs. intense vigilance. Animals usually keep eating during routine vigilance, while intense vigilance constrains food intake as chewing is arrested (Périquet et al., 2012). We

can therefore not rule out qualitative alterations in foraging and vigilance behavior. Finally, although Lapalala was a lion-free environment, the presence of other predators, such as wild dog, brown hyena and leopard (Dalerum and Belton, 2015) may have balanced the anti-predator responses of prey in several ways due to their diverse hunting techniques (Schmitz, 2008).

Despite these caveats, we highlight that our data set comprised data collected over several years, and we found consistent results across several different data types. We therefore argue that our study provided clear observations that the additions of lions to a reserve did not have any substantial effects on the behavior, physiology and foraging ecology of these two common African ungulates. However, stable isotope data suggested a potential habitat shift in the presence of lions, with could have involved animals utilizing areas of lower productivity. Our results provide support for context dependent, complex and potentially conflicting responses of anti-predatory responses to apex predator presence. We suggest that further research is needed to identify at what scales anti-predator responses occur, at what spatial and temporal scales they have ecological and demographic effects, and how spatial and temporal variation in environmental conditions interact with prey life history in shaping indirect predation effects.

DATA AVAILABILITY STATEMENT

The dataset for this study is available in figshare (https://figshare.com, doi: 10.6084/m9.figshare.7334261).

AUTHOR CONTRIBUTIONS

MC contributed to field data collection, laboratory analyses, data analyses, and wrote the initial drafts of the manuscript. LB contributed to field data collection and analyses of behavioral data. AG was responsible for analyses of endocrine samples. IG contributed to field data collection and laboratory analyses. GH was responsible for analyses of stable isotope samples. LS contributed to field data collection. FD conceptualized the study, coordinated and conducted field data collection, coordinated laboratory analyses, designed and conducted data analyses, and wrote parts of the manuscript. All authors contributed to complete the final draft of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2018.00191/full#supplementary-material

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Predator Cues Increase Silkmoth Mortality

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While prey responses to predators reduce the threat of consumption, the physiological costs of these responses can be considerable. This is especially true for organisms that lack effective anti-predator defenses and must rely on camouflage or mimicry for protection. The luna moth, *Actias luna*, is a large saturniid native to Eastern North America that is preyed on and parasitized by a wide variety of predators and parasitoids. We report the results of two separate experiments assessing the responses of *Actias* larvae to predatory wasps (*Vespula maculifrons*) that were rendered non-lethal but remained able to move freely, as well as in a control (wasp-free) treatment. We determined whether these responses were predator-specific by also testing the response of *Actias* larvae to a similarly-sized but harmless scavenging fly. In both experiments, (A) *Actias* larvae in the wasp treatment died at a higher rate than those in the control treatments; and (B) larval survival in the fly and control treatments did not differ. Despite similar *Actias* survival in the fly and control treatments, fly-treatment larvae that died appeared to respond similarly to flies as other larvae did to wasps. In both years, larvae that died in the fly and wasp treatments gained virtually no weight between the start of the experiment and their death, suggesting that they may have succumbed to starvation. Our results, replicated over 2 years, illustrate the high cost of anti-predator responses and are the first report of lethal risk effects in caterpillars.

Keywords: *Actia luna*, *Vespula maculifrons*, predation risk, predation, non-consumptive effect, anti-predator behavior

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INTRODUCTION

Predation risk can affect various aspects of prey behavior and physiology (Lima and Dill, 1990; Werner and Peacor, 2003; Adamo, 2012; Sheriff and Thaler, 2014). These changes are generally seen as adaptive since they reduce the likelihood of capture and/or consumption. Because they require prey to alter their investment in activities such as foraging, however, these changes can also negatively affect growth, physical condition, fecundity, and, in the most extreme cases, survival (Zanette et al., 2011; Siepielski et al., 2014; Duong and Mccauley, 2016). Exposure to predator cues decrease tadpole survival (Hettyey et al., 2015), for instance, and have a similar effect on dragonflies (McCauley et al., 2011) and grasshoppers (Schmitz et al., 1997).

Lepidopteran larvae are important terrestrial herbivores attacked by a wide variety of predators and parasitoids. Because they are slow-moving and lack a hard exoskeleton, they often rely on camouflage to avoid detection (Lichter-Marck et al., 2015). Foraging-related movement greatly increases caterpillar predation risk (Bernays, 1997), and camouflaged species may be especially likely to forego feeding in response to risk (Ruxton et al., 2004). Even individuals that resume feeding may suffer from the combined impact of reduced energy intake and the physiological costs of stress responses (Sheriff and Thaler, 2014).

The luna moth *Actias luna* (Lepidoptera: Saturniidae; *Actias* hereafter) is a silkmoth native to eastern North America (Wagner, 2005). The solitary larvae of this species require 3–6 weeks to mature, during which time camouflage provides their primary protection against predators and parasitoids (Tuskes et al., 1996). [Sourakov (2018); p. 488] characterized *Actias* defenses against predation as "…relying mostly on cryptic colouration and being motionless when not feeding," although they possess spines that may reduce their vulnerability to vertebrate predators (Sourakov, 2018). Vespid wasps (Hymenoptera: Vespidae) are generalist predators that hunt caterpillars (Stamp and Bowers, 1988; Lichtenberg and Lichtenberg, 2003); we have repeatedly observed yellowjacket wasps (*Vespula maculifrons*; wasps hereafter) attacking and killing *Actias* larvae in the field (A. Baranowski, *personal observation*).

We conducted two separate experiments measuring the survivorship of *Actias* caterpillars in the presence of wasps rendered unable to either sting or bite. We determined whether these responses were predator-specific by also exposing caterpillars to (1) wasp-sized scavenging flies that had been similarly treated; and (2) a no-insect control. We hypothesized that survivorship would be lowest in the wasp treatment, and higher in both the fly and no-insect treatments.

METHODS

2016 Experiment

In July 2016, a newly-emerged captive female Actias luna was mated to a wild male at East Farm (Kingston RI), an agricultural research facility managed by the University of Rhode Island. Eggs were incubated in a 473 ml polypropylene deli cup (Pactiv brand, Lake Forest, IL) and began hatching 10 days later. Hatchlings were offered hickory (Carya glabra) foliage, with waste removed and new foliage added as needed. Larvae were reared communally within the cup until the 2nd instar. To prevent overcrowding, larvae were then transferred to a 6 l polypropylene bin (Sterilite brand, Townsend, MA) until they averaged 1.5 cm in length, when they were transferred to a 12 l bin. Twenty-six days after hatching, 54 larvae were individually weighed (mean 0.673 ± 0.045 [SE] g) and transferred into individual 6 l bins containing hickory foliage kept hydrated using water-filled floral tubes. Once all larvae had been transferred, each bin was randomly assigned to one of three 18-bin treatment groups: a predator that had been rendered non-lethal, a similarly-treated harmless detritivore, and no-insect control.

Bins in the non-lethal predator ("wasp") treatment each contained a single adult *V. maculifrons* collected from either flowers or overripe fruit; prior to the experiment, we had repeatedly observed wasps attacking and dismembering free-living *Actias* larvae. Captured wasps were first anesthetized by brief chilling in a freezer; when the adults were motionless, we applied one drop of UV-bonded plastic (Bondic brand, Aurora, ON) to both the mandible and stinger. Once each drop was applied, we immediately hardened it via exposure to a UV light. This procedure rendered each wasp "non-lethal," alive and mobile but unable to either sting or bite potential prey; their non-lethal nature was confirmed via our repeated handling of test specimens with no stings or bites. We explored whether our addition of the glue affected wasp behavior via a pilot experiment in which we visually assessed the behavior of non-lethal and lethal wasps added singly to plastic bins. When resting, the non-lethal wasps spent more time grooming their mandibles than the lethal wasps; there were no other noticeable differences in time of flight or exploratory behavior. The wasp in each bin was checked daily and replaced with a new wasp when it died.

Bins in the harmless detritivore ("fly") treatment each contained a single adult scavenging fly (families Caliphoridae and Sarcophagidae), of similar size as the wasps, collected from trash or reared from eggs. As with the wasps, we added UV-bonded plastic to the mouthparts and terminal abdominal segment of each fly. Each fly was handled the same way we handled the wasps. Flies were checked daily and replaced with a new one as needed.

Bins in the no-insect ("control") treatment each received a single section of bamboo toothpick of wasp length, with one dot of UV-bonded plastic dots added to the end. Each bamboo toothpick was replaced every 2 days to simulate the level of disturbance received by the other two treatments.

After the experiment started, each bin was checked daily; food was replaced, waste removed, and treatments renewed as necessary. Larvae were weighed weekly and at either pupation or death; time (days) to either event was recorded for each larva. Each treatment was replicated 18 times for a total of 54 larvae.

2017 Experiment

To ensure that our results were robust, we repeated the experiment in 2017. The two experiments were identical except for the following differences. We collected eggs from three pairings (=broods) of different captive Actias females with wild males; eggs and larvae from the three different broods were held in separate containers. Brood one larvae hatched on July 13–18, brood two larvae hatched on August 3, and brood three larvae hatched on August 8. Larvae were reared on Juglans nigra throughout the experiment because of greater vegetation availability, and cut foliage was kept hydrated using microcentrifuge tubes filled with agar water (3 g/L agar:water). This latter procedure kept foliage fresh while preventing floral tube leakage. Larvae reaching their 3rd instar were transferred in groups of 25 to individual 6 l bins to prevent overcrowding. Data collection for experiment #2 was the same as for experiment #1, with the exception that the brood identity of each larva was recorded. A total of 86 larvae were used in the experiment, with each treatment replicated 28–29 times. Of these larvae, 21 larvae were from brood one ($n = 7$/treatment), 40 from brood two ($n =$ 13–14), and 25 from brood three ($n = 7$–9).

Statistical Analysis

For experiment #1, we analyzed treatment-level differences in larval outcome [died, pupated] by fitting a GLM with a binomial distribution and logit link (maximum likelihood estimation method). We used GLM with a normal distribution and identity link to assess differences in percentage weight gain at, and time

to, death or pupation, analyzed separately for each outcome. All p-values were obtained using likelihood-ratio χ^2 tests.

For experiment #2, we assessed the individual effects of treatment, brood identity, and their interaction on larval outcome by fitting a GLMM with a binomial error distribution and logit link function. Because the Hessian matrix suggested quasi-complete separation, we reran the model using bias-adjusted estimates (Firth adjusted maximum likelihood). Brood identity was used as a random effect in the model, and p-values were obtained by performing likelihood-ratio χ^2 tests. A linear mixed effects modeling approach was also used to analyze weight at and time to pupation (for surviving larvae), or weight at and time to death. These variables were analyzed separately for each outcome; treatment was coded as a fixed effect and brood identity as a random effect. Chi-square and p-values were obtained as above.

All analyses were conducted using JMP 9.0.0 (SAS Institute, Cary NC).

RESULTS

2016 Experiment

More wasp-cue larvae died than in the other two treatments. Only 17% of wasp-exposed larvae pupated, vs. 50% of larvae in the fly-cue and control treatments [**Figure 1A**; $X^2_{(2df)} = 6.04$, $p = 0.049$]. Of the larvae that pupated, mass at pupation and time to pupation did not differ among the three treatments (both $p > 0.20$; **Table 1**).

Although the treatments did not affect pupated larvae, there was a marginal between-treatment difference in the time to death of larvae dying prior to pupation (**Figure 1B**). Control larvae that died prior to pupation lived seven and 4 days longer than larvae in the fly- and wasp-cue treatments, respectively [$X^2_{(2df)} = 5.15$, $p = 0.076$]. Larvae that died prior to pupation also gained similar amounts of weight prior to their death [**Figure 1C**; $X^2_{(2df)} = 4.30$, $p = 0.116$].

2017 Experiment

Although more larvae in all three treatments survived to pupation (39 and 61% survival in 2016 and 2017, respectively), only the wasp-cue treatment differed from the control [**Figure 1D**; $X^2_{(2df)} = 13.30$, $p = 0.001$]. As in 2016, there were no treatment-level differences in time to, or weight gain at, pupation (both $p > 0.3$; **Table 1**).

There were substantial treatment-level differences between larvae that died before pupation. Larval longevity was greatest in the control treatment and lower in the fly- and wasp-cue treatments [**Figure 1E**; $X^2_{(2df)} = 6.18$, $p = 0.045$]; a similar pattern was seen in percentage weight gain prior to death [**Figure 1F**; $X^2_{(2df)} = 6.36$, $p = 0.042$].

The three broods differed overall and in their treatment response (**Supplementary Information**). Overall survival was highest for brood three [88%; "brood": $X^2_{(2df)} = 15.3$, $p < 0.001$], while brood one responded most strongly to the treatments ["treatment*brood": $X^2_{(4df)} = 12.9$, $p = 0.012$]. Brood three also took longer to pupate [$X^2_{(2df)} = 14.8$, $p < 0.001$] and gained more weight prior to pupation [$X^2_{(2df)} = 9.2$, $p = 0.010$]; this likely reflects the greater number of brood three larvae surviving to pupation.

While we did not take any data on predator or prey behavior, the wasps and flies appeared to behave similarly in both experiments. For the first several hours following their individual addition to a *Actias*-containing plastic bin, both types of insects spent most of their time flying between perches where they sat while attempting to groom their mouthparts. Wasps appeared more agitated than flies during the grooming period and would often buzz their wings while grooming; this behavior was never observed with flies. After this first period, both wasps and flies were predominantly found walking on the walls of the plastic bin with occasional short (5–8 cm) flights between walls. Neither type of insect appeared interested in the *Actias* larva and were only rarely observed in physical contact with it.

DISCUSSION

Our two experiments, conducted in different years with different populations, found that predation risk decreases *Actias* survival by ∼55% (66 and 43% in 2016 and 2017, respectively) relative to control treatments, while exposure to a similarly-sized and -treated detritivorous fly did not. This appears to be the first direct evidence that risk alone can increase prey mortality in lepidopterans.

The impact of predator cues may reflect the heavy reliance of *Actias* larvae on camouflage for predator defense. While their spines and strong grip on twigs and branches may deter vertebrate predators (Sourakov, 2018), *Vespula* sp. wasps dismember larger caterpillars *in situ* (Lichtenberg and Lichtenberg, 2003). Feeding by caterpillars greatly increases their vulnerability to wasp predation (Bernays, 1997), and *Actias* that perceive risk "freeze" in place (A. Baranowski, *personal observation*). Confining larvae and wasps together (the drawbacks of which are discussed below) decreases or stops feeding, as indicated by the minimal weight gain of larvae dying in the wasp treatment (**Figure 1F**). Exposure to foraging honeybees similarly reduces feeding, and thus plant damage, by *Spodoptera exigua* caterpillars (Tautz and Rostás, 2008). The fact that honeybees pose no threat to *S. exigua* suggests that hymenopteran buzzing, especially in combination with volatile and visual cues, may be a general risk cue for caterpillars (Tautz and Markl, 1978).

The risk-induced increase in *Actias* mortality is consistent with findings from aquatic predator-prey systems in which predator cues reduced the survivorship of both tadpoles (Hettyey et al., 2015) and larval dragonflies (McCauley et al., 2011). In a terrestrial system, Stamp and Bowers (1991) used data on weight gain of buckmoth (*Hemileuca lucina*) caterpillars in the presence and absence of wasps to infer the risk-induced increase in caterpillar mortality. They estimated that exposure to wasps reduced survival by 20.3% via reductions in food intake that slowed growth and increased the larval period. Our study builds on theirs by providing the first directly-measured evidence that risk increases mortality in a terrestrial predator-prey system. More generally, the strong responses of multiple lepidopteran

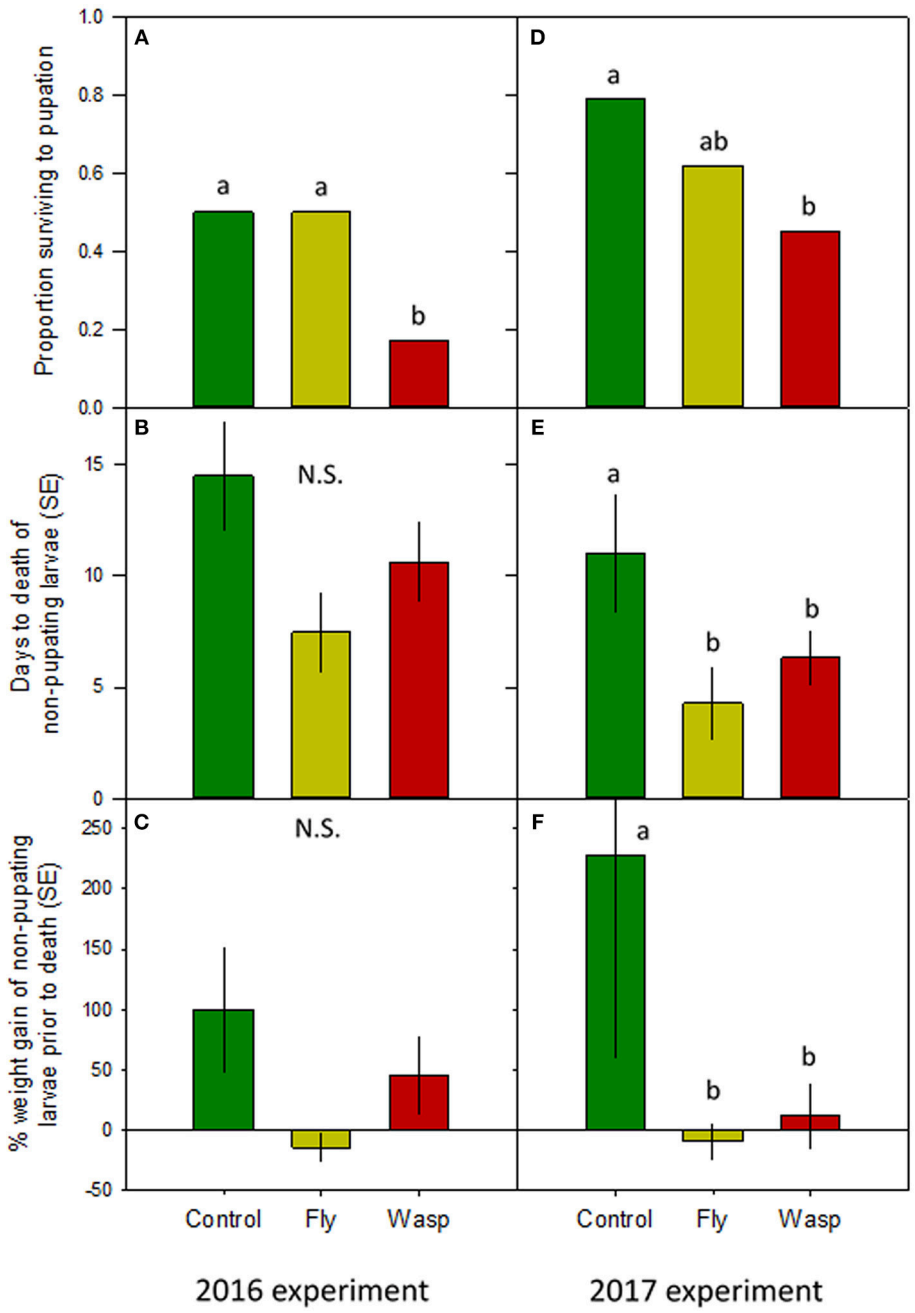

FIGURE 1 | Impact of varying risk cues on *Actia luna* larval development. Left-hand panels: 2016 experiment (n = 18/treatment); right-hand panels: 2017 experiment (n = 28–29/treatment). **(A,D)**: Proportion of *A. luna* larvae surviving to pupation. **(B,E)**: Mean ± SE days from the start of the experiment to death of non-pupating larvae. **(C,F)**: Percentage ± SE weight gain from hatching to death of non-pupating larvae. Green bars: no risk cues; yellow bars: risk cues from harmless scavenging fly; red bars: risk cues from *Vespula* sp. predatory wasp. Lower-case letters indicate treatments similar at $\alpha = 0.05$ (*post-hoc* Tukey HSD); N.S. = no significant differences among treatments.

species to risk cues (Tautz and Markl, 1978; Stamp, 1997; Johnson et al., 2007) suggests that similar results may occur in a range of systems and play an important but relatively unappreciated role in plant-herbivore-natural enemy interactions in natural and managed ecosystems.

Low mortality in the fly treatment (**Figures 1A,D**) can be interpreted as suggesting that *Actias* differentiate between predators and other similarly-sized but harmless flying insects. This interpretation agrees with work in both aquatic and terrestrial systems (e.g., Bass and Gerlai,

TABLE 1 | Weight at pupation (g) ± SE and days to pupation ± SE for larvae surviving to pupation in the 2016 (top) and 2017 (bottom) experiments.

Experiment	Treatment	Pupal weight (g)	Days to pupation
2016	Control	2.5 + 0.12	20.7 + 0.94
	Non-lethal fly	2.4 + 0.12	19.3 + 0.94
	Non-lethal wasp	2.7 + 0.21	21.0 +1.63
2017	Control	2.5 + 0.14	18.5 + 0.61
	Non-lethal fly	3.0 + 0.15	20.2 + 0.67
	Non-lethal wasp	2.8 + 0.21	22.1 + 0.85

There were no significant ($\alpha = 0.05$) treatment-level differences in either variable in either experiment.

2008; Zanette et al., 2011) showing that prey can distinguish between cues from similarly-sized dangerous and harmless species. It is not, however, consistent with our data on larvae that died prior to pupation. If *Actias* perceived flies as less risky, the time to death (**Figures 1B,E**) and weight gain prior to death (**Figures 1C,F**) of fly-exposed larvae should be either similar to, or slightly less than, the control treatment. Instead, both metrics were identical to those seen in the wasp treatment: fewer larvae died in the fly treatment, but those that did appeared to respond as strongly to flies as their counterparts did to wasps. This may suggest that individual *Actias* have different "risk thresholds" that determine their reaction to cues (i.e., the shy-bold continuum; Sih et al., 2012). Larvae with high risk thresholds would err on the side of boldness and continue to forage even when a predator might be present. Conversely, larvae with lower thresholds would cease feeding even when exposed to low-risk cues such as the buzzing of a fly. Such risk thresholds would also explain the similar size and larval period of successfully-pupating larvae; individuals that did not perceive their environment as risky should have similar times to and size at pupation. If true, then our work may point more toward *Actias* larvae distinguishing between different risk levels rather than discriminating between predatory wasps and similarly-sized but harmless flies.

The brood-level differences we observed suggest the potential for ecologically-relevant intraspecific variation in risk responses (Bolnick et al., 2011). Since the broods emerged at different times, we cannot rule out the possibility that our results are explained by phenology rather than genetic differences. Our increasing awareness of the impact of maternal stress on offspring phenotypes (Sheriff et al., 2018), however, argues strongly for additional research into this topic.

The fact that risk alone is sufficient to reduce caterpillar survival suggests several important areas of future research. Perhaps the most important is the nature of the predator cue; while other researchers have found "buzzing" important in caterpillar risk assessment (Tautz and Markl, 1978; Tautz and Rostás, 2008), chemical and visual cues may well complement and enhance auditory inputs. In retrospect, it would have been useful to include a "no-contact" treatment in which the non-lethal wasps were prevented from having the opportunity to physically touch the caterpillar; this would have allowed us to determine whether direct contact between the predator and its prey contributed to the observed effect. It is also important to consider how continuously confining caterpillars and cues together in an enclosed space might have affected our results. Although many studies have used continual risk exposure to understand its effects (reviewed in Ferrari et al., 2009), the variability of risk in natural settings should affect caterpillar responses. Finally, our work highlights the need for future studies linking risk-mediated changes in foraging time (Johnson et al., 2007) and assimilation efficiency (Thaler et al., 2012) to individual survival.

AUTHOR CONTRIBUTIONS

EP and AB designed the experiment, AB conducted the experiment, and EP and AB jointly analyzed the data and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

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The Neurobiology of Fear Generalization

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The generalization of fear memories is an adaptive neurobiological process that promotes survival in complex and dynamic environments. When confronted with a potential threat, an animal must select an appropriate defensive response based on previous experiences that are not identical, weighing cues and contextual information that may predict safety or danger. Like other aspects of fear memory, generalization is mediated by the coordinated actions of prefrontal, hippocampal, amygdalar, and thalamic brain areas. In this review article, we describe the current understanding of the behavioral, neural, genetic, and biochemical mechanisms involved in the generalization of fear. Fear generalization is a hallmark of many anxiety and stress-related disorders, and its emergence, severity, and manifestation are sex-dependent. Therefore, to improve the dialog between human and animal studies as well as to accelerate the development of effective therapeutics, we emphasize the need to examine both sex differences and remote timescales in rodent models.

Keywords: fear generalization, fear memory, neural circuits, animal models, sex differences

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INTRODUCTION

Fear is a primitive emotion that is conserved throughout the animal kingdom (Walters et al., 1981; LeDoux, 2012; Adolphs, 2013). Survival in the wild is critically dependent on the flexible assessment of threatening stimuli, which entails the processing, integration, and synthesis of information acquired by multiple sensory modalities. Because aversive experiences are never completely identical, animals must generalize their fear of a past experience to future encounters that bear a sufficient degree of similarity to the original event. Like other memory-related processes, generalization is modulated by a number of intrinsic factors, including internal states (estrous and circadian cycles; Hull, 1943; Toufexis et al., 2007; Koch et al., 2017), previous experience (Lashley and Wade, 1946), genetic background (Temme et al., 2014), and sex differences (Day et al., 2016; Keiser et al., 2017). Generalization is also influenced by external factors including the type and intensity of aversive stimulation (Baldi et al., 2004), early-life stress (Elliott and Richardson, 2018), as well as the saliency of particular elements in the environment (Huckleberry et al., 2016). Finally, generalization is sensitive to the passage of time, as memories naturally lose both their precision and strength (McAllister and McAllister, 1963; Winocur et al., 2007; Jasnow et al., 2012; Pollack et al., 2018). Given the large number of variables that impinge on the generalization of fear, it has been challenging to develop an overarching neurobiological framework with robust explanatory power. However, recent studies have begun to provide some compelling new insights. Furthermore, whereas generalization has adaptive value, overgeneralization is maladaptive, and is a major feature of anxiety- and stress-related disorders such as

post-traumatic stress disorder (PTSD; Elzinga and Bremner, 2002; Lissek et al., 2010; Dunsmoor and Paz, 2015). Therefore, a better understanding of the neurobiology of generalization is essential from a translational perspective.

In this review article, we explore the neurobiology of fear generalization within a broader historical, theoretical, and behavioral context. We then outline how the neural circuits involved in fear generalization may shift with the passage of time. Finally, we examine our current understanding of the neurotransmitter systems and cellular signaling pathways that contribute to fear generalization, and discuss how this information may be used to develop new therapeutic approaches for treating disorders of fear memory.

ADAPTIVE vs. MALADAPTIVE FEAR GENERALIZATION

What defines the boundary between adaptive and maladaptive fear generalization? From an ethological perspective, generalized responses that promote survival of an organism are defined as adaptive, whereas behaviors that contradict the mandate of self-preservation are maladaptive (Johnson et al., 1992; McEwen, 1998; Cooper and Blumstein, 2015). However, this delineation must be qualified by several caveats.

First, the environmental context in which a generalized fear response occurs is a critical parameter, because a behavior that is adaptive in one environment may be maladaptive in another. For example, increased defensive behaviors and a reduction of foraging in areas of high predatory threat are adaptive for rodents. However, deployment of an enhanced defensive response in environments lacking an elevated imminence of threat is maladaptive because it unnecessarily compromises both the acquisition of resources and allostasis, which refers to the set of adaptive processes that maintain homeostasis (Fanselow, 1994; McEwen, 1998; Blanchard and Blanchard, 2008). The same inference can be drawn for humans as well as for laboratory mice, where test subjects that are conditioned in a particular context or to a particular cue generalize fear in different contexts or to different cues (Kaczkurkin et al., 2016), but see (Elzinga and Bremner, 2002). Cues and environments exist on a perceptual continuum, and maladaptive fear generalization occurs when an abnormal stimulus-response gradient emerges to produce defensive behaviors in environments or to cues which have never been explicitly associated with threat or danger.

In addition, sexually dimorphic generalization may serve an equally adaptive function within each sex for various behaviors (Darwin, 1888; Kelley, 1988). With regard to fear generalization, female mice that have been exposed to contextual fear conditioning tend to freeze in the first retrieval context in which they are tested, whether or not it is identical to the training context (Keiser et al., 2017). One possible interpretation of this behavior is that the consequence of making a "mistake" (i.e., not exhibiting an optimal defensive strategy) in a potentially life-threatening environment is evolutionarily more costly for female mice in terms of future reproductive success (Kelley, 1988). However, this example also illustrates that the evolutionary benefit of a given behavioral pattern is not definitively clear (for review see Bangasser and Wicks, 2017). Finally, although a particular behavior may be maladaptive for an individual it may actually benefit the population (for review see Miller and Polack, 2018).

For these reasons, it is not possible to demarcate adaptive and maladaptive behavior in absolute terms. Therefore, we favor a normative definition in which performance of sex-matched, wild-type animals in a given behavioral task serves as a reference for what constitutes adaptive behavior, with phenotypic outliers representing maladaptive states. Other research groups have sought to formalize the identification of maladaptive generalization states by stratifying animal behavior across a variety of behavioral paradigms (Cohen et al., 2003, 2004; Cohen and Zohar, 2004; Richter-Levin et al., 2018). As more studies begin to implement this strategy, a major challenge will be to establish agreed upon criteria for clearly defining the boundaries that separate normal from pathological fear generalization.

THEORETICAL FRAMEWORK

For well over a century, research has examined the behavioral correlates of stimulus generalization and discrimination. In the 1920s, the seminal studies of Pavlov demonstrated that animals trained in an auditory conditioning paradigm exhibit generalization of their conditioned response (CR) to a range of auditory stimuli (Pavlov, 1927). Subsequent work suggested that a failure to discriminate between the conditioned stimulus (CS) and similar, but non-identical stimuli is a result of: (1) an active process of inhibitory weakening (Spence, 1936); (2) the failure to form a strong association between the CS and unconditioned stimulus (US), indicating that the "dimensions" of a stimulus are not well-learned (Lashley and Wade, 1946; Rescorla and Wagner, 1972); and (3) forgetting, or the failure of retrieval (Bouton et al., 1999). Although generalization likely arises from a weighted sum of these processes, many of the studies covered in this review article have explored generalization within the boundaries of each independently. For example, changes in several brain regions have been shown to actively promote or inhibit discrimination (Duvarci et al., 2009; Cullen et al., 2015; Ferrara et al., 2017). Moreover, generalization can be partially alleviated by greater learning about the CS (Biedenkapp and Rudy, 2007; but see Poulos et al., 2016). However, the neurobiological contributions of "forgetting" to generalization are more difficult to evaluate [(Rescorla, 1976; Pearce, 1987; Riccio et al., 1992), but see (Ishikawa et al., 2016; Richards and Frankland, 2017)].

Our understanding of the neurobiology of fear generalization within the aforementioned theoretical constructs is further complicated by the temporal evolution of associative memories, whereby memories become less precise and rely more heavily on cortical areas over time (Bergstrom, 2016; Jasnow et al., 2017; Sekeres et al., 2017; Asok et al., 2018b). When considering these temporal factors, we are left with a challenging question: what are the neural and molecular mechanisms that control the generalization of fear memories at remote timescales? A number of conceptual frameworks originally developed to explain the shift of associative memories from limbic to cortical structures

have also been applied to generalization. In particular, three key theories have prevailed: systems consolidation theory, multiple trace theory, and trace transformation theory.

In *systems consolidation theory*, episodic memories are transferred to the neocortex from the hippocampus, such that the expression of remote memories may no longer be hippocampus-dependent (Dudai, 2004; Dudai et al., 2015). However, a number of studies have challenged this view by showing that the hippocampus continues to play a role in the retrieval of remote fear memories (Rekkas and Constable, 2005; Lehmann et al., 2007; Clark and Sutherland, 2013). Moreover, neocortical areas may also be recruited during initial consolidation, though in an immature form (Zhao et al., 2005; Takehara-Nishiuchi et al., 2006; Vetere et al., 2011; Kitamura et al., 2017), a concept that other theoretical frameworks have attempted to incorporate (Asok et al., 2018b).

According to *multiple trace theory* (Moscovitch and Nadel, 1999; Moscovitch et al., 2005), neocortical and hippocampal areas are rapidly recruited to a memory trace, but these memories become less detailed and accurate over time. However, the act of retrieval produces a new memory trace and serves to strengthen hippocampal and neocortical connections as well as strengthen the overall memory (Moscovitch et al., 2005). Likewise, the *transformation hypothesis* suggests that context-specific episodic memory is always hippocampus-dependent, but details are lost over time as a particular memory becomes more schematic (see Broadbent and Clark, 2013). However, the transformed schematic representation is less precise and relies less on the hippocampus. Indeed, certain features of a memory persist longer than others and are differentially consolidated across the brain (Malin and McGaugh, 2006; Wiltgen et al., 2010). Thus, while certain brain areas may be especially suited for encoding specific aspects of a fear memory [e.g., foot-shock or context (Malin and McGaugh, 2006)], the subsequent retrieval of the memory may rely more heavily on another set of brain regions at recent vs. remote time points (Frankland et al., 2004).

These theories of long-term memory outlined above are, however, limited in their ability to provide a comprehensive framework for understanding fear generalization. For example, what we know about remote episodic memory is largely predicated on hippocampal-based mechanisms, despite the fact that generalization clearly involves the amygdala, frontal cortex, and other brain regions. Along these lines, these theories of long-term memory also do not embrace the fundamental circuit-wide nature of memory and generalization at recent vs. remote timescales, which may be completely independent of the hippocampus. Finally, and perhaps most important, these theories do not fully explain how memory becomes less precise over time (Bouton et al., 1999; Wiltgen and Silva, 2007), an issue that would need to be addressed by any robust theoretical model of generalization.

METHODOLOGICAL APPROACHES IN THE STUDY OF FEAR GENERALIZATION

Although there is considerable variation in methodology across studies, behavioral studies in rodents have focused on generalization to either contextual or discrete cues. In context generalization experiments, a rodent is typically exposed to contextual fear conditioning, which entails the presentation of an aversive US such as a foot shock in a conditioning context—a previously neutral environment. Subsequent re-exposure of the animal to the conditioning context (CTX+) without delivery of the US evokes a species-specific defensive reaction such as freezing, which refers to the cessation of all movement except for respiration, and is generally accepted as a proxy for fear (Blanchard and Blanchard, 1969). In turn, freezing in the CTX+ in the absence of a US can be measured at various time points after the initial CS-US pairing. When assessed at 24 h, which is perhaps the most commonly used interval of time in these experiments, freezing is an index of long-term associative memory, with longer intervals (several weeks or longer) corresponding to remote associative memory.

To measure contextual fear generalization, animals are fear conditioned and then exposed to a different context that was never paired with a shock (CTX−; Rohrbaugh and Riccio, 1968; Ruediger et al., 2011). Freezing in the CTX− is an index of fear generalization and can also be evaluated at multiple time intervals, although the degree to which the CTX+ and CTX− environments share similarities (e.g., odor, lighting, and chamber shape) can vary substantially between studies, and can greatly impact experimental outcomes, as we discuss later. Furthermore, recent work has found that similarity between olfactory and tactile elements of the CTX+ and CTX− are more important than visual cues for generalization in males relative to females [(Huckleberry et al., 2016), but see (Bucci et al., 2002; Murawski and Asok, 2017)]. Less is known about whether particular stimulus elements are more critical in females (e.g., odors given maternal roles), but within a species the most salient sensory elements are likely similar between males and females (Dunsmoor et al., 2011; Lissek et al., 2013).

In contrast, fear generalization to discrete cues commonly involves exposing animals to an aversive US paired with a stimulus presented through one sensory modality such as a neutral tone or odor, which then becomes a CS. When subsequently presented with a cue that resembles the CS, animals exhibit a defensive response whose magnitude with respect to the original CR is dependent upon the perceptual similarity of the two stimuli (Shaban et al., 2006; Zhang et al., 2017). In psychometric terms, the strength of the defensive response varies as a function of the degree to which the new CS approximates the original CS (see **Figure 1**). Thus, a narrow generalization gradient (high discrimination) is signified by a maximal defensive response that only occurs within a narrow range of stimuli that are very similar to the CS, whereas a broad generalization gradient (low discrimination) is indicated by the ability of progressively dissimilar stimuli to elicit a defensive response. It is important to note that the type of conditioning (e.g., auditory trace fear conditioning vs. unpaired controls) can influence generalization. Given that discrete cues are always presented in a particular context, the type of conditioning can influence both the associative value of the CS and the associative value of the context. Thus, discrete cue conditioning paradigms that manipulate how the context is presented, either

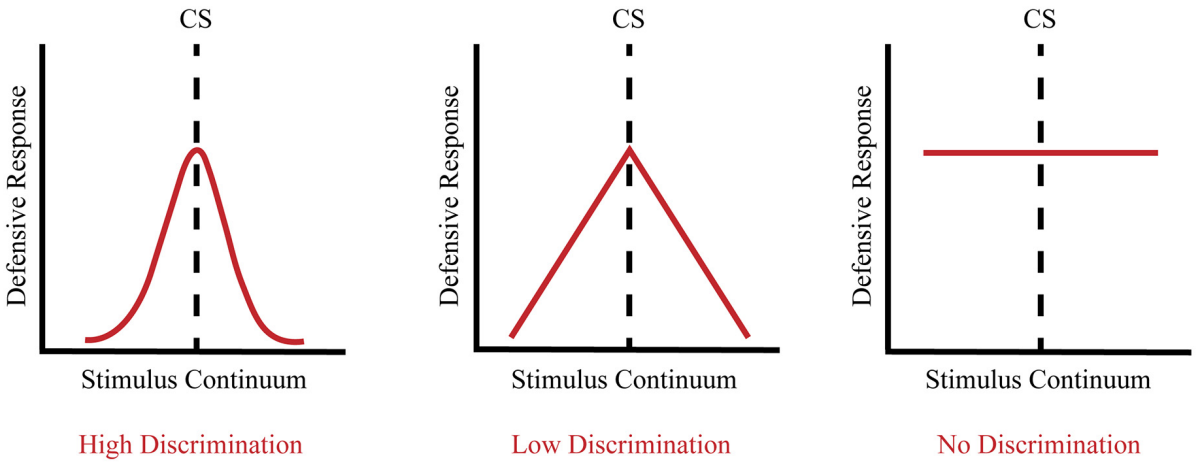

FIGURE 1 | Fear generalization occurs along a continuum. High discrimination is a product of a heightened defensive response to the conditioned stimulus (CS) and a low defensive response to non-target CSs, reflecting a narrow generalization gradient (left panel). Low discrimination is a product of a heightened defensive response to the CS as well as an elevated defensive response to stimuli that approximate the CS, reflecting a broad generalization gradient (middle panel). No discrimination is a product of a heightened defensive response to the CS as well as stimuli that markedly differ from the CS, reflecting an elevated flat generalization gradient (right panel).

in the foreground or in the background of the discrete cue, can differentially influence fear discrimination (Rescorla, 1976; Pearce, 1987; Desmedt et al., 2003).

It is more straightforward to parametrically examine generalization using a discrete cue vs. a context. For example, one can alter the frequency of a tone by defined gradations and observe an animal's response during different cue presentations (Guttman and Kalish, 1956). In an analogous manner, a structurally related series of odorants that differ by a single carbon group can produce a generalization gradient (Pavesi et al., 2013). This is not as easily accomplished in contextual generalization experiments, because contextual representations reflect a combination of stimulus elements (e.g., spatiotemporal elements, as well as tactile, olfactory, visual, and auditory inputs) that are bound into a unitary representation (Sutherland and Rudy, 1989; O'Reilly and Rudy, 2001; Rudy, 2009). Furthermore, given the number of potentially salient features in a contextual fear conditioning chamber, the extent to which an animal may attend to one element over another is poorly understood, although computational models of serial element processing have been proposed (Krasne et al., 2015).

Yet, in both types of generalization, behavior can be modulated by a number of external factors, including strength and duration of the US, strength of the CS-US association, similarity between the CS and generalization stimuli, as well as a number of internal factors such as genetic background, sex, and circadian cycle. Moreover, these parameters can interact with one another. For example, pre-exposing male mice to a conditioning context (CTX+) can enhance the strength of recent fear memories during single-trial conditioning (McHugh and Tonegawa, 2007; Brown et al., 2011), but it also produces generalization to the CTX− (Radulovic et al., 1998; Rudy and O'Reilly, 1999). In addition, whereas pre-exposing females to the CTX+ reduces generalization without altering the strength of recent fear memories, pre-exposing males to the CTX− enhances generalization to the pre-exposed context (CTX−; Keiser et al., 2017). These observations are further complicated by the fact that generalization is dependent on the test order of the different contexts, whereby extinction produced by testing in an non-reinforced context may influence the generalization of fear in a subsequent test context (Wood and Anagnostaras, 2011; Huckleberry et al., 2016; Keiser et al., 2017).

The diverse behavioral outputs observed in the latter experiments are ostensibly a reflection of adaptive tuning mechanisms that are modulated by a number of critical parameters, including animal species and strain. Furthermore, it is important to emphasize the potential contributions of sex differences. For example, when considering external factors that contribute to fear generalization in males relative to females the recruitment of different brain regions (e.g., amygdala vs. hippocampus) may be an important variable (Keiser et al., 2017). In addition, new studies are beginning to identify how active or passive defense strategy selection may differ between sexes (Gruene et al., 2015; Shansky, 2018). It is possible that ovarian hormonal state in females may alter the functional connectivity of neural circuits during specific temporal windows, leading to differential effects on stress reactivity and memory (Andreano et al., 2018), which in turn could affect generalization. Regardless of the range of parametric factors that mediate the generalization of recently acquired fear memories, animals may generalize their fear to the CTX− at remote time-points (Balogh et al., 2002; Wiltgen and Silva, 2007; Poulos et al., 2016; Pollack et al., 2018; but see Biedenkapp and Rudy, 2007; Vanvossen et al., 2017). Despite methodological differences across studies, sex differences in generalization at recent and remote time-points, whether to cues or contexts, are likely a product of alterations in information processing within fear circuits. Cellular and molecular changes within these neural circuits that control normal fear learning and

memory likely serve as key conduits for promoting or inhibiting fear generalization between sexes across time.

NEURAL CIRCUITS OF FEAR GENERALIZATION

Fear memories rely on discrete neural circuits which shift as a function of the type of CS-US pairing (e.g., discrete cues in trace or delay conditioning vs. contextual conditioning; for review see Maren, 2001; Tovote et al., 2015). US foot-shock information from peripheral sensory inputs enter the ventroposterior nucleus of the thalamus (VPN) as well as the posterior intralaminar nucleus of the thalamus (PIN). Accordingly, studies have found that electrolytic lesions of the PIN disrupt fear conditioning (Lanuza et al., 2004, 2008). US information from the PIN and the posterior insular cortex (PIC) is then relayed to the lateral nucleus of the amygdala (LA), which is a critical site of plasticity in fear learning and memory regardless of the type of CS-US pairing (Goosens and Maren, 2001). However, US pathways also show selectivity for the type of CS-US pairing in that lesions of the PIC only disrupt auditory, but not contextual, fear memories (Brunzell and Kim, 2001; Davis, 2006), which likely reflects multimodal information processing.

Auditory Fear Circuits

During auditory fear conditioning, auditory information is relayed from lemniscal and extralemniscal pathways to the auditory thalamus. This information from the auditory thalamus is then relayed to the LA by either a direct pathway arising from extralemniscal projections originating in the medial part of the medial geniculate nucleus (mMGN) and PIN, or by an indirect pathway, which arises out of the lemniscal pathway and projects from the ventral MGN to the primary auditory cortex, and subsequently to the auditory association cortex and then LA (Weinberger, 2011). Inputs to the LA from the mMGN are critical for fear memories and, as discussed later, molecular perturbations in the mMGN produce fear generalization (Nabavi et al., 2014; Ferrara et al., 2017). Auditory CS and US foot-shock information is thought to converge in the LA and in parts of the central nucleus of the amygdala (CeA; Paré et al., 2004). The Excitatory and inhibitory balance of discrete populations of neurons in the LA for tones, and basal amygdala complex for contexts given inputs from the ventral hippocampus (Canteras and Swanson, 1992; Maren and Fanselow, 1995), has been implicated in fear generalization as similarity of the CS− approaches the CS+ (Tovote et al., 2015; Rajbhandari et al., 2016; Grosso et al., 2018). The LA provides inputs to the CeA, of which the medial division (CeAm) contains the primary outputs to structures which mediate behavioral and neuroendocrine aspects of fear (e.g., the periaqueductal gray; PAG; Gross and Canteras, 2012). Interestingly the lateral division of the CeA (CeAl) also receives direct inputs from the thalamus (Linke et al., 2000), provides tonic inhibition of the CeAm, and is associated with fear generalization to auditory CSs (Ciocchi et al., 2010). Moreover, recent studies have suggested that corticotropin releasing factor in the CeAl may be important for modulating fear generalization under conditions of low-associative strength (Sanford et al., 2017). However, the CeAl and basolateral amygdala (BLA) complex also send projections to the bed nucleus of the stria terminalis (BNST), a region implicated in anxiety-like behaviors and contextual fear (Dong et al., 2001; Davis et al., 2010; Asok et al., 2018a). Lesions of the BNST enhance the precision of recent auditory memories while reducing fear generalization (Duvarci et al., 2009).

Contextual and Olfactory Fear Circuits

The dorsal hippocampus is critical for the formation of a unitary contextual representation during contextual fear conditioning (Maren et al., 1997; Holland and Bouton, 1999; Rudy et al., 2004). Information from sensory and association cortices is relayed to post-rhinal (POR) and peri-rhinal (PER) cortices, followed by the medial and lateral entorhinal cortices [MEC and LEC, respectively (Lee and Lee, 2013)]. This information from different MEC and LEC layers then flows into the hippocampal formation, with segregated inputs to the dorsal dentate gyrus, dorsal hippocampal CA3 subfield, dorsal CA1 subfield, and dorsal subiculum (dSub). Indeed, distinct outputs from CA1 and dSub to MEC are important for the acquisition and retrieval of recent fear memories, respectively (Roy et al., 2017). The DG and CA3 have received considerable attention for their role in fear generalization because of their contributions to pattern separation and pattern completion (see McHugh et al., 2007; Rolls, 2013). Mice with deletion of the N-methyl-D-aspartate receptor (NMDAR) in CA3 exhibit generalization during short-term, but not recent, fear memory tests, suggesting that CA3 has an important role in the rapid formation of contextual representations (Cravens et al., 2006). Recent studies have suggested that while neuronal ensembles in the DG show context selectivity during the retrieval of recent fear memories, there is a substantial loss of DG selectively at remote time-points which parallels fear generalization (Matsuo, 2015; Yokoyama and Matsuo, 2016). It is worth noting that the DG is one of the few sites in the brain that exhibits neurogenesis, and manipulations that promote neurogenesis improve contextual discrimination—a finding which suggests that enhancing DG function may increase remote memory precision (Sahay et al., 2011; Nakashiba et al., 2012; Besnard and Sahay, 2016). Given the importance of olfactory cues to rodents and their relevance to disorders such as PTSD (Rolls et al., 2013; Cortese et al., 2015), as well as the influence of neurogenesis in the olfactory bulb, it will also be interesting to determine whether manipulating neurogenesis in the olfactory bulb modulates fear generalization to odors (see Tong et al., 2014).

Hippocampal-Thalamic-Prefrontal Circuits

The functional distinction between the dorsal hippocampus and ventral hippocampus has long-been debated, but there is agreement that both divisions are essential in the consolidation of contextual fear memories (Fanselow and Dong, 2010; Zhu et al., 2014). Moreover, the ventral hippocampus and its connections may be important for the maintenance of memory precision (Ciocchi et al., 2015; Cullen et al., 2015; Jimenez et al., 2018). The ventral hippocampus has reciprocal connections with the medial

prefrontal cortex (mPFC; anterior cingulate, prelimbic, and infralimbic regions), the BLA, the retrosplenial cortex, and the insular cortices (Pitkänen et al., 2000; Cenquizca and Swanson, 2007). Interestingly, neuronal activity in the mPFC increases in parallel with the emergence of fear generalization at remote time-points (Cullen et al., 2015), suggesting the possibility of an active role in promoting generalization. Moreover, the mPFC is reciprocally linked with vCA1 by the nucleus reuniens (NR), and enhancing activity of mPFC inputs to the NR decreases contextual fear generalization (Xu and Südhof, 2013). Thus, the mPFC ↔ NR ↔ vCA1 circuit is likely to play an important role in the modulation of memory precision as well as the generalization of fear during the natural course of systems-consolidation (Rozeske et al., 2015; Ramanathan et al., 2018). In humans, other brain regions such as the striatum, insula, and PAG have also been implicated in the generalization of recent fear memories (Dunsmoor et al., 2011).

Considerations in the Neural Circuits of Fear Generalization

Generalization gradients exist in core sensory cortices which process discrete CSs such as odors and tones. The generalization of contextual fear appears to follow a similar organizational structure, but involves a more elaborate network to account for multimodal sensory and representational processing. How fear generalization gradients emerge and shift across time at the neural circuit level is an important area of future research. For example, understanding how contextual information differentially engages the dorsal hippocampal, ventral hippocampal, and medial prefrontal circuits at recent and remote time-points may provide important insights into a global framework for how fear generalization to complex representations occurs. Similarly, identifying whether these shifts are paralleled in sensory cortices which represent the elemental components of a contextual representation, such as auditory information in direct and indirect thalamic relay pathways to the LA, will help to build a global framework of the brain-wide circuits which modulate fear generalization (Weinberger, 2011; Shang et al., 2015). However, an important consideration in this work is the interaction between remote fear generalization and systems consolidation. Beyond neural circuits, this interaction raises a fundamental biological question: are the molecular and genetic mechanisms that regulate fear generalization at early time-points similar to those at remote time-points?

While most of the neural circuits and molecular mechanisms enumerated above promote generalization, it is also clear that generalization is an active process in which a consolidated memory is prevented from undergoing accurate retrieval. For example, inactivation of the ACC or vCA1 has no effect on contextual fear memory at remote time-points when mice are tested in the training context, but enhanced freezing to a novel context is suppressed (Frankland et al., 2004; Cullen et al., 2015). Thus, the ability to discriminate between an aversive context and neutral context at remote time-points is actively inhibited by the ACC and vCA1. Similarly, blocking protein synthesis in the MGN enables tone discrimination that is otherwise not observed in an auditory fear conditioning paradigm (Ferrara et al., 2017). Also, as mentioned previously, lesions of the BNST reduce fear generalization (Duvarci et al., 2009), implying an unidentified role for this brain region and its outputs in the active suppression of discrimination.

Finally, the substrates of generalization are nested within the neural circuits that support fear memory. For example, the acquisition, extinction, and generalization of fear are all regulated by NMDARs functioning in excitatory neurons of the PFC (Vieira et al., 2015). It is therefore possible that experimental manipulations which target associative fear memories may exert uncharacterized effects on generalization, and would merit further exploration. However, the molecular and cellular mechanisms that support various components of associative fear memory including generalization are not entirely identical. For example, pharmaco-genetic deletion of a subset of excitatory and inhibitory neuronal ensembles in the amygdala impairs generalization, but not fear memory (Grosso et al., 2018), demonstrating that these processes are separable.

MOLECULAR AND CELLULAR MECHANISMS OF FEAR GENERALIZATION

Adding to the complexity of circuit-level processes are the many distinct molecular and cellular pathways that also contribute to generalization. These cellular and molecular pathways represent conduits for information that do not operate in isolation from one another. For example, the cannabinoid CB_1 receptor is found in GABAergic and glutamatergic neurons in the CNS, thus allowing the endocannabinoid system to influence the activity of both inhibitory and excitatory synapses (Kano et al., 2009). Moreover, certain neurons may release both gamma-aminobutyric acid (GABA) and glutamate (Shabel et al., 2014), and changes in the excitatory and inhibitory balance within neurons may be important for certain behaviors (Froemke, 2015; Mongillo et al., 2018). Furthermore, signaling pathways intersect not only at the cellular level, but also at the circuit level. For instance, glucocorticoid and beta-adrenergic signaling across the limbic system cooperate in the regulation of long-term memory (Rodrigues et al., 2009; Roozendaal et al., 2009; McIntyre et al., 2012). Nevertheless, for organizational purposes and the sake of simplicity, we focus on particular contributions of discrete pathways.

Excitatory and Inhibitory Neurotransmission

Activity within neural circuits involved in the storage and processing of memory is governed by a balance of excitatory and inhibitory neurotransmission (Froemke, 2015; Mongillo et al., 2018). Altering this balance impinges on circuit-level functions, leading to distinct alterations in behavioral outputs. Not surprisingly, in addition to their central roles in associative fear memory, both excitatory and inhibitory neurotransmission are also important in fear generalization.

Glutamate is the major excitatory neurotransmitter in the brain, and direct evidence for the role of glutamatergic signaling in fear generalization is provided by studies that target ionotropic glutamate receptors. The NMDAR, in particular, plays a key role in synaptic plasticity and memory (Tsien et al., 1996). Conditional deletion of an obligatory subunit (NR1) of the NMDAR in excitatory CaMKIIα-positive principle neurons within the PFC causes a time-dependent increase in generalization to auditory cues, which is driven by ineffective CS− learning (Vieira et al., 2015). Thus, NMDAR activation in excitatory neurons is critical for stimulus discrimination because it promotes a reduction in defensive behavior when an animal is presented with a non-reinforced CS−. In addition to mPFC-dependent mechanisms for generalization, glutamatergic signaling at excitatory NR1 subunit-containing NMDARs in the hippocampus is important for pattern separation and contextual fear memory (McHugh et al., 2007). Specifically, while contextual fear conditioning and discrimination between very different contexts is intact following NR1 deletion in dentate granule cells, KO mice cannot easily discriminate between perceptually similar contexts. Also, selective inactivation of NMDARs in the LA reveals a role for NMDA signaling in auditory fear generalization (Jones et al., 2015).

Complementing the latter studies, more recent work has found that injection of NMDA into the rodent prelimbic cortex to activate NMDARs during the consolidation or retrieval phases of contextual fear conditioning induces fear generalization (Vanvossen et al., 2017). However, the effect is only observed for strong fear conditioning, whereas NMDA injection during a weaker training protocol actually enhances contextual discrimination, a finding that is consistent with the idea that the mPFC is involved in promoting retrieval of weaker memories (Rudy et al., 2005). Thus, there exists a complex relationship between the magnitude of aversive stimuli and prelimbic mPFC activation with respect to different components of associative fear memory.

Although most studies that implicate glutamatergic signaling in fear generalization focus on NMDAR-dependent mechanisms, AMPA-dependent signaling is also important. For example, peptide-mediated blocking of the removal of AMPA receptors in the dorsal hippocampus maintains long-term contextual fear memory and inhibits generalization, which in turn correlates with inhibition of synaptic depotentiation (Migues et al., 2016). Another study found that upregulation of synaptic expression of GluR1-containing AMPA receptors in the amygdala may drive the generalization of auditory fear (Ferrara et al., 2017). Together, these studies point to an elementary role for glutamatergic signaling in both contextual and cued generalization, but also underscore the complex relationship between excitatory neurotransmission and behavior.

Counterbalancing the actions of glutamate is GABA, the major inhibitory neurotransmitter in the brain. GABAergic neurons in the amygdala and hippocampus play a critical role in the formation of fear memories (Fendt and Fanselow, 1999). Whereas ionotropic $GABA_A$ receptors mediate fast inhibitory signaling, metabotropic $GABA_B$ receptors exert a slow inhibitory tone over synaptic circuits (Chua and Chebib, 2017; Frangaj and Fan, 2018). Several lines of genetic evidence demonstrate that GABAergic transmission is involved in the generalization of both contextual and cued fear memory. For example, deletion of $GABA_{B(1a)}$ receptors in mice is associated with increased contextual generalization, but has no impact on acquisition or maintenance of fear memory (Cullen et al., 2014). Likewise, deletion of the $GABA_B$ receptor subtype leads to generalization of cued fear without affecting retrieval by CS+ presentation, an effect that is evident for high- but not low-intensity foot shocks (Shaban et al., 2006). Similarly, deletion of $GABA_A$ receptor δ subunit causes an increase in generalization to auditory cues (Zhang et al., 2017). It will be interesting to see if perturbation of $GABA_A$ receptors within discrete circuits also has an impact on the generalization of contextual fear. Finally, deletion of glutamic acid decarboxylase (GAD65), an enzyme responsible for synthesizing GABA, causes generalization to auditory cues, although GAD65 KO mice show normal contextual fear learning (Bergado-Acosta et al., 2008; Sangha et al., 2009). Together, these studies indicate that GABAergic transmission contributes to both cued and contextual fear generalization, but like glutamatergic signaling, the relationship between inhibitory neurotransmission and behavior is both subtle and complex.

Monoaminergic Signaling

Among the classical monoamine neurotransmitters, dopamine appears to be the most significant modulator of fear generalization, although serotonin and noradrenaline (NA) can also regulate the processing of fear memory. Dopamine is critically involved in motivation, salience, reward learning, and prediction error (Schultz et al., 1997; Bromberg-Martin et al., 2010). In addition, a number of genetic and pharmacological studies have demonstrated a contribution for dopamine signaling in fear memory, which is mediated by dopamine receptors expressed in the hippocampus, amygdala, PFC, and striatum (Civelli et al., 1993; Pezze and Feldon, 2004). For example, mice lacking the dopamine D1 receptor (D1R) in granule cells of the dentate gyrus and the striatum exhibit poor retention of contextual fear (Ikegami et al., 2014; Sarinana et al., 2014). However, studies examining the impact of global D1R deletion on contextual fear memory have yielded mixed results (Ortiz et al., 2010; Abraham et al., 2016), which may be explained by methodological differences as well as the possibility of recruitment of compensatory pathways. Importantly, with respect to generalization, mice lacking D1R in the dentate gyrus are unable to discriminate between the training context and a novel context after exposure to contextual fear conditioning (Sarinana et al., 2014), while a modest increase in contextual fear generalization is observed when D1R is globally deleted (Abraham et al., 2016). Finally, cued fear memory may or may not be affected by D1R deletion (Ortiz et al., 2010; Sarinana et al., 2014; Abraham et al., 2016), which may be attributed to methodological differences in the studies. It remains to be seen whether these manipulations have an impact on generalization to discrete cues.

In contrast to D1R deletion, pharmacological studies reveal a role for dopamine D2 receptors (D2Rs) in cued fear

generalization. For example, cannulated delivery of the D2R antagonist, raclopride, into the CeA or BNST is sufficient to increase generalization to auditory cues (De Bundel et al., 2016). Specifically, in the latter study, raclopride increases generalization to the CS− tone, while the dopamine receptor agonist, quinpirole, had the opposite effect. On the other hand, in a human fMRI study, pharmacological blockade of dopamine D2Rs produces a reduction in stimulus generalization (Kahnt and Tobler, 2016). Differences in drug specificity or route of delivery may explain these discordant effects. Nevertheless, the authors suggest that the hippocampus flexibly modulates the width of the stimulus generalization gradient, and that the hippocampus can provide active inhibition of generalization, by recruiting a dopamine-dependent process during retrieval. Given that the visual discrimination task employed in the latter study is based on positive reward, it would be interesting to evaluate the effects of D2R blockade in an aversive task in humans.

NA is a neurotransmitter involved in the consolidation of emotional memories during attentional processes, which in turn are essential for maintaining precision of memory (McGaugh, 2013). In rats exposed to contextual fear conditioning, pharmacological enhancement of noradrenergic transmission enhances the consolidation of memory, but also increases generalization of the freezing response to a neutral context (Gazarini et al., 2013). However, the latter effect on generalization was not observed when NA activity was induced after retrieval of fear memory, indicating an important role for NA presumably by interacting with stress hormones at the time of fear learning (McReynolds et al., 2010).

Finally, serotonin signaling is mediated by a large family of serotonin receptors and transporters that exerts complex, often paradoxical effects on both cued and contextual fear memory (Homberg, 2012; Burghardt and Bauer, 2013). However, far less is known about the impact of serotonergic signaling on generalization, which may reflect the complex relationship between serotonin and fear memory. One of the rare examples of research focusing on serotonin-dependent generalization found that male mice lacking the serotonin 1A receptor (5-HT1AR) exhibited heightened generalization of contextual fear, which is proposed to be a hippocampus-dependent effect (Klemenhagen et al., 2006).

Hormones

Contextual generalization occurs more rapidly in female rats compared to males, and is partly mediated by estrogen (Lynch et al., 2013). In ovariectomized female rats, treatment with either an estrogen receptor (ER) agonist or estrogen itself enhances generalization to a neutral context in a passive avoidance task, an effect that is mediated by cytosolic or nuclear (but not membrane-bound) ERs in the dorsal hippocampus (Lynch et al., 2016b). These results provide yet another example of how retrieval of an aversive memory can be dissociated from generalization to a neutral context, because the latter manipulations that enhance generalization did not affect memory retrieval in the training context. Importantly, because the generalization of fear is associated with anxiety-related disorders, which have a disproportionately greater impact on women than men (Kessler et al., 2005, 2012; Tolin and Foa, 2006), estrogen-dependent mechanisms identified in rodents are likely to be clinically relevant. As with estrogen in females, testosterone in males is likewise capable of modulating the processing of fear memory. Gonadectomized male rats exhibit generalized fear to a neutral context in a passive avoidance task, which is ameliorated by injection with testosterone (Lynch et al., 2016a).

Corticosterone, the major glucocorticoid in rodents, is a steroid hormone that is released by the hypothalamic-pituitary-adrenal (HPA) axis during stress (de Kloet et al., 2005), and its effects on learning and memory are well documented (Schwabe et al., 2012; Meir Drexler and Wolf, 2017). In terms of fear generalization, a number of studies have implicated glucocorticoid-dependent signaling. For example, glucocorticoid receptors in the ventral hippocampus or BLA are important for contextual fear, and infusion of corticosterone into the hippocampus after fear conditioning prevents mice from discriminating between correct and incorrect predictors of threat (Donley et al., 2005; Kaouane et al., 2012). Although not all studies demonstrate an effect of corticosterone (Bueno et al., 2017), there is significant variation with regard to many key variables, including conditioning parameters, corticosterone regimen, species and strain differences, etc.

Finally, noradrenergic neurons in the locus coeruleus (LC) respond to orexin, a neuropeptide hormone produced by hypothalamic neurons and involved in the regulation of wakefulness, arousal, feeding behavior, and energy homeostasis (Sakurai, 2007). In conjunction with fear conditioning, optogenetic stimulation of orexinergic projections from the lateral hypothalamus to LC potentiates freezing to a novel context or cue (Soya et al., 2017). Furthermore, orexin neurons modulate a number of signaling pathways described above, including dopaminergic and cholinergic signaling (Sakurai, 2007).

Transcriptional Regulatory Mechanisms

In addition to intercellular signaling, there are a number of intracellular, transcription-based mechanisms that contribute to fear generalization. For example, the cyclic-AMP response element binding (CREB) protein, an inducible transcription factor necessary for the consolidation of fear memories, has been shown to have regionally specific effects on fear generalization. Viral-based overexpression of CREB in the auditory thalamus not only enhances cued fear conditioning, but also increases generalization to the tone (Han et al., 2008). In the mPFC, depletion of the CREB binding protein (CBP), a transcriptional coactivator of CREB and histone acetyltransferase, reduces memory precision and enhances generalization of recent auditory fear memories (Vieira et al., 2014). Interestingly, this generalization emerges after discrimination training, which is consistent with the view that prefrontal circuits may have multiple roles across time (Frankland et al., 2004; Malin and McGaugh, 2006). More recently, the transcription factor Klf9 has been implicated as a stress- and sex-dependent regulator of fear generalization in male mice (Besnard et al., 2018).

Other Signaling Pathways

Finally, limited evidence has supported a role for an assortment of other cellular signaling pathways in the generalization of fear, including nitric oxide, endocannabinoid, as well as cholinergic and neuropeptide Y (NPY) systems. For example, nitric oxide deficiency caused by deletion of the neuronal isoform of nitric oxide synthase (nNOS) increases generalization to odor in both male and female mice, and also inhibits olfactory fear memory (Pavesi et al., 2013). Activation of the cannabinoid system by cannabidiol (CBD) treatment in rodents has no impact on explicit contextual fear memory at 24 h, but generalization to a distinct context is significantly reduced (Stern et al., 2017). Pharmacological targeting of muscarinic acetylcholine receptors modulates generalization of fear with respect to an odor that was previously paired with a foot shock, although the particular brain regions involved in this olfactory paradigm remain to be defined. In another study, lesions of cholinergic inputs from the basal forebrain to the vmPFC results in contextual fear generalization (Knox and Keller, 2016), which the authors suggest is caused by impaired synchronization between the hippocampus and mPFC during fear learning. Finally, NPY is a component of a neuropeptide system that is highly expressed in limbic areas of the brain, where it regulates fear- and anxiety-related behavior (Tasan et al., 2016), while mice lacking NPY or one of its receptors (Y_2) exhibit heightened generalization to auditory cues (Verma et al., 2012).

Considerations in the Molecular and Cellular Mechanisms of Fear Generalization

The signaling pathways implicated in the generalization of fear are both diverse and complex, yet they ultimately converge on the excitatory/inhibitory activity of specific cell types within specific brain regions. While much of our current understanding of molecular and cellular mechanisms of fear generalization is derived from the study of proximal time-points, future studies will need to explore how these signaling pathways operate at the level of distinct neural circuits at remote time-points, and as a function of sex. Furthermore, it will be important to investigate how these signaling events impinge on translational, transcriptional, and post-transcriptional processes to modulate the balance between excitatory and inhibitory signaling to produce adaptive or maladaptive behavioral outputs.

AN INTEGRATED PERSPECTIVE

The generalization of fear is governed by a variety of molecular, cellular, and circuit-level mechanisms that promote the deployment of an optimal defensive response in the face of perceived threat or danger (Maren et al., 2013). Ultimately, the tuning of generalization gradients is a reflection of a complex interplay of multiple internal and external factors, and is shaped by evolutionary imperatives. Although many questions remain as to how this tuning is normally accomplished at a molecular and neural circuit level, and how aberrant tuning might contribute to psychopathology, the evolutionary benefit of generalization itself is clear (e.g., cautious foraging for resources in high-risk environments). Furthermore, the molecular pathways and neural circuits that enable the generalization of experience are governed by positive and negative feedback loops which themselves are dynamic. Indeed, the evolution of complex systems relies on biological networks whose structures are inherently dynamic (Kitano, 2004), and the generalization of fear memories is a prime example of how such networks produce adaptability.

One notable characteristic of the molecular and neural mechanisms described in this review article, whether they promote or inhibit generalization, is that they reflect active processes. As is the case with all aspects of associative memory, including acquisition, consolidation, retrieval, extinction, and forgetting, the generalization of fear is governed by active mechanisms that require significant amounts of energy to regulate highly evolved molecular interactions (Davis and Zhong, 2017). However, it is important to acknowledge the potential contributions of passive mechanisms, despite the fact that these are poorly understood. Given that biological networks are subject to the laws of entropy, it is possible that certain neural circuits are inherently more insulated from the effects of signal degradation than others. Therefore, components of a memory trace may rely on neural circuits that are subject to different rates of decay, leading to imprecise memory (Mensink and Raaijmakers, 1988). Moreover, the entropic decay of elements contained within a memory trace would offer a parsimonious mechanism by which the capacity to generalize may have been shaped by evolution. Furthermore, if synaptic consolidation represents a subroutine of systems consolidation (Dudai et al., 2015), it is likely that even subtle losses in fidelity of the signaling events that govern synaptic plasticity are manifested in higher-order neural functions. Thus, a major focus in the immediate future should be with the identification of whole-brain activity patterns associated with different types of fear generalization (e.g., to contexts and discrete cues) across time.

What can we learn from this discussion of the neurobiology of fear generalization? First, under a certain set of conditions, fear generalization at recent and remote time-points is modulated by the strength of learning (Biedenkapp and Rudy, 2007; Poulos et al., 2016). However, the loss of memory precision driven by forgetting or the inhibitory weakening of the initial memory trace seems to be a continual process. This weakening and loss of precision occurs both in micro-circuits within a brain region (e.g., the central amygdala, dentate gyrus, etc.) as well as in macro-circuits between brain regions (e.g., ventral hippocampus to mPFC; Ciocchi et al., 2010; Cullen et al., 2015). In particular, amygdalar, prefrontal, hippocampal, and thalamic areas appear to be especially important for fear generalization, and a delicate balance between excitatory and inhibitory transmitters, receptors and synapses is critical. Moreover, the neural circuits initially involved in hippocampal-related processes such as pattern separation and pattern completion (Rolls, 2013) may have a different contribution to fear memories and generalization at recent vs. remote time points (Kitamura et al., 2017; Khalaf et al., 2018). Thus, the generalization of fear which occurs at remote timescales likely results from an interaction between the

initial associative strength, systems consolidation, and the natural weakening or forgetting of the original memory.

FUTURE DIRECTIONS

Understanding the neurobiology of fear generalization is a critical step in the development of novel therapeutic approaches for treating psychiatric disorders such as PTSD. Because fear generalization is conserved across species, animal models are indispensable in the search for causative and potentially exploitable relationships between molecular, cellular, and circuit-level events that influence behavior. With this idea in mind, we emphasize several ideas, both old and new, that should be considered in the design of future studies.

First, given the sexual dimorphisms observed in many psychiatric illnesses, the use of both male and female animals is of paramount importance. Fortunately, at least for several types of fear-related behaviors observed in female C57BL/6 mice, strict monitoring of estrous phase may not be necessary (Meziane et al., 2007; Keiser et al., 2017), and therefore naturally cycling females can be used. In addition, the persistent nature of PTSD and anxiety disorders emphasizes the need to examine remote time-points and other processes such as fear relapse in behavioral experiments (Goode and Maren, 2014; Goode et al., 2018). Also, because psychiatric disorders such as PTSD are highly heritable (Duncan et al., 2018), it will be critical to develop and characterize animal models with genetic alterations at defined loci, whether borne out by GWAS studies or candidate approaches. In this regard, it would be helpful to revisit mouse genetic models that exhibit alterations in fear memory, yet have not been evaluated in remote generalization experiments, given the overlapping circuitry that regulates these processes. Furthermore, because memory traces evolve over time with regard to both their precision and how they are stored in neural circuits, we emphasize the need to evaluate remote changes at the electrophysiological and structural level. For example, optogenetic interrogation of cortical ensembles that are activated during remote fear generalization should help to elucidate the nature of systems consolidation. Finally, cued fear conditioning may be more relevant to short-lasting fear, while contextual conditioning may be more relevant to longer-lasting anxiety states (Davis et al., 2010; Shackman and Fox, 2016; Asok et al., 2018a). Although studies of both types of fear memory have generated a trove of basic neurobiological knowledge, it is likely that contextual models will prove to be especially useful. The breadth and depth of sensory and cognitive experiences associated with a traumatic event in humans suffering from PTSD may be approximated more effectively by a multimodal CS rather than a discrete sensory cue.

In terms of developing new and effective treatments for fear-related disorders, circuit-level approaches such as transcranial magnetic stimulation (Kozel, 2018) and deep brain stimulation (Bina and Langevin, 2018) are promising, although they lack the specificity afforded by pharmacological approaches. The molecular and cellular pathways involved in the processing and storage of fear memories can already be targeted at multiple levels by a vast and extant pharmacopeia with undiscovered capacity to modulate the generalization of fear. However, a given drug target that is expressed throughout the brain can serve distinct functions depending on its subcellular localization in particular brain areas (Engin et al., 2018), making it difficult to modulate specific neural circuits with strictly pharmacological approaches. Therefore, to achieve both molecular and circuit-level specificity, it will be important to capitalize on new technologies that allow cell-type specific targeting of compounds (Nassi et al., 2015; Shields et al., 2017). Well-designed pre-clinical animal studies using targeted delivery of potential therapeutic drugs to examine their effects on fear memory and generalization, across long timescales and as a function of sex, will provide a critical stepping-stone in translating novel compounds from bench to bedside.

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AA and JR wrote the manuscript. EK provided important conceptual insight and helped prepare the manuscript.

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frontiers in Behavioral Neuroscience

REVIEW
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Sensory Ecology of Predator-Induced Phenotypic Plasticity

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Ecological communities are organized in trophic levels that share manifold interactions forming complex food webs. Infochemicals can further modify these interactions, e.g., by inducing defenses in prey. The micro-crustacean *Daphnia* is able to respond to predator-specific chemical cues indicating an increased predation risk. *Daphnia* shows plastic responses by adapting its morphology, behavior, and physiology, increasing organism, and population fitness. This stabilizes community structures. This review will describe the progress that has been made in understanding the high degree of plasticity observed in the model crustacean *Daphnia*. I summarize current knowledge on the processes of predator detection, ranging from the nature of biologically active chemical cues to the underlying neurophysiological mechanisms. With this, I aim to provide a comprehensive overview on the molecular mechanisms of *ad hoc* environmental phenotypic adaptation. In times of climate change and pollution understanding information transfer in aquatic systems is valuable as it will allow us to predict whether and how community structures are being affected.

Keywords: phenotypic plasticity, daphnia, protocerebrum, deutocerebrum, chemoreceptors, neckteeth, inducible defenses

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INTRODUCTION–SENSORY SYSTEMS

Organisms have evolved the capacity to respond to changes in the environment by phenotypic adaptation. Fluctuations in the biotic environment are dominated by the ever-changing presence and absence of con- and heterospecific organisms, prey, and predators. Therefore, detecting mates, prey or predators, and responding appropriately is critical for organisms' survival. Species have evolved dedicated sensory systems that are optimized to their particular ecology. Chemical perception is of central importance, alongside vision, mechanoreception, and electrical senses (Wisenden, 2000; Ferrari et al., 2010). Chemical cues are of particular relevance in aquatic environments as they can be identified regardless of turbidity and are reliable in space and time. Many prey organisms are able to detect the presence of predators even against a background of high chemical diversity. This allows the prey to respond to predator presence with plasticity of their phenotype and increase survival chances. Yet, for any kind of plasticity to be adaptive in the context of an increased risk of predation, the correct interpretation of the environmental challenge is pivotal, as a mal-adapted phenotype could in fact increase its predation risk. Understanding the sensory mechanisms and neuronal pathways underlying predator perception is also critical in the light of anthropogenic disturbances, which often interfere with neuronal signaling cascades.

This review focuses on the neuronal mechanisms underlying predator-induced morphological plasticity. Other defensive strategies like behavioral and life history adaptations, have so far received less attention. I will introduce the concepts of phenotypic plasticity and inducible defenses and explain their ecological relevance.

I summarize the general insights that underlie chemical predator perception, neuronal wiring, neurophysiology, and neuronal plasticity.

This review centralizes on the perception and neuronal processing of kairomones. In the classical and broad sense kairomones are defined as interspecific chemical cues perceived by a benefiting prey to reduce the negative impact of a natural enemy (Ruther et al., 2002). As there are already numerous, valuable, and detailed reviews on the perception of chemical alarm pheromones (Sorensen and Stacey, 2004; Døving and Lastein, 2009; Dew et al., 2014; Ahuja et al., 2015; Lastein et al., 2015; Wisenden, 2015), these and the perceptive mechanisms will not be reviewed here.

I detail current knowledge of predator detection and neuronal signaling of predator-induced morphological plasticity in the freshwater crustacean *Daphnia*. This zooplankter is an important component of freshwater food webs, showing a strong plasticity against a range of predators. Several *Daphnia* genomes have now been sequenced (Colbourne et al., 2011; Ye et al., 2017, www.fleabase.org) and accordingly molecular biology applications (e.g., *in situ* hybdridization, RNAi, etc.) are becoming available (Kato et al., 2012; Nakanishi et al., 2014; Naitou et al., 2015; Nong et al., 2017). Many studies have investigated the changes in gene expression levels that affect morphological defense expression in *Daphnia pulex* (Schwarzenberger et al., 2009; Spanier et al., 2010; Miyakawa et al., 2015; Rozenberg et al., 2015, 2016; Hales et al., 2017), while only a few have looked at the (neuro-) physiological changes (Hanazato, 1991; Barry, 1999, 2002; Weiss et al., 2012a; Miyakawa et al., 2015).

PHENOTYPIC PLASTICITY

Genotypes equipped with adaptive strategies to increase individuals' fitness can help organisms to conquer environments with fluctuating conditions. From an ecological and evolutionary perspective, phenotypic plasticity is a powerful, and widespread means of organismal adaptation. Phenotypically plastic organisms may respond to environmental extremes, and Bradshaw was one of the first to recognize the importance of genetic variation of plasticity in an evolutionary context (Bradshaw, 1965). She postulated that plasticity should be understood as a trait that underlies evolutionary trajectories, such as selection. Moreover, phenotypic plasticity can be discussed as a means of evolution affecting biodiversity by enabling better niche use or the exploitation of several niches. Evolution of adaptive phenotypic plasticity has led to the success of organisms in novel habitats, and potentially contributes to genetic differentiation and speciation (Miner et al., 2005; West-Eberhard, 2005; Theißen and Melzer, 2016). An expanding body of work examines how plasticity can affect all levels of ecological organization through effects on demographic parameters, but also through direct and indirect species interactions, such as competition, predation, and coexistence (reviewed in Miner et al., 2005). For example, there are recent arguments that predator-induced plasticity makes evolutionary change possible and that evolution may be preceded by genetic assimilation (Reger et al., 2018).

Despite the growing body of work, the question of how plasticity is realized remains and the underlying molecular mechanisms are often unexplored. To elucidate these pathways, it is important to first distinguish whether the phenotypic adaptations are induced by biotic or abiotic environmental cues. The abiotic environment can affect the phenotype by physical laws (Kelly et al., 2012). So, for example, temperature can cause phenotypic changes through enzyme kinetics and diffusion rates (Kelly et al., 2012). Similarly, low nutrient availability can impact growth and morphology. Cold acclimation is known to result in metabolic responses involving increases in mitochondrial amount and capacity (Healy et al., 2017). Other examples of abiotic induction of plasticity are photoperiod-induced life history shifts in aphids (Simon et al., 2011) and timing of metamorphosis in amphibians (Wright et al., 1988).

Biotic environmental challenges originate from con- and heterospecifics and are in general signaled through chemical cues: predators release semiochemicals (kairomones), which indicate predation risk and conspecifics may release density cues, which modify and fine tune the response (Tollrian et al., 2015). The phenotypic change is realized through a complex interaction between these environmental chemical cues and organismal sensors. For example, prey species sense the chemical cues released by their predators. The active sequence of events from cue release → cue perception → signal transmission → endocrine signaling → phenotype adaptation is mostly elusive (Beldade et al., 2011; Morris and Rogers, 2014). One particular form of phenotypic plasticity are inducible defenses, where the phenotype is adapted to an increased predation risk.

INDUCIBLE DEFENSES

Predation is a major factor driving adaptation and predator-induced defenses are an intriguing form of phenotypic plasticity that can decrease the likelihood of predator encounter or detection, and reduce the effectiveness of predator attacks. Ecologically, inducible defenses have been discussed to be of high importance as they dampen predator–prey oscillations, thereby stabilizing population dynamics (Verschoor et al., 2004). Many different defensive strategies expressed against an increased predation risk have been described (reviewed in Tollrian and Harvell, 1999). Defenses may occur in the form of behavioral or morphological adaptations or shifts of life history parameters, and many taxa show a variety of different anti-predatory adaptations (reviewed in Weiss et al., 2018).

For precise development of the appropriate defense, prey organisms must be able to accurately distinguish between predators; the expression of a defense that is not effective against the predator may pose a disadvantage and thereby reduce organism fitness (reviewed in Weiss and Tollrian, 2018). In addition, prey organisms must also be able to respond to multiple predators simultaneously as in most ecosystems predation is not limited to a single predator. All this is further complicated, as

the expression of inducible defenses is not simply adapted to the predator but also to the predation risk (Tollrian et al., 2015; Crane and Ferrari, 2017). It was shown that, with growing conspecific numbers conspecific chemical cues indicate that the predation risk decreases, upon which defense expression is reduced (Peacor, 2003; Tollrian et al., 2015).

The fact that organisms are able to distinguish between predators, con- and heterospecifics and perform predation risk assessment in combination with a fine-tuned defense expression, shows that dedicated sensory systems must have evolved, enabling correct interpretation of the environment. The freshwater crustacean *Daphnia* has been especially well-studied for its capacity to express a diversity of inducible defenses.

INDUCIBLE DEFENSES IN *DAPHNIA*

Inducible defenses in *Daphnia* are manifold. Some *Daphnia* species show behavioral adaptions to fish predation (**Figure 1**). They perform fish-induced diel vertical migration, seeking refuge in the deeper water strata during the day, in order to escape visual predators (Ringelberg, 2010). Intriguingly, this defensive strategy is induced by two co-occurring cues: the predator and light conditions. Also, reduced swimming speeds have been reported to occur countering predation by the tactile predator the phantom midge larvae *Chaoborus* (Dodson et al., 1997). Shifts of life history parameters, are similarly expressed against fish predation (Brett, 1992; Hanazato et al., 2001, **Figure 2**; Boersma et al., 2009). Here somatic growth is traded with reproduction and the presence of fish induces early maturation at a smaller size and the production of more and smaller offspring in subsequent generations (Brett, 1992; Tollrian, 1995; Carvajal-Salamanca et al., 2008; Boersma et al., 2009).

Morphological defenses in *Daphnia* have been in the focus of ecological research for decades (Krueger and Dodson, 1981; Tollrian, 1993; Stollewerk, 2010; Tollrian and Leese, 2010; Weiss et al., 2012b); including helmet development in *D. cucullata* (Tollrian, 1990, **Figure 3A**) and neckteeth expression in *D. pulex* against *Chaoborus* spec. predation (Krueger and Dodson, 1981; Tollrian, 1993; Weiss et al., 2016, **Figure 3B**), crest development in *D. longicephala* against the heteropteran backswimmer *Notonecta* spec. (Grant and Bayly, 1981; Weiss et al., 2015, **Figure 3C**), head- and tail-spine development in *D. lumholtzi* against fish predation (Tollrian, 1994, **Figure 3D**) and crowns of thorns in *D. atkinsoni* against the tadpole shrimp *Triops* spec. (Petrusek et al., 2009).

PREDATOR-SPECIFIC CHEMICAL CUES–IDENTIFICATION OF FRIENDS AND FOES

Sensory information often plays a pivotal role in shaping species interactions (Hay, 2009). Species acquire information about their biotic and abiotic environment by detecting specific chemical cues (Atema et al., 1988). Organisms with poor vision and those in turbid environments are particular beneficiaries of chemical cues. Furthermore, chemical cues can be transmitted over long temporal, and spatial scales (Wisenden, 2000). A network of chemical cues is speculated to significantly complicate our current knowledge of trophic interactions (Burks and Lodge, 2002; Pohnert et al., 2007). It is therefore obligatory to not only describe trophic interactions *per se* but also identify the modifying agents. The different type of cues are distinguished based on their origin; so that the sources need to be determined prior to correct categorization into either alarm cues or predator specific cues (Hazlett, 2011; Wisenden, 2015). Sometimes, this even requires the chemical identification of the compound(s). While alarm cues are chemicals of conspecific prey, and released upon mechanical damage of the same, predator specific cues are generally unintentionally released by the predator. Predator cues can also be but do not have to be associated to foraging activities (Mitchell et al., 2017). While there are numerous reviews on alarm cues (Hazlett, 2011) some with the special focus on fish (Døving and Lastein, 2009; Lastein et al., 2015; Wisenden, 2015), predator specific cues are less well-reviewed and will therefore be the focus here.

Interspecific chemical cues released by predators induce the development of defensive features in prey organisms (Tollrian and Harvell, 1999; Weiss et al., 2012b). These, so-called, kairomones are released by a sender serve as an advantage for a receiver, as in this context they indicate an increased predation risk. Kairomones decrease the efficiency of the predator (Jeschke et al., 2002) thereby affecting the dynamics of entire food webs (Kats and Dill, 1998; Dicke and Grostal, 2001; Verschoor et al., 2004; Vos et al., 2006).

When these substances are associated with foraging activities, the value of this cue as a signaling agent increases significantly for the prey organisms and evolution of sensitivity to such cues should be favored. Unfortunately, many of the infochemicals's structure and/or composition remain elusive. Kairomones have disparate chemistry, for example they can be proteins that signal the development of inducible defenses in ciliates (Kusch and Heckmann, 1992), which develop lateral wings in the presence of the predatory ciliate *Lembadion*. Alternatively, aliphatic sulfates released by *Daphnia* inducing defensive coenobia in algae (Yasumoto et al., 2005), and composites like the recently described copepodamides induce the production of the paralytic shell fish toxin in the dinoflagellate *Alexandrinum minutum* (Selander et al., 2015). Trigonelline and homarine were shown to induce fear in mud crabs (Poulin et al., 2018). In rodents, fear responses are induced by volatile molecules such as TMT, 2-PEA, and 2-PT, which result from meat digestion in the predator (Pereira and Moita, 2016).

These signals appear to be enormously diverse in chemical and temporal structure, and the ability to identify and quantitate them, especially from the aquatic environment, has been a major challenge in chemical ecology. The identification of such signaling agents is however a pivotal component in our understanding of predator perception. Of course, chemoreceptors play a critical role, where the identification and deorphanization of an explicit chemoreceptor requires knowledge of the ligand.

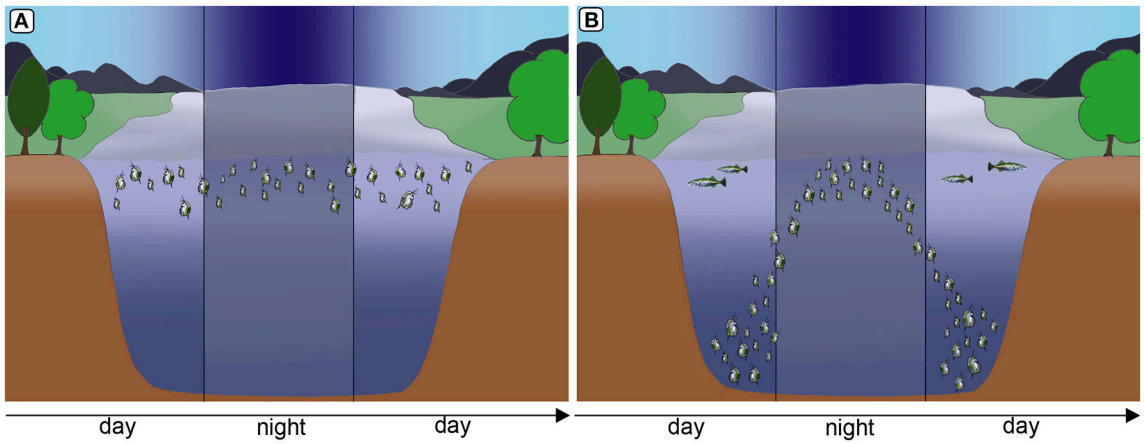

FIGURE 1 | Diel vertical migration as a form of behavioral defense in *Daphnia* to counter fish predation. In the absence of fish predation *Daphnia* remain in warm, nutritious water strata **(A)**. Under predation, *Daphnia* migrate to deeper water strata, seeking refuge from visual predators. However, during the night they migrate to nutrient rich and warmer strata for feeding **(B)**. Images by Weiss.

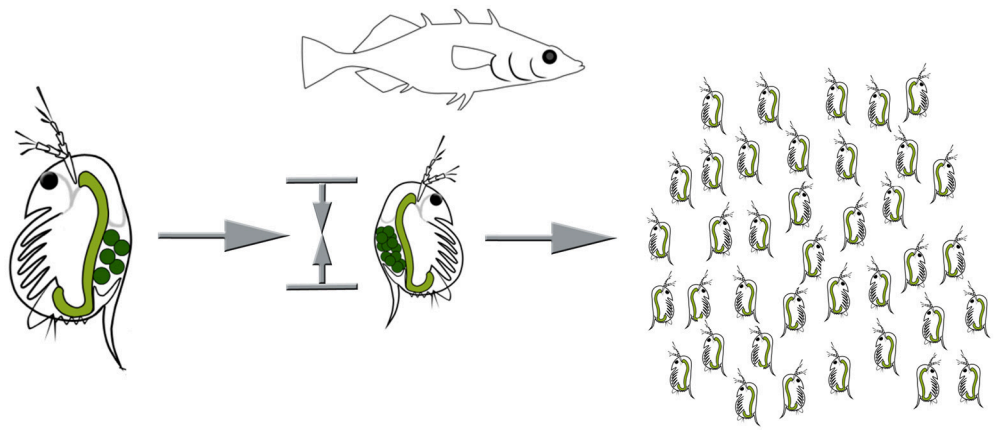

FIGURE 2 | Life history adaptations under fish predation. Resources are shifted from somatic growth to reproduction, resulting in earlier sexual maturity at a smaller size, and an increased number of smaller offspring. Images by Weiss.

THE *CHAOBORUS* KAIROMONE

Fourth instar phantom midge larvae of the genus *Chaoborus* (Diptera) prey upon juvenile *D. Pulex*. The larvae grasp the daphniid prey with their feeding basket composed of head appendages. The prey is captured and consumed by alternating movements of the mandibles. All digestible parts are consumed, and the indigestible components are egested. The kairomone is released during the egestion process. It comprises a family of compounds consisting of long-chained (≥C14) fatty acids coupled to the N-terminus of an L-glutamine residue. Lipidated -L-glutamine conjugates released by feeding *Chaoborus* larvae trigger defensive neckteeth formation in juvenile *Daphnia* (Weiss et al., 2018).

These molecules carry characteristics of suitable aquatic infochemicals: they are water-soluble and thus pass from the emitting organism (i.e., predator) to the receiving organism (e.g., prey). Additionally, because of their origin from active digestion they are good indicators of predation risk.

Regarding the origin of the kairomone, it is anticipated that the larvae take up fatty acids from their diet (in this case the *Daphnia,* yet *Chaoborus* also consumes e.g., ciliates and this rearing medium also induces defensive neckteeth, Tollrian personal communication) and use them for glutamine assimilation in the mid-gut as seen in many caterpillars or other dipteran species like *Drosophila* (Mori and Yoshinaga, 2011; Yoshinaga, 2016). Glutamine seems to function as a nitrogen storage as it is mandatory for many biosynthetic pathways. This also explains why co-evolution leading to suppression of chemical release is hampered. Unsurprisingly, substances whose production cannot be avoided are exploited as reliable interspecific information cues by the different prey species. The active components of the *Chaoborus* kairomone are only produced during active feeding, however the type of diet is irrelevant (Tollrian, personal communication). For categorization purposes, this means that the *Chaoborus* kairomone is not a dietary alarm cue, but an activity dependent compound produced by the predator during digestion. It is

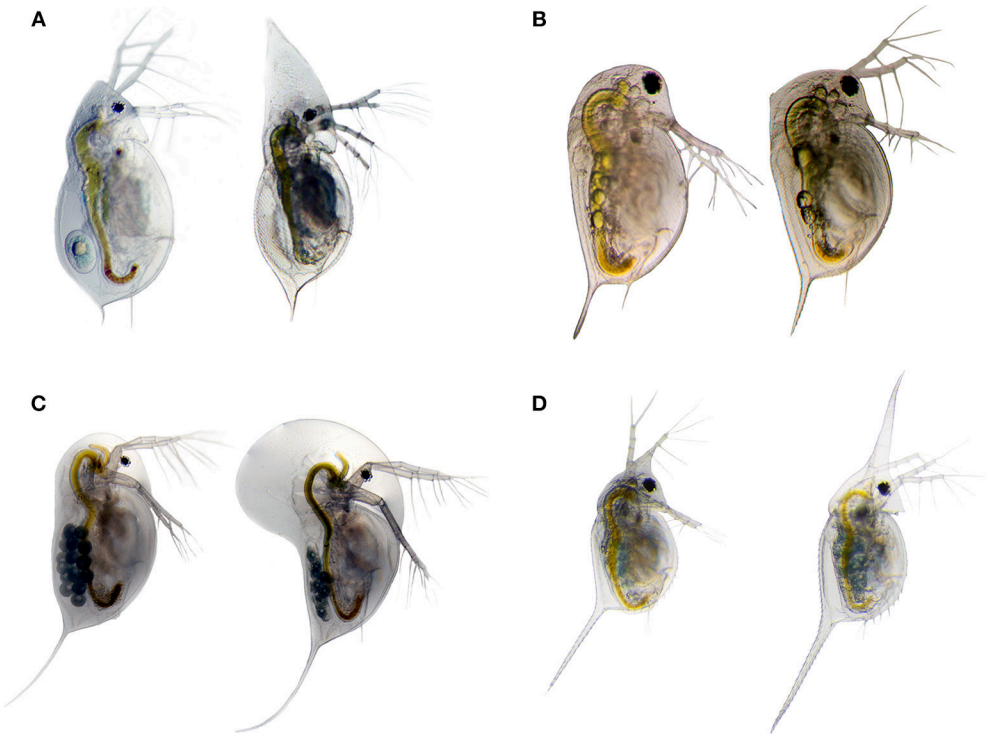

FIGURE 3 | Inducible morphological defenses are manifold in the genus *Daphnia*. The listed examples show helmet expression in *D. cucullata* **(A)**; crest expression in *D. longicephala* **(C)**; head- and tail-spine formation *D. lumholtzi* **(D)**, and neckteeth expression in *D. pulex* **(B)**. Undefended morphotypes are displayed on the left side, and the defended morphotype on the right side. Images by Becker & Weiss.

beneficial for the predator only in the context of metabolism. A benefit in the context of information transfer is only advantageous for the receiver, i.e., the prey.

Having the information of the inducing agent is critical for the identification of respective chemoreceptors and the signaling pathways used to detect such cues within environmental noise.

CHEMORECEPTORS

By 1991 Buck and Axel had identified a set of ca. 1,000 olfactory receptors (ORs) in rats (Buck, 1996). These belong to a class of rhodopsin-like G-protein coupled receptors (GPCRs) and have been identified in all vertebrates. No homologs of these receptors have been detected in any protostome. In insects, a distinct group of ORs and gustatory receptors (GRs) have been described that are not homologs to the vertebrate ORs (Vosshall et al., 1999). They represent an individual gene family, with the GRs being the ancestral type. These are ionotropic receptors (ligand-gated ion channels), similar to traditional ionotropic glutamate receptors such as kainite, N-methyl-D-aspartate (NMDA), and AMPA receptors. Compared with vertebrate ORs, insect ORs show an inverted membrane topology with extracellular carboxyl and intracellular amino terminals (Galizia and Sachse, 2010). Insect ORs function as heteromultimers composed of at least one ligand-specific OR and the co-receptor Orco (Vosshall et al., 1999; Galizia and Sachse, 2010; Wicher, 2018). Neither Orco nor ORs are present in the genome of the crustacean *D. pulex*, indicating that ORs are insect specific. However, GRs were found in *Crustacea*, just as in insects (Peñalva-Arana et al., 2009).

Another example of chemoreceptor proteins is a subset of ionotropic receptors (IRs) found in all protostome clades, known as the IRs. These probably evolved from the non-NMDA ionotropic glutamate receptors in ancient protostomes (Croset et al., 2010).

For a comprehensive review of the different chemoreceptor proteins and their evolution please refer to (Vosshall et al., 1999; Derby et al., 2016; Brand et al., 2018).

Our understanding of how predator kairomones are perceived and processed is very limited and dedicated chemoreceptors have predominantly been identified in mice (Isogai et al., 2011). In this study, con- and heterospecific chemical cues stimulated neurons in the vomeronasal organ (VNO) were screened for chemoreceptor expression (Isogai et al., 2011). It was thus possible to detect the activation of neurons with specific receptors during chemical cue exposure. The authors found that within the 250 receptors that are expressed in the VNO of mice, 71 receptors in total exclusively respond to heterospecific cues, only 11 of these receptors also responded to conspecific cues (Isogai et al., 2011). Intriguingly, some receptors were exclusively responsive to different types of predators so that two receptors responded to snake cues, while others responded to owl cues. Each predator species tested activated a distinct subset of receptors, pointing to the capacity of mice to be able to distinguish between predators (Isogai et al., 2011).

In general, chemical cues bind to chemoreceptors that are located on some kind of chemoreceptive organ innervated by olfactory receptor neurons (ORNs).

Olfactory wiring is achieved as one ORN expresses one functional receptor type, and all ORNs expressing the same receptor type coalesce in one glomerulus of the odor information processing region e.g., the olfactory lobes in arthropods (Strausfeld and Reisenman, 2009), or the olfactory bulb in mammals (Nagayama et al., 2014).

CHEMORECEPTORS IN *DAPHNIA*

Compared with other taxa (like insects and mammals) not much is known about chemoreceptors in *Daphnia*. There is a known repertoire of 58 chemoreceptors that cluster in 3 distinctive superfamilies and share sequence homology with insect gustatory receptors (Grs). No genes encoding proteins similar to the insect odorant receptors (Ors) were found, which might indicate a quite recent expansion of this gene lineage concomitant with the evolution of hexapods (Peñalva-Arana et al., 2009). This is further supported by the observation that, as yet, Ors are also absent in other crustacean genomes. Yet, it is highly questionable that *Daphnia* relies on these 58 Grs only. Recently, it was reported that crustacean olfactory receptors are orthologs of insect olfactory IRs and the *Daphnia* genome holds an abundant number of IRs (Croset et al., 2010). In fact, it was shown later in lobsters that two IR subunit genes *PargIR25a* and *PargIR93a* are expressed in most or all spiny lobster ORNs, as confirmed by *in situ* hybridization (Corey et al., 2013). Other encoded IR subunits are expressed only sparsely, suggesting an ORN-specific expression pattern. This suggests that, as in insects, the odorant specificity of individual lobster ORNs is determined by a specific set of expressed subunits and that these subunits are composed of *IR25a* and/or *IR93a* co-receptors (Croset et al., 2010; Corey et al., 2013).

Thus, it is likely that IRs play a general role in initiating chemosensory signaling in crustaceans and also in *Daphnia*. Yet, limited attempts have been undertaken for heterologous expression and deorphanization of the chemoreceptors that have been identified by sequence homology with other arthropods. It is also likely that there are additional classes of chemoreceptors yet to be discovered (Derby et al., 2016; Harzsch and Krieger, 2018).

For a precise description of how environmental chemical cues are decrypted, it is pivotal to deorphanize receptors specific for kairomones but also disentangle the underlying neuronal structures involved in kairomone perception such as nervous fibers and the computational steps in higher brain areas.

NEURONAL WIRING OF OLFACTORY RECEPTOR NEURONS

Neural circuits are both anatomical and functional entities and the involved neurons never function in isolation. Such neuronal circuits process specific kinds of information and their identification is crucial for the understanding of how sensory information is encoded. There is a general concept of odorant coding found in vertebrates and invertebrates. Odorants are first detected by chemoreceptors located on ORNs. The axons of the neurons then organize centrally into glomeruli organized by olfactory receptor type (Vosshall et al., 1999; Derby et al., 2016; Harzsch and Krieger, 2018). A glomerulus is a roundish substructure that contains most synapses within the antennal lobe or olfactory bulb (depending on taxon). In vertebrates and invertebrates alike, projection neurons (PNs) relay olfactory inputs to higher-order brain areas like the mushroom bodies and the lateral horns (in insects). These brain areas permit associative learning or mediate innate behaviors (reviewed in Galizia and Rössler, 2010).

While the projection neurons are uni- and multi-glomerular and suspected to be of cholinergic pharmacology (Galizia and Rössler, 2010), there are also local neurons (LNs) that interconnect the glomeruli and are of the amacrine type (Homberg et al., 1989). They are diverse in morphology and controlled by inhibitory neurotransmitters like GABA or excitatory neurotransmitters like acetylcholine. For further detail please see Galizia and Rössler (2010); Galizia and Sachse (2010); Derby et al. (2016); Harzsch and Krieger (2018).

NEURONAL AND CELLULAR WIRING IN *DAPHNIA*

In order to understand the cellular mechanisms of plasticity, an overview of the overall nervous system and the functioning of the individual components is necessary. Even if the neuroanatomy of the *Daphnia* nervous system appears comparatively simple, it is known to be able to discriminate a vast array of intra- and interspecific signals. The *Daphnia* brain is of classical arthropod organization and consists of three regions, the protocerebrum, the deutocerebrum, and the tritocerebrum (syncerebrum) as described in other branchiopod crustacean species (Harzsch and Glötzner, 2002; Kirsch and Richter, 2007; Fritsch and Richter, 2010; Kress et al., 2016). In the protocerebrum, the optical neuropils are connected via the optical tracts with its remaining scaffold (Weiss et al., 2012c). The deutocerebrum receives nerve fibers from the antennule (Weiss et al., 2012c, **Figure 4A**). Nerves originating from the tritocerebral ganglia enter the antenna, the labrum, and the alimentary tract (Weiss et al., 2012c). The tritocerebrum is thus also involved in functions of the stomatogastric nervous system (Heribert, 1915; Bullock, 1965; Weiss et al., 2012c). The protocerebrum is the anterior-most neuropil and comprises the largest portion of the brain (**Figures 4B–D**). The deutocerebrum is proximal to the protocerebral neuropil. The deutocerebral neuropil is less distinctive than the protocerebrum and consists of a pair of undifferentiated neuropils (Hallberg et al., 1992; Harzsch, 2006). The question now is, how are such adaptive processes encoded on the neuronal level. The receptors for the detection of predator cues were shown to be located on the first antennae (Weiss et al., 2015, **Figure 4A**). From here neurites extend to the deutocerebrum of the brain (Weiss et al., 2012c, **Figures 4A,D**). Yet, olfactory glomeruli in the deutocerebrum have not been detected Hallberg et al., 1992; Weiss et al., 2012c and the precise wiring underlying predator detection in *Daphnia* is unknown.

Likewise, the chemical and functional description of individual brain cells and specialized brain centers is yet understudied. For example, there is a group of serotonergic cells located in the protocerebrum that probably control phototactic behaviors (Rivetti et al., 2018, **Figure 4B**) and may therefore also be involved in predator-induced diel vertical migration patterns, which are triggered by changes in light intensity (Ringelberg, 2010).

On the cellular level outside the nervous system, a group of large polyploid cells located in close association with the morphological defense structure in various daphniid subgenera was suggested to be involved in the development of defensive traits (**Figure 4E**). These cells were speculated to serve as central control stations secreting proliferation agents (Beaton and Hebert, 1997) like dopamine inducing the mitotic activity in the vicinity of these cells (Weiss et al., 2015). Nonetheless, it remains only speculative how these cells are controlled and how they determine the development of phenotypically plastic morphological defenses.

NEUROPHYSIOLOGY OF PREDATOR-INDUCED DEFENSES IN *DAPHNIA*

Neurophysiological stimulation studies have shown that the cascade underlying predator perception and defense expression, comprises multiple signaling components, including the involvement of cholinergic, glutaminergic and GABAergic signaling (Weiss et al., 2012a; Miyakawa et al., 2015). In general, acetylcholine in the brain alters neuronal excitability, influences synaptic transmission, induces synaptic plasticity, and coordinates firing of groups of neurons (Picciotto et al., 2012). As a result, it changes the state of neuronal networks throughout the brain and modifies their response to internal and external inputs, which is the classical role of a neuromodulator (Picciotto et al., 2012). Glutamate is a dominant neurotransmitter in nervous systems and activates neurons (Meldrum, 2000). Neuronal receptors for glutamate are divided into two groups: the metabotropic glutamate receptors, which are members of the G-protein coupled receptor family, and ionotropic glutamate receptors, which are members of the ligand-gated ion channel family. Ionotropic glutamate receptors are further divided into three groups whose names are derived from specific agonists: NMDA-type, (±)-α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA)-type and kainate-type (Meldrum, 2000). These subtypes are expressed mainly in central nervous systems and are involved in various biological processes, including memory and learning, in many animal species (Malenka and Nicoll, 1999). Genes coding for these receptors were identified using microarrays and their involvement in defense expression was further validated with a functional analysis using the receptors' antagonists (Miyakawa et al., 2015).

GABA is a major inhibitory neurotransmitter, reducing a nerve cell membrane potential and thereby decreasing its excitability (Wu and Sun, 2015). GABA is always functional in the nervous system, fine-tuning neuronal responses and controlling neuronal firing rates (Wu and Sun, 2015). In predator-exposed *D. pulex,* genes for G-protein coupled GABA receptors were differentially expressed (Miyakawa et al., 2015). Only the $GABA_B$ receptor type is metabotropic and therefore G-protein coupled. While Barry (2002) antagonized the actions of GABA using picrotoxin, a non-competitive antagonist of the ionotropic $GABA_A$ receptor (Barry, 2002), neurophysiological stimulation with GABA did not validate direct involvement of GABergic signaling (Weiss et al., 2012a). Indirect GABAergic signaling is reasonable, but not observable with simple neurophysiological stimulation in bioassays. Rather this requires e.g., patch-clamp measurements of ion currents in culture cells, or optogenetic strategies applied *in vivo* (Spoida et al., 2014).

NEURONAL PLASTICITY

Ever since the pioneering work of Drs. Hubel and Wiesel more than 40 years ago, neurobiologists have appreciated that the environment plays an essential role in shaping neural connectivity. These observations are framed by the term neuronal plasticity, which is known as the ability of the brain to change throughout an individual's life. This includes changes in gray matter, constant removal and creation of synapses depending on the activity level, or dendritic outgrowth adjustment all according to neuronal activity levels. This kind of activity-dependent plasticity is a form of functional and structural neuroplasticity arising from cognition and experience. It is thus the basis for learning and the formation of memories. Also, neuronal plasticity is a result of changes in gene expression patterns triggered by dedicated signaling cascades activated by signaling molecules such as calcium, dopamine, and glutamate.

Within an ecological perspective it has been seen that both relative brain size and structure are statistically correlated with environmental parameters. These include spatial complexity of the natural and social environment, water depth, light environment, and predation (Samuk et al., 2018). For example, an analysis of 623 pairs of predator and prey species of fish found that on average, prey species tend to have larger brains than the species that prey upon them, perhaps suggesting a "cognitive arms race" (Samuk et al., 2018).

Other studies described how predator exposure can change brain morphology e.g., predator exposed nine-spined sticklebacks grow larger *bulbi olfactorius* (Gonda et al., 2011), but the opposite effect has also been observed where predator-exposed brains become shorter and narrower. The underlying cause of this change in morphology, however, was not investigated. It remains elusive, from where the structural change originates, so that an increase in brain size could indicate an increased number of nerve cells or an increased number of cellular connections.

NEURONAL PLASTICITY IN *DAPHNIA*

If and how the neuroanatomy of the brain changes during predator exposure requires investigation. So far, only the involvement of NMDA receptors, which only respond

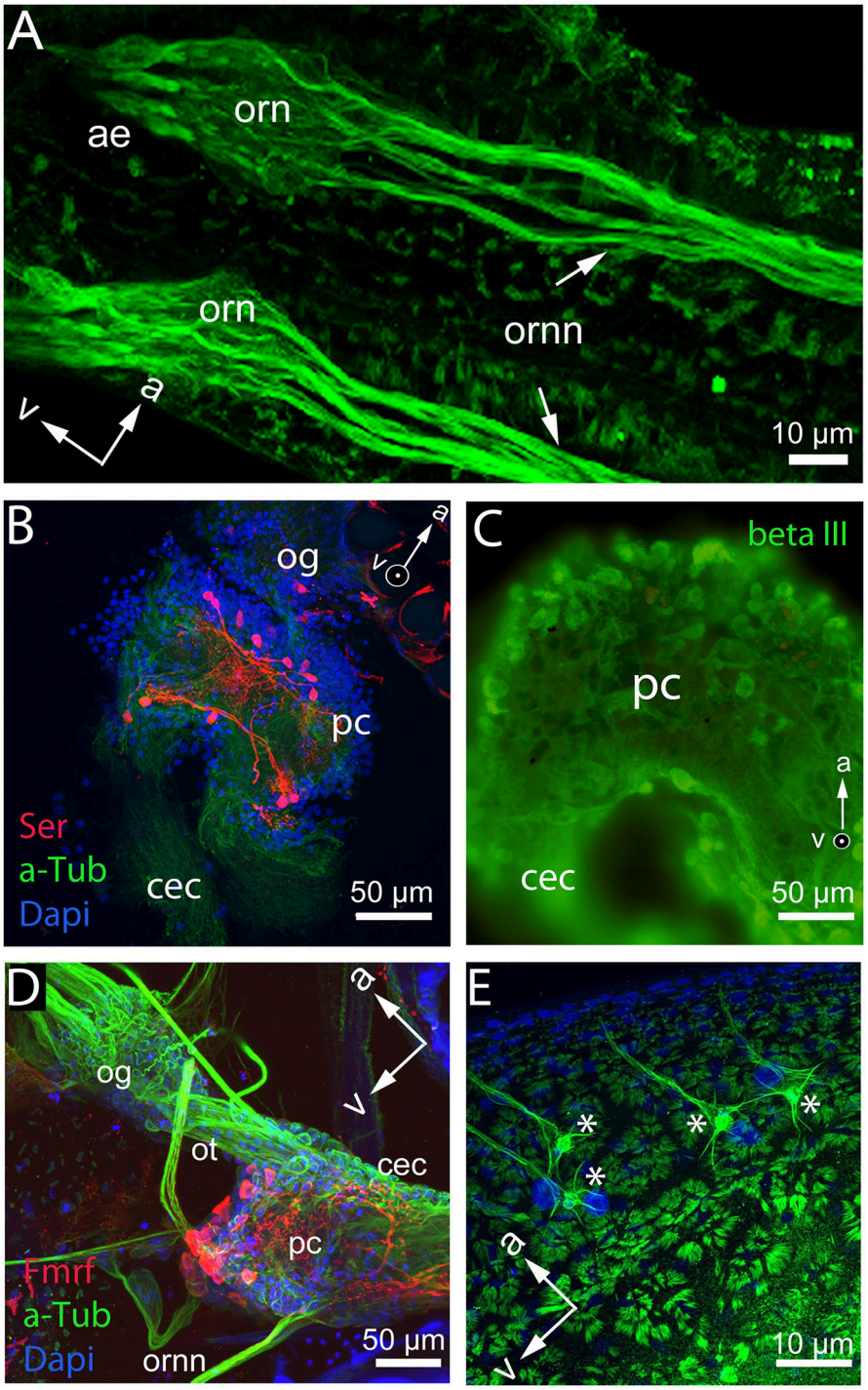

FIGURE 4 | Whole mount and brain mount preparation of the *Daphnia* nervous system displaying differential functional qualities of cells. **(A)** Base of the antennules and cuticular insertion of the aesthetascs (ae) with olfactory receptor neurons (orn) and olfactory receptor neuron neurites (ornn) extending from the first antennule to the deutocerebrum of the brain in *D. longicephala*. **(B)** Protocerebrum (pc) and optic ganglia (og) of *D. magna* show neurons stained with anti-serotonin antibody (red). Cytoskeleton is stained with anti-alpha tubulin (green) and nucleoli with dapi (blue). **(C)** Protocerebrum overview of the *D. longicephala* brain stained with an antibody detecting neuron-specific beta III tubulin. **(D)** Whole mount preparation of a *D. longicephala* head displaying the optic ganglia (og) connected via the optic tract (ot) with the protocerebrum (pc) extending into the circumesophageal connective that connects to the tritocerebrum (not shown). The olfactory receptor neurons connect to the central brain via the deutocerebrum. Nucleoli are stained in dapi displayed in blue, the cytoskeleton is stained with anti-alpha tubulin in green, and red displays cells with anti-Fmrfamide reactivity. **(E)** Polyploid cells (asterisks) lining the region of crest expression in *D. longicephala* stained with anti-alpha tubulin. Cells are characterized by a large nucleus stained with dapi (blue), lateral extensions, and one dominant extension innervating the epidermal cells of the crest. Orientation of the preparations is marked by arrows in the anterior (a) and ventral (v) directions. Images by Ioannidou & Weiss.

on over-activation due to the magnesium block and are known to be essentially involved in long-term potentiation in associative learning, points to a degree of synaptic neuronal plasticity.

While the perception of predators is performed by the above described actions of the nervous system, also epigenetic changes are anticipated to contribute to the modification of an organism's phenotype. Such changes can also be inherited to subsequent generations rendering these better adapted to environmental conditions.

EPIGENETICS OF PREDATOR INDUCED DEFENSES

Epigenetics study the emergence of different phenotypes that result from a single genotype (Bonasio, 2015). Up to date, only little attention has been paid to epigenetic modifications and how these may affect ecological interactions. Well-known changes are described by non-coding RNAs, histone modifications and cytosine methylation (Harris et al., 2012). Ultimately, all these mechanisms lead to changes in gene expression patterns, but do not change the DNA sequence itself. Epigenetic changes are of particular interest not only because they are affected by environmental conditions but also because of their heritability. This can either take place during meiosis or mitosis. During mitosis epigenetic changes are responsible for the maintenance of discrete transcriptional states and e.g., control cell identity over multiple rounds of cell division (Duncan et al., 2014; Bonasio, 2015). During meiosis epigenetic changes can be transferred to subsequent generations. So environmental changes may be epigenetically imprinted and passed on to offspring even after the initial stress has disappeared (Harris et al., 2012). The role of epigenetics in the context of defense systems and adaptive morphotypes is not yet fully exploited. Parthenogenetic organisms like *Daphnia* are discussed as valuable models for such endeavors (Harris et al., 2012; Robichaud et al., 2012), as they have the epigenetic repertoires (e.g., differential methylation Asselman et al., 2015 and histone modification Robichaud et al., 2012) as well as the explicit ability to express context dependent phenotypes within (Weiss and Tollrian, 2018) and across generations (Agrawal et al., 1999).

OUTLOOK

The ability of many organisms to adjust to the predation risk and to be able to distinguish between predators shows a distinct capacity to sense and interpret the environment. This is pivotal for an individual's fitness and ultimately for ecosystem stability.

In recent years, it has become increasingly clear, that many anthropogenic agents released into the environment affect the sensory systems of marine and freshwater species.

For example, many studies have demonstrated that elevated pCO_2 levels in the oceans and in freshwater ecosystems affect organismal neurobiology (Nilsson et al., 2012; Hamilton et al., 2014; Weiss et al., 2018). In many cases this prevents the correct interpretation of the environment and can lead to inappropriate responses (Chivers et al., 2014; Weiss et al., 2018). This may render prey species more susceptible to predators, which can have cascading effects on the ecosystem level. Likewise, a number of laboratory studies suggest that anthropogenic pollutants can disrupt chemoreception, even at low, non-toxic concentrations, but there are few tests of whether real-world variation in water quality affects chemoreception (Troyer and Turner, 2015).

These observations demonstrate the necessity to further analyze chemical signaling cues together with the sensory mechanisms that mediate environmental adaptations. With next-generation sequencing strategies, genome mining for e.g., chemoreceptors is possible and also pivotal for any molecular investigations. The availability of novel genome-editing strategies (Crispr/Cas9, TALEN, RNAi) (Kato et al., 2012; Nakanishi et al., 2014; Naitou et al., 2015) in combination with optogenetic applications (Herlitze and Landmesser, 2007; Deisseroth, 2011) and electroantennograms (Simbeya et al., 2012) will allow us to further decipher the molecular mechanisms underlying predator-induced phenotypic plasticity.

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The author confirms being the sole contributor of this work and has approved it for publication.

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frontiers in Behavioral Neuroscience

MINI REVIEW
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Neuronal Plasticity in the Amygdala Following Predator Stress Exposure

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Predation causes robust long-term stress-related effects on prey individuals even if they do not get consumed by the predator. Here I review the role of basolateral amygdala (BLA) neurons in the mediation of non-consumptive effects of predation. This brain region is critical for the generation and maintenance of fear response across many phylogenetic groups. The exposure to cues of predator presence activates neurons within the BLA. Hormones secreted during stressful episodes cause long-lasting structural changes in BLA neurons, causing facilitation of endocrine response during subsequent exposure to stressful episodes like later predator exposure. Some studies also suggest that BLA is involved in creating anticipatory defensive behavior in response to the expectation of change in the environment.

Keywords: amygdala, fear, stress, glucocorticoids, predator-prey

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PREDATOR CUES CAUSE ENVIRONMENT-DEPENDENT FLEXIBILITY IN PREY RESPONSE

Predation has been considered by both ecologists and neurobiologists as a fundamental biological process (Clinchy et al., 2013). The approaches taken by these disciplines somewhat differ. For an ecologist, predation is a consumptive and acute process. A predator stalks a prey. The encounter evokes an immediate fight-or-flight response. A chase ensues. Prey is either eaten up, contributing to mortality rates; or the victim escapes to partake in other processes of life like reproduction and foraging. Mortality rates can then be modeled to predict population dynamics for predator-prey dyads, and so on. The crucial thing here is the assumption that the predation does not lead to an effect on prey beyond the encounter itself. Effects of predation can be summed up by consumptive rates of capture. Can the elk population in Yellowstone be modeled once wolves are introduced? Can we predict cyclicity of wolf population as a function of prey density? These sorts of questions dominate the to-do list of an ecologist. For a neurobiologist, however, predation is very much a non-consumptive process. Prey animals in this narrative develop a fear of cues of predator presence, like urine or body odors. These signals initiate an innate aversion that reduces the chance of a predator encounter. The fear can be generalized by the process of association whereby previously emotion-neutral environmental stimuli gain emotional valence ("Avoid that waterhole because last time I smelled a predator there"). Fear causes chronic effects like lower investment in reproductive hormones, greater activation of the stress axis and lower growth rates. Thus, the impact of predation includes chronic effects of fear itself beyond the direct mortality of the prey. Which brain regions should be lesioned to observe less fear of predators? Which molecules should be over-expressed to increase fear? These sort of questions are a mainstay for a neurobiologist of fear. So, in short, analysis of predation takes the form of acute consumption of one organism by another on the one hand; and chronic non-consumptive proximate changes on the other. This division reflects the

difference in scholarly tradition. This division does not reflect the divergence of the underlying biological process itself. Thus, multiple research teams have successfully integrated acute and chronic effects of the predations (Clinchy et al., 2011; Buck and Ripple, 2017; Hermann and Landis, 2017).

There are two inter-related processes that mediate non-consumptive predation effects on the prey. First, coping with predators evoke a fight-or-flight response which requires the initiation of stress endocrine axis (Harris and Carr, 2016). Repeated or prolonged activation of stress endocrine response causes allostatic load on the neuroendocrine mechanism (McEwen, 1998). For example, excessive stress hormones over long-time cause atrophy of hippocampal neurons that provides negative feedback on the release of adrenal hormones, causing an increase in stress hormones and further exacerbating hippocampal atrophy (Magariños and McEwen, 1995). Similarly, excessive adrenal glucocorticoids suppress the immune system which is adaptive during acute phases of stress but become maladaptive during chronic episodes. Non-consumptive effects in this framework are then the unavoidable cost of mounting defense to the predators. A second framework can also be proposed based on predictive regulation of behavior and physiology by the brain. The brain can detect environmental cues of predator presence and then change the physiological landscape in anticipation of need for the higher defense. Thus, environmental adversity can percolate from mother to child through changes in maternal care and enhances stress responsivity (Liu et al., 1997). Alternatively, stress hormones *in utero* can change the growth trajectory of offspring (Dantzer et al., 2013). Non-consumptive effects in this framework reflect environment-dependent plasticity in the prey behavior. Both allostatic load and environment-dependent plasticity converge on the same proximate mechanisms and are thus inter-related.

THE CONTRIBUTION OF BASOLATERAL AMYGDALA IN MEDIATION OF DEFENSIVE BEHAVIORS

Amygdala is a collection of structurally heterogeneous brain regions in the medial temporal lobe (Sah et al., 2003). These brain regions are divided into three broad groups that include basolateral, cortical and centromedial amygdala. Basolateral complex amongst these group has been widely studied in the context of fear or conditioning of fearful stimuli to innocuous environmental cues (LeDoux, 1998). Molecular evidence suggests that broad features of amygdala were present in concestor basal to the vertebrates (Martínez-García et al., 2002; Medina et al., 2011). Homologs of basolateral amygdala (BLA) have been found in amphibians and birds, supporting an ancestral origin of this brain structure and its associated functions (Cheng et al., 1999; Moreno and González, 2007).

Exposure to predator cues activate neurons within BLA (**Table 1**). This has been studied using Fos, an immediate early gene product that is often used as a proxy for recent neuronal activity. Exposing rats to cat odors causes Fos expression in the BLA (Dielenberg et al., 2001). Similarly, exposure to ferret odor leads to recruitment of CaMKII positive neurons suggesting an influx of Ca^{2+} during the odor exposure (Butler et al., 2011). Electrophysiological studies also reveal neurons within BLA selectively fires to exposure of anesthetized rats to cat urine (Karst et al., 2002). All these chemosensory cues cause defensive behavior in rats. Similarly, infection with *Toxoplasma gondii* reduces fear to cat urine in rats and in parallel causes dendritic retraction within BLA neurons (Mitra et al., 2013). This suggests that inflammation associated with infections can influence processing of the fear within BLA. On the other hand, this observation can also be interpreted in view of direct effect of parasite presence within the BLA.

But the neuronal activation of BLA neurons during exposure does not separate incidental activation from involvement in the mediation of fear response. Predator odors do cause Fos expression and neuronal firing in a wide range of brain regions including the medial amygdala and other components of medial hypothalamic zones (Dielenberg et al., 2001). It is plausible that activation of BLA neurons relates more to Pavlovian conditioning rather than unconditioned fear itself (Takahashi et al., 2008). This possibility is shown by the observation that temporary or permanent lesions of BLA cause disruption of conditioned fear to cat odors in rats (Takahashi et al., 2007).

Nonetheless, several lesions studies show the possible role of BLA in unconditioned behavioral response to predators. Early resection studies of a medial temporal lobe in monkeys, for example, showed the development of "psychic blindness" in subjects, as a response, to stimuli which were loaded with aversion in non-manipulated animals (Lanska, 2018). Fiber sparing lesions of BLA reduces aversion to predator odors in rats in parallel to causing deficits in the conditioning process (Bindi et al., 2018). Disruption of cholinergic projections to the BLA in rats reduces unconditioned freezing to cat fur (Power and McGaugh, 2002). Similarly, optogenetic inhibition of anterior cingulate cortex projections to BLA potentiates freezing of mice to fox urine, and extraneous activation of this pathway reduces freezing (Jhang et al., 2018). These experiments suggest that BLA and its communication from upstream brain regions do change prey response to predator chemosensory cues. Apropos, BLA send moderate density of efferent fibers to medial amygdala. Anterograde tracer *Phaseolus vulgaris* leucoagglutinin travels to dorsal portion of medial amygdala in rats (Pitkänen et al., 1995). Sensory information reaches independently to basolateral and medial amygdala. It is plausible, and currently untested, that intra-amygdaloidal connections between basolateral and medial amygdala can allow an inter-dependent representation of the emotional valence in the predator associated olfactory cues.

Moreover, BLA might have a heterogeneous role in defensive behaviors dependent on specific predator odors used in the experiments. For example, chronic exposure to odor from ferret anal glands induces a set of physiological changes that are similar to those of chronic stress with robust BLA involvement (Campeau et al., 2008). This includes atrophy of thymus and hypertrophy of adrenal glands. Odors from ferret anal glands, and cat urine also activate BLA neurons apart from other brain regions in medial hypothalamic system

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TABLE 1 | Role of basolateral amygdala (BLA) in defensive behaviors.

Animal model; and treatments	Observation(s)	References
Wistar rats; Exposure to worn cat collar	Expression of immediate early gene (c-Fos) in BLA; in addition to regions of medial hypothalamic zone	Dielenberg et al. (2001)
Long-Evans rats; Exposure to ferret-scented towel	Increase in BLA neurons colabeled with CaMKII and c-Fos	Butler et al. (2011)
Wistar rats; Acute brain slices washed with glucocorticoid receptor agonist	Increase in voltage-activated Ca+2 influxes in BLA projection neurons	Karst et al. (2002)
Wistar rats; *Toxoplasma gondii* infection	Parallel decrease in BLA dendritic length and circulating baseline corticosterone	Mitra et al. (2013)
Wistar rats; BLA cytotoxic lesion	Reduction in innate and learned fear response to a live cat	Bindi et al. (2018)
Sprague-dawley rats; Disruption of cholinergic projection to the BLA	Reduced unconditioned fear to the car hair	Power and McGaugh (2002)

(Campeau et al., 2008; Govic and Paolini, 2015). A reversible lesion of BLA reduces unconditioned fear to cat fur (Vazdarjanova et al., 2001). Optogenetic inactivation of anterior cingulate cortex, a brain region with selective efferent reaching BLA, enhances unconditioned fear to fox urine (Jhang et al., 2018). In contrast, several studies fail to find significant effects of BLA lesions on unconditioned defensive behaviors upon exposure to 2,3,5-trimethyl-3-thiazoline (Müller and Fendt, 2006; Rosen et al., 2008); a component of fox fecal odor. The difference between 2,3,5-trimethyl-3-thiazoline and other predator odors suggest a disparity in how these heterospecific cues are processed by BLA. Alternatively, it is possible that 2,3,5-trimethyl-3-thiazoline is perceived as a noxious odor rather than a predator related semiochemical (Fendt and Endres, 2008) and thus does not activate brain regions like BLA or medial hypothalamic zone.

THE RELATIONSHIP BETWEEN BLA AND ALLOSTATIC LOAD DURING REPEATED STRESSORS

Exposure to predators induce physiological activation of stress endocrine axis through activation of hypothalamus-pituitary-adrenal axis (H-P-A axis; McEwen, 1998). Adrenal stress hormones include epinephrine from adrenal medulla and glucocorticoids from the adrenal cortex. Adrenal hormones prepare the body for metabolic demands placed by the need to fight-or-flight from the predators. Stress endocrine axis returns to the baseline once acute predator event has been successfully resolved (or the prey has been eaten, in which case the issue becomes moot!).

The homeostatic process works very well regarding switching the body between with-predator and san-predator episodes as far as such encounter are few and far between. Yet repeated or prolonged activation of stress endocrine axis takes a toll on neural mechanisms that control stress endocrine response through wear and tear (McEwen, 1998). Epinephrine does not readily cross the blood-brain barrier. But glucocorticoids can cross the barrier and bind to their receptors within a wide variety of brain regions. Central glucocorticoid signaling is important in maintaining the homeostatic regulation of stress hormones during acute events. For example, occupied glucocorticoid receptors within hippocampus lead to negative feedback on further release of adrenal hormones thus returning the physiological landscape to pre-stress baselines (Herman et al., 2003; Jankord and Herman, 2008). Yet the same neural machinery undergoes wear and tear during chronic stress; lowering the negative feedback, increasing glucocorticoids, exacerbating damage in the brain, further reducing homeostatic control. Thus, the relation between brain and stress hormone undergoes a drastic alteration from a situation of the sporadic stress of predator presence to a landscape dominated by frequent exposure to predators or cues of their presence. This reflects non-consumptive effects brought about by an allostatic load of repeated cycles of stress endocrine activation. BLA has a crucial role in this alteration.

BLA neurons undergo structural plasticity during stressful episodes (Vyas et al., 2002, 2003; Mitra et al., 2005). BLA contains a robust number of glucocorticoid receptors which bind to circulating stress hormones (Joëls and Karst, 2012). These receptors are important in creating long-lasting allostatic load. This is borne out by experiments involving exogenous supplementation of glucocorticoids, which elicits long-lasting dendritic expansion and spinogenesis in projection neurons of the BLA (Mitra and Sapolsky, 2008). Dendritic remodeling of BLA neurons can be prevented when experimental opportunities are created for competitive binding away from endogenous glucocorticoid receptors (Mitra et al., 2009c; Mitra and Sapolsky, 2010b). Similarly, exogenous glucocorticoids increase voltage-dependent calcium currents within individual BLA neurons (Karst et al., 2002; Joëls and Karst, 2012). This is important because calcium currents facilitate the formation of long-term potentiation and persistent synaptic changes. High levels of glucocorticoids also increase excitatory glutamatergic transmission within BLA, especially when it is coupled with prior exposure to epinephrine (Karst and Joëls, 2016). These studies collectively argue that chronic or severe stress leaves long-lasting footprints within the structure and function of BLA neurons (**Figure 1**). It is plausible that glucocorticoid-induced changes in BLA dendritic structure lead to greater excitability of BLA neurons through increasing number of synapses on the BLA neurons. Greater excitability of BLA neurons could, in turn, also increase dendritic complexity of BLA neurons through greater availability of calcium ions. The relationship between structure and electrophysiology remains unresolved at present.

These long-lasting footprints can change the response of animals to subsequent predator encounter. Several correlative studies point towards this direction. Rats show significant

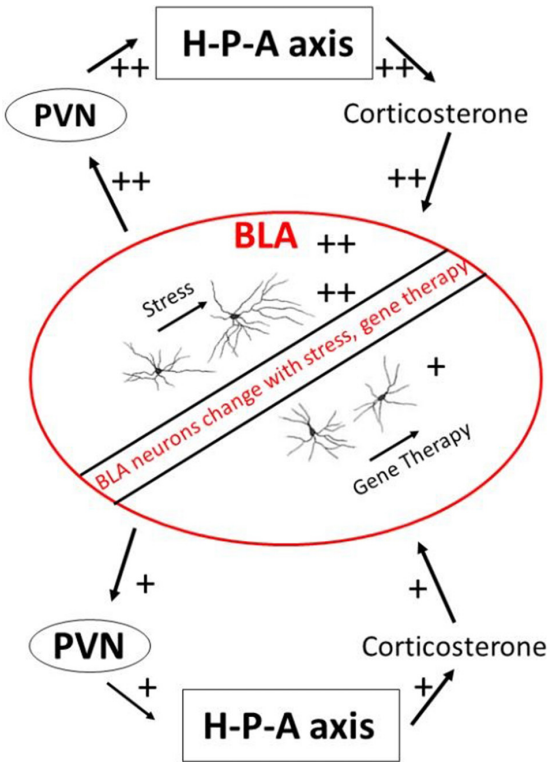

FIGURE 1 | A schematic view of the BLA and stress endocrine response. BLA, basolateral amygdala; H-P-A axis, hypothalamus-pituitary-adrenal axis; PVN, paraventricular nucleus of hypothalamus.

inter-individual variations in terms of long-term behavioral change resulting from acute exposure to a predator (Adamec and Shallow, 1993). Some individuals develop persistent anxiety, while anxiety in others remains undifferentiated from controls after a few weeks. Animals that show recalcitrant anxiety to cat presence in this paradigm have larger and more complex BLA dendritic trees compared to animals that do not show anxiogenesis (Mitra et al., 2009a). Similarly, the dendritic complexity of BLA neurons shows a predictive association with basal levels of circulating glucocorticoids in animals previously exposed to predator odor (Hegde et al., 2017). These studies are limited in the sense that they cannot be used to create cause-and-effect relationships. For example, it remains unclear if the BLA dendritic architecture is a primary change leading to change in glucocorticoids or change in behavior. Or if glucocorticoids lead to a parallel change in behavior and dendritic architecture through independent means. Nonetheless, these studies show that long-term non-consumptive consequences of predator exposure on stress hormones, behavioral change, and BLA structure coelute with each other.

There is an added possibility that changes within the BLA caused by stress hormones can change future secretion of stress hormones themselves through the effects of BLA on downstream paraventricular hypothalamic nucleus responsible for stress endocrine activation. Some paraventricular hypothalamus cells do receive direct projects from BLA, although these projections are sparse (Herman et al., 2003; Jankord and Herman, 2008). Yet there are at least two known polysynaptic pathways that lead from BLA to the paraventricular hypothalamus and thus to downstream modulation of stress hormones. The first pathway encompasses excitatory synapses made by BLA neurons on the central and medial amygdala, which then project to bed nucleus of stria terminalis and onward to the paraventricular hypothalamus (Dong et al., 2001). A smaller efferent route also leads from BLA directly onto bed nucleus of stria terminalis. These connections suggest that a change in BLA structure or function by stress can amplify response during subsequent exposure to stress, e.g., successive predator exposures. Some evidence of such metaplastic response comes from studies of BLA ablation. Lesions of BLA does not cause changes in basal glucocorticoids or those induced during acute stressors (Feldman et al., 1994). But inactivation of BLA causes excess secretion of adrenal glucocorticoids after a novel stressor (Bhatnagar et al., 2004). Studies have also directly compared effects of reducing excitation in BLA neurons on the secretion of adrenal steroids. A spatially-constrained over-expression of SK2 potassium channels within BLA, which should increase inhibition, leads to reduced glucocorticoids secretion in the periphery (Mitra et al., 2009c). Similarly, competitively blocking binding of glucocorticoid receptors within BLA results in lower stress hormone secretion from adrenals (Mitra et al., 2009b; Mitra and Sapolsky, 2010a). These observations point to an exciting possibility that changes within BLA caused by stress and changes to stress hormone axis caused by BLA represent a positive feedback loop that can rapidly change the metabolic state of animals during repeated predator encounters.

Exposure to ferret odor in early juvenile period increases social play in female rats but decreases play in males, when measured 3 weeks later (Stockman and McCarthy, 2017). Exposure to cat cues also lead to more risk assessment, higher avoidance and greater activity-suppression in female rats than males (Shepherd et al., 1992). Overexpression of corticotropin-releasing hormone during development also leads to gender-specific effects on response to presence of a predator. Under basal conditions, without overexpression of corticotropin-releasing hormone, only female mice show greater emotional reactivity after exposure to a cat (Toth et al., 2016). This gender difference is obliterated by overexpression of corticotropin-releasing hormone. These studies suggest substantial sexual dimorphism in effects of predator exposure on later behavior. Similarly, stress experienced by predator cues can interact with physiological state of the animals. For example, exposure to cat litter at start of inactive phase of the diurnal cycle causes more pronounced changes in behavior of rats compared to the same stressor at start of active phase (Cohen et al., 2015).

ROLE OF THE BLA IN MEDIATION OF ENVIRONMENT-DEPENDENT BEHAVIORAL FLEXIBILITY

BLA, apart from its place in allostatic change, is also involved in matching defensive behaviors to changing environments.

Several studies have used cues of environmental adversity to study the relationship between BLA and plasticity in the fear response. Early postnatal life has often been used in these studies because it represents a critical period of brain development and behavioral flexibility. Fear to predators in rat pups emerges around postnatal day 10, a period that is similar to the emergence of stress hormone secretion and amygdala development (Moriceau et al., 2004). Exogenous glucocorticoids at postnatal day 8 are sufficient to atypically cause the emergence of fear to a potential predator and activity within the BLA. Similarly, removal of adrenals and thus loss of glucocorticoids at postnatal day 12 prevents BLA activation and fear to predator usually present at this age (Moriceau et al., 2004). This process of fear emergence through glucocorticoid recruitment of BLA can be buffered through parental presence (Moriceau et al., 2006). Maternal presence attenuates stress endocrine response in early postnatal window resulting in a period where aversive Pavlovian conditioning procedures paradoxically lead to approach rather than avoidance of conditioned stimuli in an amygdala-dependent manner.

A similar interaction effect can also be seen when pregnant mice mothers are exposed to predator odors and offsprings are tested. Offsprings of predator odor-exposed mothers show enhanced fear to cat urine. Offsprings from predator odor-exposed mothers also show more robust secretion of adrenal glucocorticoids when they are exposed to a novel predator odor (St-Cyr and McGowan, 2015). These trans-generational effects occur concomitantly with increased mRNA abundance for corticotrophin-releasing hormone receptor in at least female offspring of predator odor-exposed mothers. The functional role of greater corticotrophin hormone receptor transcripts is not clear in this case. Yet sub-threshold doses of urocortin, an agonist for corticotrophin-releasing hormone, placed specifically within the BLA causes a long-lasting increase in anxiety and lowered neuronal inhibition in adult rats (Rainnie et al., 2004). These observations suggest that BLA and glucocorticoids can plausibly alter the magnitude of defensive responses elicited by animals as a function of predator density seen by the parents during critical developmental windows. Defensive responses can be entrained from parental experience to build anticipatory environment-dependent changes in the behavior through the interaction of BLA and glucocorticoids.

A reverse corollary can also be found whereby living in a sensorily enriched environment can dampen the defensive responses through proximate mechanisms based in the BLA. Relatively short periods of environmental enrichment in rats causes dendritic retraction in BLA neurons (Ashokan et al., 2016; Koe et al., 2016), a phenomenon that is opposite to dendritic expansion observed during exogenous glucocorticoid exposure. Similarly, this treatment reduces responsivity of stress endocrine axis. Interestingly the same enrichment paradigm also causes an increase in active risk assessment sorties made by rat towards cat urine (Mitra and Sapolsky, 2012). Cause and effect relationships in these studies remain under-investigated.

CONCLUSION

Exposure to predators cause both consumptive and non-consumptive consequences for the prey animals. BLA is likely an important mediator of non-consumptive effects of predation on prey behavior. Exposure to predator often leaves a historical trace in the functioning of the stress endocrine axis. BLA is a crucial node in proximate mechanisms of these effects through its reciprocal interaction with adrenal glucocorticoids. The strength of neuroendocrine interactions between BLA and adrenal hormones can be further modulated by the incipient environment. Exposure to predators can also initiate anticipatory investment in defensive behaviors. There is growing evidence that BLA and glucocorticoids are involved in this process. Further experiments are warranted to dissociate incidental relationship from causal sequences.

Stress generated from exposure to predators or predator cues have been used to create animal models of fear-related psychiatric disorders like post-traumatic stress disorder and anxiety disorders (Adamec et al., 2008; Zoladz and Diamond, 2016). Several observations suggest an important role of BLA in precipitation of symptoms in preclinical models. For example, excitatory neurotransmission within BLA is required for anxiety-like symptoms and concurrent neural activation in rats after exposure to a live cat (Adamec et al., 2005; Blundell and Adamec, 2007). Similarly, high-frequency BLA stimulation reduces anxiety-like behavior in rats after exposure to cat urine (Dengler et al., 2018); an effect similar to informational lesion observed in deep-brain stimulation paradigms. Relationship between predator exposure, BLA and psychiatric conditions remains currently understudied; and represents an important opportunity to understand clinically abnormal fear in the future.

AUTHOR CONTRIBUTIONS

RM contributed to the organization, literature search and writing of this manuscript.

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Prolonged Bat Call Exposure Induces a Broad Transcriptional Response in the Male Fall Armyworm (*Spodoptera frugiperda*; Lepidoptera: Noctuidae) Brain

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Predation risk induces broad behavioral and physiological responses that have traditionally been considered acute and transitory. However, prolonged or frequent exposure to predators and the sensory cues of their presence they broadcast to the environment impact long-term prey physiology and demographics. Though several studies have assessed acute and chronic stress responses in varied taxa, these attempts have often involved *a priori* expectations of the molecular pathways involved in physiological responses, such as glucocorticoid pathways and neurohormone production in vertebrates. While relatively little is known about physiological and molecular predator-induced stress in insects, many dramatic insect defensive behaviors have evolved to combat selection by predators. For instance, several moth families, such as Noctuidae, include members equipped with tympanic organs that allow the perception of ultrasonic bat calls and facilitate predation avoidance by eliciting evasive aerial flight maneuvers. In this study, we exposed adult male fall armyworm (*Spodoptera frugiperda*) moths to recorded ultrasonic bat foraging and attack calls for a prolonged period and constructed a *de novo* transcriptome based on brain tissue from predator cue-exposed relative to control moths kept in silence. Differential expression analysis revealed that 290 transcripts were highly up- or down-regulated among treatment tissues, with many annotating to noteworthy proteins, including a heat shock protein and an antioxidant enzyme involved in cellular stress. Though nearly 50% of differentially expressed transcripts were unannotated, those that were are implied in a broad range of cellular functions within the insect brain, including neurotransmitter metabolism, ionotropic receptor expression, mitochondrial metabolism, heat shock protein activity, antioxidant enzyme activity, actin cytoskeleton dynamics, chromatin binding, methylation, axonal guidance, cilia development, and several signaling pathways. The five most significantly overrepresented Gene Ontology terms included chromatin binding, macromolecular complex binding, glutamate synthase activity, glutamate metabolic process, and glutamate biosynthetic process. As

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a first assessment of transcriptional responses to ecologically relevant auditory predator cues in the brain of moth prey, this study lays the foundation for examining the influence of these differentially expressed transcripts on insect behavior, physiology, and life history within the framework of predation risk, as observed in ultrasound-sensitive Lepidoptera and other 'eared' insects.

Keywords: bat, moth, neurophysiology, stress, predation, *Spodoptera frugiperda*, transcriptomics, ultrasound

INTRODUCTION

Predator-induced stress has long fascinated biologists for its integrated, scalable effects on prey physiology, behavior (Slos and Stoks, 2008), and even spatiotemporal population demographics (Clinchy et al., 2013). Though a mechanistic understanding of the physiological responses that are induced by predation related stress in vertebrates has been known for some time, researchers interested in similar responses in invertebrate taxa, such as insects, now seek a similar descriptive model. The study of invertebrate stress responses has a rich history, yet the diversity of molecular components induced by various stressors has thus far stymied most attempts at holistic understanding. Recently, however, Adamo (2010, 2017a) demonstrated that the early stages of stress responses in insects are homologous, and likely anciently related, to vertebrate neurotransmitter signaling and downstream neurohormonal activation. The challenge remains, then, in describing the varied taxon- and tissue-specific responses seen in insects and elucidating the mechanisms responsible for inducing them.

Often before a predator has even localized its prey, a suite of adaptive behavioral and physiological responses which improve the chances of survival (Endler, 1991) are induced in prey organisms which may be eavesdropping on mechanical, auditory, visual, and chemosensory predation cues (Adamo et al., 2013). For instance, moths and butterflies that are sensitive to ultrasound display startle responses when exposed to synthetic broad frequency ultrasound (Roeder, 1966; Ratcliffe et al., 2008, 2011; ter Hofstede et al., 2011) and recorded bat calls (Acharya and McNeil, 1998; Rydell et al., 2003; Ratcliffe and Fullard, 2005), such as changing the course of flight, ceasing flight, accelerating, performing evasive flight maneuvers (Yack, 2004; Yack et al., 2007; Pfuhl et al., 2015), and/or calling back with jamming ultrasound themselves (Corcoran et al., 2009). Upon exposure to ultrasound, non-flying noctuid moths cease movement while many aerial noctuids exhibit evasive flight maneuvers, such as erratic changes in direction, loops, increases in flight velocity, and even falling to the ground (Surlykke and Miller, 1982). Moreover, when exposed to bat calls, many female and male tympanate moths alter their mating behavior by stopping pheromone release or ceasing flight, respectively (Acharya and McNeil, 1998). These behavioral responses, especially when borne out for an extended period of time, may contribute to patterns of stressor-induced gene regulation in insects that may contribute to reports of moths that display modified fecundity and life history patterns following prolonged exposure to recorded and synthetic bat ultrasound in a laboratory setting (Huang et al., 2003; Zha et al., 2008, 2013). For instance, *Plodia interpunctella* (Lepidoptera: Pyralidae) exposed to short bursts of ultrasound near their hearing range (approximately 50 kHz) respond by modifying mating behavior (Trematerra and Pavan, 1995) and long-term exposure even affects spermatophore quality and larval numbers by up to 75% (Kirkpatrick and Harein, 1965; Huang et al., 2003) while simultaneously reducing F_1 larval weight and growth rates (Huang et al., 2003; Huang and Subramanyam, 2004). Conversely, long-term exposure to broadband ultrasound in *Helicoverpa armigera* (Lepidoptera: Noctuidae) significantly increased whole-body acetylcholinesterase activity (Zha et al., 2008), the number of spermatophores per female, and the number of eggs laid (Zha et al., 2013).

In order to maintain internal homeostasis during stressful periods, whether osmotically, metabolically, or otherwise, insects and most other forms of life evolved biomolecular signaling cascades, both intra- and extra-cellularly, that often regulate the expression of stress-related genes (Pauwels et al., 2005; Aruda et al., 2011; Yamaguchi et al., 2012; Roszkowski et al., 2016) and resulting behaviors, including vigilance (Lima, 1990; Kight and Swaddle, 2011) and modified activity patterns (Abramsky et al., 2014). Further, these responses mediate cellular metabolism and the degradative effects of prolonged and persistent stressor exposure, including oxidative damage (Slos and Stoks, 2008; Clinchy et al., 2013), protein misfolding (Fleshner et al., 2004; Even et al., 2012), and organelle turnover (Salvetti et al., 2000; Gesi et al., 2002). However, individual cells can respond to stressful conditions by activating transcriptional pathways that usually produce one or more damage-mitigating antioxidant enzymes or protein folding chaperones, such as the heat shock proteins (Hsps). Though these molecular defenses promote physiological homeostasis in the short-term, prolonged periods of stress clearly influence the life history and fitness of many species. Even though biologists have long recognized the importance of stress hormone signaling for initiating behavioral and physiological defenses to predation, the cellular- and tissue-level mechanisms by which long-term acclimation to predation risk can influence the life history and fitness of prey species remains unclear, particularly among insects.

In this study, we exposed adult male fall armyworm moths to recorded ultrasonic foraging and attack calls of three insectivorous bat species over an 8-h period to test the influence of an ecologically relevant auditory cue of predation on the cellular physiology of the noctuid brain. The fall armyworm, though a non-model species itself, is in the same family as the corn earworm (*Helicoverpa zea*), whose annotated reference genome was recently published (Pearce et al., 2017) and whose old world sister species, *H. armigera*, has long been a prominent subject in insect auditory neuroethology studies for its dramatic neurobehavioral responses to ultrasound. The fall armyworm, and many other tympanic moths, thus make prime candidates

for describing the biochemical and cellular responses that have evolved to cope with prolonged predation risk in insects. We hypothesized that a broader transcriptomic response would be induced in the brains of cue-exposed relative to unexposed individuals. Further, we predicted this response might involve transcripts pertaining to the following physiological functions: (1) intracellular secondary messenger systems, (2) antioxidant and Hsp activity, and (3) gene regulation.

MATERIALS AND METHODS

Fall armyworm larvae were purchased from Frontier Agricultural Sciences (Newark, DE, United States) under USDA APHIS PPQ 526 permit (P526P-04080) and were shipped over-night as second and third instar larvae. Upon arrival at the Illinois Natural History Survey, Prairie Research Institute, University of Illinois at Urbana-Champaign in Champaign, IL, United States, larvae were transferred to individual 59 mL (2 oz.) plastic cups filled with 10–15 mL of standard lepidopteran diet and reared in an environmental chamber (Percival Scientific, Perry, IA, United States) at 30 ± 1°C and 75 ± 5% RH, with a photoperiod of 16 h light/8 h dark. Larvae fed *ad libitum* on a modified standard larval lepidopteran diet (Sims, 1998; Cohen, 2001; Elvira et al., 2010) prepared every 2 weeks. This diet consisted of 13 g agar, 770 mL distilled water, 31.5 g vitamin-free casein, 24 g sucrose, 27 g wheatgerm, 9 g Wesson's salt mix, 10 g alphacel, 5 mL 4 M potassium hydroxide, 18 g Vanderzant's vitamins, 1.6 g sorbic acid, 1.6 g methyl paraben, 3.2 g ascorbic acid, 0.12 g streptomycin salt, 4 mL wheatgerm oil, and 2 mL 10% formaldehyde. We blended the casein, sucrose, wheatgerm, Wesson's salt mix, alphacel, 220 mL distilled water, and potassium hydroxide on high for 5 min, to which we added 550 mL of mildly boiling distilled, deionized water mixed with agar. We then blended the mixture for another 5 min and allowed it to cool to 60°C before we added Vanderzant's vitamins, sorbic acid, methyl paraben, ascorbic acid, streptomycin, wheatgerm oil, and formaldehyde and blended for a final 5 min. We poured 10–15 mL of the cooled diet into each 2 oz. rearing cup and allowed them to solidify in a cold-room for at least 30 min.

We then placed a larva into each filled cup and secured a lid in which two holes had been punched using a No. 1 insect pin. Once a larva cleared its gut before pupation, we transferred it to a shallow Tupperware container (29.4 cm × 15.1 cm × 10.5 cm) filled with 3.5 cm of loose potting soil (SunGro Horticulture, Vancouver, BC, Canada). Once per day, this soil was sifted gently by hand to extract any pupae, which were placed in a separate 30.48 cm^3 mesh cage (BioQuip Products, Inc., Compton, CA, United States) with a mesh-size of 51.15 holes/cm^2 within the environmental chamber until emergence.

Upon emergence, adults were transferred to a similar mesh cage and allowed to mate. Twice daily, we saturated the sides of the mesh cage with a 10% sucrose solution to allow feeding. To avoid the possible confounding effects of shipment and the change in diet undergone by the generation of larvae received from Frontier Agricultural Sciences, F_1 eggs were collected daily from within this cage and placed in small plastic containers within the rearing chamber. Once hatched, we reared F_1 larvae as above until emergence as adults.

Predator Cue Exposure

A random sample of four control and four experimental F_1 adult males (sex determined by visual inspection of terminal pupal abdominal segment) were selected for use in trials 24–48 h post-eclosion. Females were not used, as female noctuid moths broadcasting pheromones are often sedentary (Stelinski et al., 2014) and may be preyed upon less frequently by aerial-hawking insectivorous bats. Three individual recordings were sampled at 480 kHz, 16-bit format and concatenated with 10 s of silence between each call. The calls consisted of (1) a 4.27 s *Molossus molossus* (Chiroptera: Molossidae) attack call, (2) a 1.51 s *Myotis nigricans* (Chiroptera: Vespertilionidae) foraging call, and (3) a 2.92 *Saccopteryx bilineata* (Chiroptera: Emballonuridae) foraging call. These three neotropical bat species were selected specifically because the neurophysiological response of *S. frugiperda* auditory neurons to these species' calls have been explicitly described (Mora et al., 2014), they each represent a ubiquitous species throughout much of *S. frugiperda*'s range in the Americas (Mora et al., 2004; Jung et al., 2007; Surlykke and Kalko, 2008), and they likely represent novel predators for the lab-reared, United States-based *S. frugiperda* colony used in this study. Further, these species produce calls of varying amplitudes and frequencies that together span the known response curve of the *S. frugiperda* tympanum (Mora et al., 2004, 2014). Specifically, *M. molossus*, *M. nigricans*, and *S. bilineata* broadcast at 20–50 (Mora et al., 2004), 50–85, and 45–55 (Jung et al., 2007) kHz, respectively, whereas *S. frugiperda* responds optimally to sounds within 20–50 kHz (Mora et al., 2014). The individual sound files were processed in Audacity v. 2.1.0. to reduce background ultrasound by applying a 20-dB noise reduction filter to frequencies lower than 30 kHz with moderate sensitivity (10.0) and re-sampled each file at 195.3125 kHz to meet the limitations of our playback system. This down-sampling attenuated frequencies greater than 75 kHz (Tucker-Davis Technologies, personal communication), but reproduced the bat calls faithfully within the 20–50 kHz optimal hearing range reported for noctuid moths (Fullard, 1988; Norman and Jones, 2000). The resulting 38 s file was then broadcast on a loop for the 8-h duration of each experimental trial while control trials consisted of an identical setup with no sound played whatsoever. Calls were broadcast via a Tucker-Davis Technologies (TDT; Alachua, FL, United States) System 3 amplifier powering an ES1 electrostatic free-field speaker (TDT) that was situated 30 cm from the center of the cage in a soundproof, anechoic chamber at the Beckman Institute, University of Illinois at Urbana-Champaign in Urbana, IL, United States. The RPvdsEx software suite v. 80 (TDT) was used to process and playback the audio file via the TDT RP2.1 processor, ED1 Electrostatic Speaker Driver, and SA1 Stereo Amplifier tandem setup. Each of the four, 8-h replicate exposure and control trials took place on alternating nights in September 2017 from 22:00 to 05:00.

Sample Preparation and Sequencing

Post-exposure, each moth was placed into a 2 mL vial and immediately immersed in liquid nitrogen. After 30 s, the moth

was removed from the vial and transferred quickly to a Petri dish on dry ice. After the head was removed, we immersed it in RNAlater stabilization solution (Life Technologies). Upon immersion, scales on the head capsule were removed by scraping with scalpel, and a 1 mm × 1 mm section of cuticle was cut to expose the brain tissue directly to RNAlater. We then dissected the brain from the head capsule, rinsed it with fresh RNAlater solution, placed it in a 2 mL microtube of fresh RNAlater solution, and stored it at 2°C until all samples had been collected.

RNA was extracted from each brain using a PicoPure RNA Isolation Kit (Arcturus Bioscience). RNA was eluted in 30 μL of RNase-free water and stored at −80°C until further analysis. Before freezing, 3.5 μL aliquots were removed from each extract and used for RNA quantification via a NanoDrop (Thermo Fisher Scientific) spectrophotometer and a Qubit fluorometer (Life Technologies) using a Qubit RNA HS Assay Kit (Life Technologies). After a 1:10 or 1:15 dilution based on each sample's concentration, we submitted these subsamples to the Functional Genomics Unit of the University of Illinois at Urbana-Champaign's (UIUC) Roy J. Carver Biotechnology Center to confirm RNA quality with a Bioanalyzer RNA 6000 Pico chip (Agilent).

We then submitted each RNA extract to the UIUC Roy J. Carver Biotechnology Center's High-Throughput Sequencing and Genotyping Unit for library preparation and sequencing. Strand-specific cDNA libraries were prepared using an Illumina TruSeq Stranded mRNA Sample Prep Kit (dUTP based) according to manufacturer specifications and quantified by quantitative polymerase chain reaction (qPCR). The eight samples were multiplexed on a single lane of an Illumina 2500 sequencer and the RNA fragments were sequenced using Illumina's HiSeq SBS Sequencing Kit v4 for 101 cycles with a 100 nt paired-end read length.

Raw mRNA Read Preprocessing

Sequence files were demultiplexed with Illumina's bcl2fstq v. 217.1.14 conversion software. To ascertain raw read quality, we used FastQC v. 0.11.2 (Andrews, 2010) with default settings on each set of reads. We then preprocessed the raw reads by performing adapter trimming, quality filtering, and *in silico* normalization. Adapter trimming and quality filtering was achieved using Trimmomatic v. 0.33 (Bolger et al., 2014) in palindrome mode to search for and remove adapter sequences and low quality bases. To remove redundant reads and improve transcriptome assembly performance, the remaining reads were then digitally normalized to a coverage depth of 50× via the Trinity transcriptome assembly suite v. 2.1.1 (Grabherr et al., 2011; Haas et al., 2013).

De novo Transcriptome Assembly, Annotation, and Quality Assessment

To our knowledge, there is no publicly available annotated reference genome for *Spodoptera frugiperda*; therefore, we chose to build a *de novo* transcriptome assembly with the pre-processed reads using the Trinity assembler v. 2.1.1 (Grabherr et al., 2011). We designated the sequence-specific strand orientation to 'reverse-forward' (RF) when possible. The quality of the resulting transcriptome was then assessed using TransRate v. 1.0.1 (Smith-Unna et al., 2016) and BUSCO v. 3 (Simão et al., 2015). We then utilized the Annocript v. 2.0 automated transcriptome annotation algorithm (Musacchia et al., 2015) to complete sequence-similarity searches on each assembled transcript against the National Center for Biotechnology Information (NCBI)'s non-redundant nucleotide database using BLAST+ v. 2.2.30 (Camacho et al., 2009). We selected the UniRef90 protein database (Boutet et al., 2016) to screen for computationally derived protein annotations. Annocript first downloaded the UniRef90 database, stored it in a MySQL v. 7.3 (Oracle Corporation, Redwood City, CA, United States) database, and indexed it for faster searches (Camacho et al., 2009). Annocript carried out BLASTX searches against the UniRef90 database and reported those hits with an e-value < 1e-5. Annocript output a tab-delimited feature map file containing the collated annotation information for each putative assembled transcript.

Read Alignment, Abundance, and Differential Expression Analysis

Following annotation, we indexed the transcriptome in Kallisto (Bray et al., 2016) using the 'kallisto index' command before aligning each sample's reads against the index using the 'kallisto quant' command to select 250 bootstrap replicates each. In R v. 3.5.1 (R Core Team, 2014), we utilized the packages 'edgeR' v. 3.12.1 (Robinson et al., 2009) and 'limma' v. 3.26.9 (Ritchie et al., 2015) to import the estimated read counts and perform DE statistical analyses. First, we used the trimmed mean of M-values (TMM) normalization method (Robinson and Oshlack, 2010) to account for small biases in each sample's overall read library size. To filter out transcripts with low or no expression estimates in one or more grouped replicates (Rau et al., 2013), we calculated the counts per million (CPM) mapped reads for each transcript and removed those with a CPM < 1.

We then visually assessed the presence of batch effects in our data by performing principal components analysis (PCA) on log-transformed CPM expression values across each sample using the 'affycoretools' v. 1.42.0 (MacDonald, 2008) package in R. To account for a large amount of expression variation observed between replicate samples (**Figure 1A**), we used the 'sva' v. 3.18.0 (Leek et al., 2012, 2010) package to explicitly model three identified surrogate variables as covariates. After adding these covariates to our dataset, we log-transformed all CPM estimates to prepare for linear modeling. We then used the 'limma' package and its 'voom' function (Law et al., 2014) to fit a negative binomial linear model and proceeded to compute pairwise t-statistics, F-statistics, and log-odds of differential expression for each transcript according to exposure type using empirical Bayes (Smyth, 2004). The resulting differentially expressed transcripts were filtered by selecting only those with false discovery rate (FDR)-adjusted p-values < 0.05 and a fold-change > 2 to account for multiple testing bias on p-value significance (Benjamini and Hochberg, 1995; Benjamini and Heller, 2007).

To produce a heatmap of gene expression across the samples, we scaled each transcript's associated fold-change to

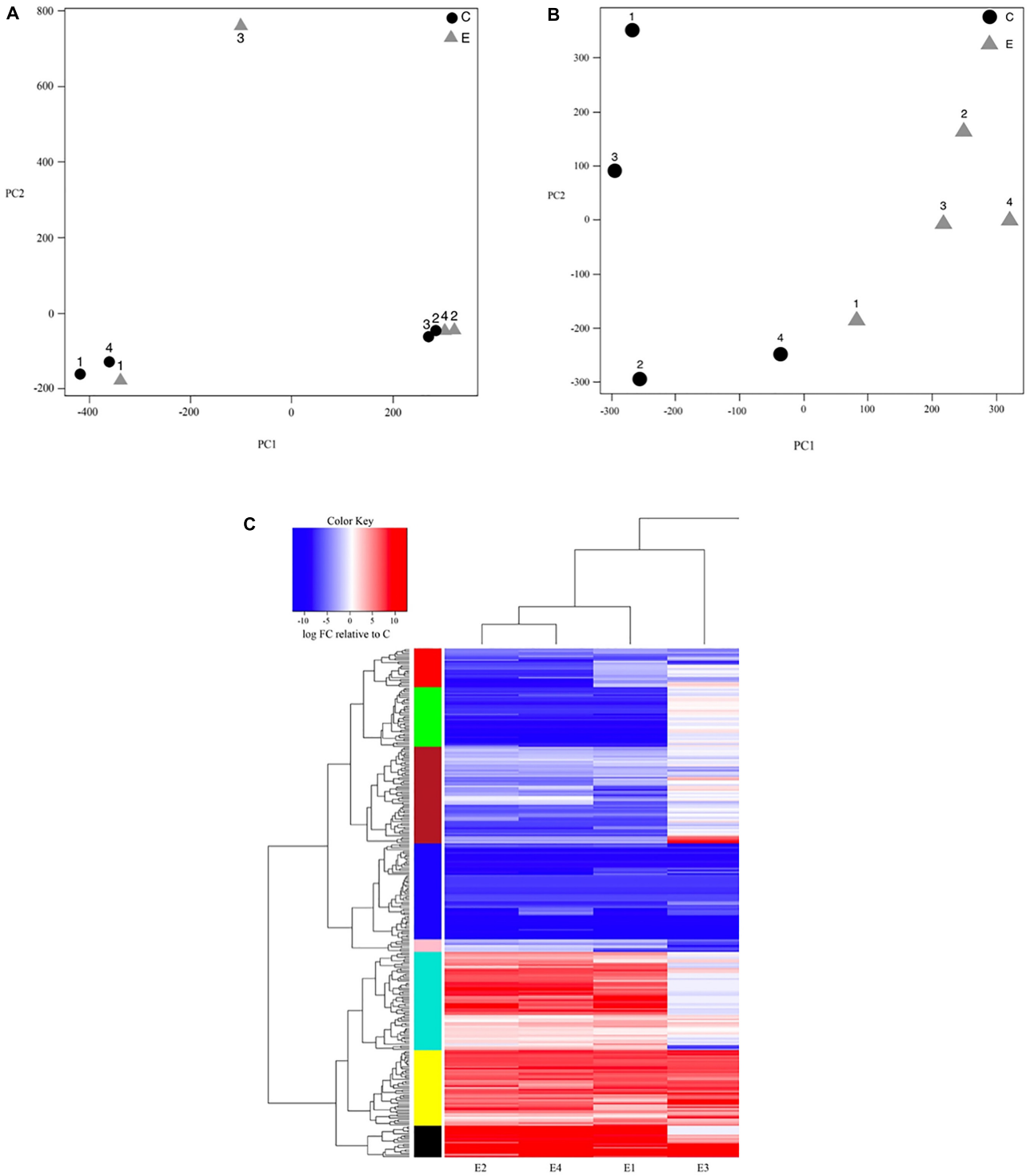

FIGURE 1 | (A) Principal components plot showing sample clustering based on the first two principal components of variation in log-based counts per million read estimates for both control (C; black circle) and bat-ultrasound exposed (E; gray triangle) *Spodoptera frugiperda* moths; numbers (1–4) represent replicate samples from each of the control and exposure groups. **(B)** Principal components plot after surrogate variable analysis was performed to account for unexpected batch effects showing sample clustering based on the first two principal components of variation in log-based counts per million read estimates for both control (C; black circle) and bat-ultrasound exposed (E; gray triangle) adult male *Spodoptera frugiperda* moths; numbers (1–4) represent replicate samples from each of the control and exposure groups. **(C)** Transcript expression heatmap detailing the up- (red) and down- (blue) regulation (log_2FC) of each transcript relative to the mean expression of the control group across bat-ultrasound exposed (E) adult male *Spodoptera frugiperda* moths; samples (horizontal axis) and transcripts (vertical axis) are clustered according to expression similarity (stacked multicolored bars).

the mean fold-change observed in all transcripts from the control group. To assess similarity in expression between samples, we used a hierarchical clustering method based on a distance matrix compiled by taking the maximal distance between any two expression values in each sample via the 'fastcluster' package v. 1.1.20 (Müllner, 2013) in R. The resultant base dendrogram of similarity between individual transcripts was then used to identify the most appropriate level at which to cluster our transcripts using the R package 'dynamicTreeCut' v. 1.63-1 (Langfelder et al., 2008). We chose to use the 'hybrid' method to first identify large, base clusters following four criteria: (1) each cluster must contain ≥2 transcripts; (2) transcripts that are too distant from a cluster are excluded, even if they occur on the same branch; (3) each preliminary cluster must be distinct from those clusters near to it; and (4) the tips of each preliminary cluster must be tightly connected. Once these clusters were identified, any transcripts not previously assigned were placed in the closest neighboring cluster. Using 'cdbfasta' v. 0.99[1], we then retrieved the sequences and Gene Ontology (GO) terms associated with these differentially expressed (DE) transcripts from our annotated transcriptome for downstream functional GO enrichment analysis. Figures were constructed using the R packages 'graphics' v. 3.2.4, 'grDevices' v. 3.2.4, 'rgl' v. 0.95.1441, and 'gplots' v. 2.17.0.

Functional Gene Ontology Term Enrichment and KEGG Pathway Analyses

To obtain a broader perspective on the function of our DE transcripts and how they may be related, we tested their associated annotated GO terms for statistically significant over- and under-representation via GO term enrichment analysis. The background set of transcripts we used to test our DE set against included all GO annotations from base transcriptome. Using the 'Biological Networks Gene Ontology' (BiNGO) plugin v. 3.0.3 (Maere et al., 2005) in the Cytoscape platform v. 3.3.0 (Shannon et al., 2003), we tested for both over- and under-representation using a hypergeometric test at an FDR-adjusted p-value < 0.05. Each differentially expressed transcript was also annotated using the automated BlastKOALA (Kanehisa et al., 2016) KEGG pathway webserver and analyzed manually for functional relevance.

Data Availability

The raw sequence reads have been uploaded to the NCBI Sequence Read Archive (SRA) database (accessions: SRR3406020, SRR3406031, SRR3406036, SRR3406052, SRR3406053, SRR3406054, SRR3406055, SRR3406059) and are also available through the BioProject accession PRJNA318819[2]. The transcriptome has been archived to NCBI's Transcriptome Shotgun Assembly database under accession GESP00000000; the version used here is GESP00000000.1. A repository containing R scripts and output files from all analyses downstream of assembly is also hosted on GitHub[3].

RESULTS

RNA Extraction, Library Preparation, and Read Processing

Each RNA extract was found to produce satisfactory yields, and these were subsequently used in downstream analyses. Total RNA concentration in each sample was generally consistent between NanoDrop and Qubit estimates, the absorbance ratios signified little if any contamination (A260/280 > 2) and the bioanalyzer assay revealed each sample consisted of high-quality RNA with negligible signs of degradation (RIN > 8; **Table 1**). Our cDNA fragment lengths after library preparation ranged from 80 to 700 bp, with an average of 300 bp. Each sample produced similar numbers of reads, ranging between 28.3 million and 31.1 million. The average quality scores for each base in each sample were ≥33 (phred-33 scaling), allowing us to proceed without sequencing error correction. Preprocessing steps led to less than 0.12% of reads being removed in each sample, and the GC content of the samples ranged from 42 to 45% post-trimming.

[1]https://sourceforge.net/projects/cdbfasta/

[2]https://www.ncbi.nlm.nih.gov/bioproject/PRJNA318819

[3]https://github.com/cinel1/FallArmyworm.git

TABLE 1 | Total RNA concentration, absorbance values, absorbance ratios, and RNA Integrity Number (RIN) for each brain tissue RNA extraction from control (C) and bat ultrasound-exposed (E) adult male *Spodoptera frugiperda* moths.

Sample ID	NanoDrop concentration (ng/μL)	Qubit concentration (ng/μL)	A260	A280	A260/280	RIN
C1	49.65	49.7	1.241	0.593	2.09	8.3
C2	54.78	58.4	1.370	0.636	2.15	8.5
C3	42.76	44.2	1.069	0.491	2.18	8.7
C4	46.21	49.1	1.155	0.539	2.14	8.3
E1	74.34	71.8	1.859	0.869	2.14	8.7
E2	52.91	56.6	1.323	0.638	2.07	8.8
E3	66.32	61.9	1.658	0.768	2.16	8.7
E4	35.29	38.1	0.882	0.419	2.11	9.0

De novo Transcriptome Assembly Statistics

Our transcriptome contained a total of 27,734 putative transcript contigs in total, ranging in length from 124 to 38,522 bp with an average of 1,399.9 bp. The contig N50 of our assembly was 2,933 bp and 40.9% of the contigs exceeded 1,000 bp in length. Out of 303 eukaryotic orthologs used as a reference in BUSCO, we identified 290 (95.7%) complete matches, with 234 single-copy and 56 duplicate hits, along with three fragmented and ten missing orthologs. Annocript annotated 10,367 (37.38%) contigs with reliable protein annotations from significant (e-value $< 10^{-5}$) BLASTX hits using the UniRef90 database. Of these hits, 97.0% and 93.6% were annotated to species of Insecta and Lepidoptera, respectively. Mapping GO annotations to these hits resulted in 6,476 GO annotations present in the transcriptome, with 4,075 gene products attributed to biological processes, 815 to cellular components, and 1,586 to molecular function. The top GO terms attributed to the largest numbers of transcript contigs included 'integral to membrane' (GO:0016021), 'nucleic acid binding' (GO:0003676), 'ATP binding' (GO:0005524), 'nucleus' (GO:0005634), and 'zinc ion binding' (GO:0008270).

Read Alignment and Abundance Quantification

On average, 36.59% ± 0.573% (95% CI) of reads from each sample mapped to the transcriptome. TMM normalization resulted in normalization factors ranging from 0.915 to 1.104, which we then multiplied by our actual library sizes to find our final effective library sizes. After filtering low and no expression transcripts with <1 CPM, 17,558 out of 27,734 (63.3%) were retained for DE analysis.

Differential Transcript Expression Analysis

Our initial PCA indicated strong, unexpected clustering of samples along the first two principal axes (**Figure 1A**), leading us to use surrogate variable analysis in effort to remove potential unaccounted batch effects. We found three significant surrogate variables that we included in our negative binomial regression model as covariates, resulting in clear clustering of samples by experimental group (**Figure 1B**). Further, we improved our detection of significant DE transcripts at a FDR < 0.05 with $\geq$2-fold change in expression from 75 to 290 transcripts after including the covariates (**Figure 1C**). Of the 290 DE transcripts, 146 (50.3%) had significant BLASTX hits (e-value $<$ 1e-5), though 44 (15.2%) had uncharacterized functions (**Tables 2**, **3**). The top 11 organisms with the highest number of hits to DE transcripts were all also lepidopteran taxa, with most pertaining to *Amyelois transitella* (Lepidoptera: Pyralidae). Of the 290 DE transcripts, 117 were upregulated while 173 were downregulated.

Upregulated Genes

Among the top 10 most highly upregulated genes were a X-linked retinitis pigmentosa GTPase regulator (RPGR) homolog and a mitochondrial calcium uniporter protein, though seven genes were unannotated, including the most highly upregulated transcript, with the remaining transcripts annotated by uncharacterized proteins. Genes also had highly variable absolute $\log_2$-transformed fold changes ($\log_2$FC) ranging from 1.29 to 11.45. Additional upregulated genes of interest include the regulatory-associated protein of TOR, axin, inositol 1,4 5-triphosphate 5-phosphatase, Hsp 67B2-like isoform X2, glutathione (GSH) *S*-transferase 2-like, and the rho GTPase-activating protein.

Downregulated Genes

The top 10 most downregulated genes included three with annotations, a 27 kDa hemolymph protein, an equilibrative nucleoside transporter, and protein polybromo-1 (Pb-1), while the remaining seven failed to be annotated. Again, absolute fold-change expression varied broadly (1.48–10.55 $\log_2$FC) though several other annotated and functionally relevant genes were downregulated. In particular, voltage-gated ion channels, DNA N^6-methyl adenine (6mA) demethylase-like isoform, histone-lysine *N*-methyltransferase, phosphatidylinositol 5-phosphae 4-kinase, two different cytochrome P450s, glutamate synthase, integrin beta, mitoferrin-1, ankyrin repeat domain-containing protein 17, arrestin, and several zinc finger proteins.

Gene Ontology Enrichment Analysis and KEGG Pathway Reconstruction

Of the 146 DE annotated transcripts, 102 (69.8%) displayed GO term sequence identity (**Figure 2A**). GO term enrichment analysis identified 15 overrepresented and 0 underrepresented GO categories in our exposed samples (FDR-adjusted *p*-value < 0.05; **Table 4**). Six of these overrepresented GO terms pertained to glutamate metabolism, biosynthesis, and synthase activity, while dicarboxylic acid biosynthesis and metabolism corresponded to two terms, and oxidoreductase, aminoacylase, flavin mononucleotide binding, chromatin binding, and macromolecular complex binding corresponded to one term each. Notably, 14 of these 15 overrepresented GO terms annotated a downregulated transcript while only a single term pertained to an upregulated transcript. Of note is that the majority of transcripts mapping to significantly enriched GO terms occurred as very low or zero transcript count observations in the exposed relative to the control group. All transcripts mapping to chromatin binding-, glutamate-, integrin-, oxidoreductase-, and aminoacylase-related GO terms exhibited this pattern of "all-or-nothing" transcript expression. As the data included considerable noise, the prevalence of this pattern among the differentially expressed GO annotated transcripts may simply be due to these patterns being the only ones strong enough to discern statistically, though their functional relevance in stress physiology requires further investigation. Our BlastKOALA KEGG pathway reconstruction of the 290 DE transcripts recovered 43 (14.8%) with functional annotations, including 37 pertaining to cellular metabolism, six related to genetic information processing, nine that function in cellular signal transduction to environmental stimuli, five related to cell growth and death, two related to glutamatergic and GABAergic

TABLE 2 | List of differentially upregulated (log_2-transformed fold change) transcripts recovered from brain tissue mRNA extractions in bat call-exposed *Spodoptera frugiperda* adult male moths relative to controls, including the most significant (e-value < 1e-5) BLASTX protein annotation from the UniRef90 database and the organism from which the annotation is derived.

Transcript ID	Log_2 fold change	Ave. expression	*P*-value	FDR-adjusted *P*-value	Top UniRef90 BLASTX Hit	E-value	Organism
TRINITY_DN2230_c0_g1_i1	11.4500	−1.4769	0.0000	0.0000	–	–	–
TRINITY_DN37212_c0_g1_i3	9.8601	−0.7439	0.0000	0.0000	X-linked retinitis pigmentosa GTPase regulator homolog	0	*Bombyx mori*
TRINITY_DN36403_c0_g1_i3	9.6654	−2.1606	0.0000	0.0000	Calcium uniporter protein mitochondrial	0	*Papilio polytes*
TRINITY_DN30280_c6_g1_i3	9.6383	−1.3637	0.0000	0.0000	–	–	–
TRINITY_DN38404_c0_g2_i3	9.5504	−1.0808	0.0000	0.0000	–	–	–
TRINITY_DN33318_c7_g1_i1	9.4738	−2.2233	0.0000	0.0000	–	–	–
TRINITY_DN33671_c2_g1_i8	9.3876	−1.2532	0.0000	0.0000	–	–	–
TRINITY_DN35646_c2_g4_i3	9.0219	−1.4826	0.0003	0.0594	Uncharacterized protein LOC105386011	1.00E-43	*Plutella xylostella*
TRINITY_DN37234_c0_g1_i10	8.6926	−2.5344	0.0000	0.0000	–	–	–
TRINITY_DN30400_c2_g1_i4	8.6099	−2.5413	0.0000	0.0000	–	–	–
TRINITY_DN38739_c1_g1_i16	8.5436	−1.2902	0.0000	0.0000	Protein polybromo-1	0	*Papilio* sp.
TRINITY_DN35646_c2_g4_i5	8.4296	−2.0614	0.0000	0.0130	Uncharacterized protein LOC105386011	4.00E-42	*Plutella xylostella*
TRINITY_DN34405_c8_g8_i2	8.4089	−2.3193	0.0000	0.0000	–	–	–
TRINITY_DN40154_c8_g1_i2	8.2644	−1.0302	0.0000	0.0030	–	–	–
TRINITY_DN37042_c2_g2_i1	8.1724	−2.2874	0.0000	0.0006	–	–	–
TRINITY_DN40225_c4_g3_i1	7.9335	−2.4214	0.0000	0.0000	–	–	–
TRINITY_DN37134_c1_g1_i13	7.7773	−0.8312	0.0000	0.0111	–	–	–
TRINITY_DN34896_c2_g1_i1	7.7609	−2.3763	0.0000	0.0017	–	–	–
TRINITY_DN37497_c1_g1_i16	7.7348	−2.7427	0.0000	0.0024	Nuclear factor 1 C-type-like	0	*Plutella xylostella*
TRINITY_DN33065_c0_g2_i1	7.7268	−2.6286	0.0000	0.0008	Aminoacylase-1-like	1.00E-92	*Amyelois transitella*
TRINITY_DN32642_c3_g3_i12	7.6793	−2.1043	0.0000	0.0000	Regulatory-associated protein of TOR	0	*Bombyx mori*
TRINITY_DN38729_c4_g1_i3	7.3105	−0.6533	0.0002	0.0484	Phosphatidate cytidylyltransferase	0	*Ditrysia* sp.
TRINITY_DN36166_c2_g1_i1	7.1246	−2.2272	0.0000	0.0000	–	–	–
TRINITY_DN29778_c0_g1_i3	7.1124	−2.3090	0.0000	0.0077	–	–	–
TRINITY_DN31620_c0_g1_i6	7.0401	−3.2045	0.0000	0.0007	Uncharacterized protein	0	*Papilio* sp.
TRINITY_DN34936_c0_g1_i3	6.9579	−2.4491	0.0000	0.0010	Myoneurin-like	1.00E-76	*Bombyx mori*
TRINITY_DN37234_c0_g1_i8	6.8524	−2.5641	0.0000	0.0012	–	–	–
TRINITY_DN29467_c1_g1_i4	6.8383	−2.4531	0.0000	0.0000	–	–	–
TRINITY_DN25058_c0_g1_i3	6.8317	−3.0539	0.0000	0.0000	Putative ecdysone oxidase	2.00E-15	*Operophtera brumata*
TRINITY_DN25058_c0_g1_i2	6.7810	−3.2099	0.0000	0.0000	Mitochondrial choline dehydrogenase	3.00E-21	*Operophtera brumata*
TRINITY_DN36104_c1_g1_i1	6.7539	−1.9239	0.0000	0.0037	Pro-resilin-like	2.00E-17	*Amyelois transitella*
TRINITY_DN37496_c0_g1_i3	6.7062	−2.3284	0.0000	0.0000	–	–	–
TRINITY_DN36563_c0_g1_i5	6.6760	−2.0072	0.0000	0.0149	Axin	0	*Papilio* sp.

(Continued)

TABLE 2 | Continued

Transcript ID	Log_2 fold change	Ave. expression	*P*-value	FDR-adjusted *P*-value	Top UniRef90 BLASTX Hit	E-value	Organism
TRINITY_DN37270_c2_g1_i4	6.6685	−2.6072	0.0001	0.0178	–	–	–
TRINITY_DN39071_c0_g1_i2	6.6680	−3.4169	0.0000	0.0088	Putative uncharacterized protein	0	*Tribolium castaneum*
TRINITY_DN39461_c2_g2_i3	6.5369	−3.3048	0.0000	0.0000	–	–	–
TRINITY_DN39385_c2_g1_i1	6.4561	0.4404	0.0000	0.0007	–	–	–
TRINITY_DN36280_c3_g1_i10	6.4352	−1.6074	0.0001	0.0339	Putative uncharacterized protein	3.00E-08	*Culex quinquefasciatus*
TRINITY_DN37496_c0_g1_i4	6.4322	−2.7123	0.0000	0.0000	–	–	–
TRINITY_DN37496_c0_g1_i6	6.3548	−2.6646	0.0000	0.0000	–	–	–
TRINITY_DN37496_c0_g2_i12	6.3394	−2.4473	0.0000	0.0000	Uncharacterized protein	2.00E-96	*Danaus plexippus*
TRINITY_DN37877_c0_g1_i2	6.3218	−3.3586	0.0000	0.0004	–	–	–
TRINITY_DN30964_c1_g2_i5	6.3073	−2.7133	0.0000	0.0002	Ester hydrolase C11orf54 homolog	3.00E-136	*Amyelois transitella*
TRINITY_DN34741_c4_g2_i4	6.2842	−3.2922	0.0001	0.0191	–	–	–
TRINITY_DN37496_c0_g1_i10	6.2575	−2.7025	0.0000	0.0000	–	–	–
TRINITY_DN40271_c4_g1_i5	6.2438	−3.0788	0.0000	0.0004	–	–	–
TRINITY_DN38404_c0_g2_i2	6.2234	−2.7265	0.0000	0.0000	Acyl-CoA synthetase short-chain family member 3 mitochondrial	0	*Amyelois transitella*
TRINITY_DN32565_c0_g2_i3	6.2016	−2.6574	0.0000	0.0000	–	–	–
TRINITY_DN39907_c0_g1_i3	6.1037	−2.8112	0.0001	0.0207	Coronin-6 isoform X1	0	*Obtectomera* sp.
TRINITY_DN37997_c0_g1_i2	6.0595	−2.4577	0.0000	0.0001	Type II inositol 1,4,5-trisphosphate 5-phosphatase	0	*Papilio* sp.
TRINITY_DN31620_c0_g1_i1	6.0511	−3.5720	0.0000	0.0002	Uncharacterized protein	0	*Papilio* sp.
TRINITY_DN37364_c0_g5_i1	5.9831	−3.5345	0.0000	0.0000	Cystinosin homolog isoform X1	3.00E-12	*Plutella xylostella*
TRINITY_DN38655_c0_g1_i1	5.9808	−3.0258	0.0000	0.0078	ATP-binding cassette sub-family G member 5	0	*Bombyx mori*
TRINITY_DN32711_c0_g1_i3	5.9679	−2.9031	0.0000	0.0007	Doublesex- and mab-3-related transcription factor 3	6.00E-134	*Amyelois transitella*
TRINITY_DN29332_c0_g1_i3	5.8838	−3.1589	0.0000	0.0027	–	–	–
TRINITY_DN35731_c2_g1_i1	5.8568	−1.1235	0.0002	0.0488	–	–	–
TRINITY_DN39575_c4_g3_i5	5.8164	−3.7350	0.0001	0.0360	–	–	–
TRINITY_DN33671_c2_g1_i7	5.7500	−3.1041	0.0000	0.0000	–	–	–
TRINITY_DN27959_c1_g1_i1	5.7076	−3.3723	0.0000	0.0004	Uncharacterized protein	7.00E-98	*Bombyx mori*
TRINITY_DN30778_c0_g1_i4	5.6997	−2.5512	0.0001	0.0233	Putative chemosensory ionotropic receptor IR75d (Fragment)	0	*Spodoptera littoralis*
TRINITY_DN33595_c2_g1_i8	5.6920	−2.9806	0.0000	0.0001	Uncharacterized protein	0	*Bombyx mori*

(Continued)

TABLE 2 | Continued

Transcript ID	Log_2 fold change	Ave. expression	*P*-value	FDR-adjusted *P*-value	Top UniRef90 BLASTX Hit	E-value	Organism
TRINITY_DN37234_c0_g2_i1	5.6403	−2.9497	0.0000	0.0023	–	–	–
TRINITY_DN37848_c1_g3_i6	5.6040	−3.0244	0.0000	0.0000	Mutant cadherin	8.00E-16	*Helicoverpa armigera*
TRINITY_DN16945_c0_g1_i1	5.4814	−3.4197	0.0000	0.0016	–	–	–
TRINITY_DN37364_c0_g1_i1	5.4556	−3.7441	0.0000	0.0000	Heat shock protein 67B2-like isoform X2	1.52E-87	*Helicoverpa armigera*
TRINITY_DN38899_c0_g1_i1	5.2744	4.5685	0.0000	0.0016	Uncharacterized protein	8.00E-168	*Danaus plexippus*
TRINITY_DN38884_c1_g1_i5	5.1372	−3.8241	0.0000	0.0009	–	–	–
TRINITY_DN33012_c0_g1_i2	5.1079	−1.6502	0.0000	0.0134	–	–	–
TRINITY_DN37390_c4_g4_i3	5.0402	−3.2212	0.0001	0.0341	–	–	–
TRINITY_DN33831_c3_g1_i12	5.0258	−3.2425	0.0001	0.0177	–	–	–
TRINITY_DN38345_c0_g1_i5	4.9378	−3.2726	0.0000	0.0016	Dorsal 1a	5.00E-100	*Spodoptera litura*
TRINITY_DN40225_c4_g3_i3	4.8838	−2.1985	0.0000	0.0100	Glutathione *S*-transferase 2-like	1.19E-127	*Spodoptera litura*
TRINITY_DN36290_c2_g1_i3	4.8566	−3.2157	0.0000	0.0003	–	–	–
TRINITY_DN39385_c0_g1_i1	4.8501	1.4926	0.0000	0.0001	–	–	–
TRINITY_DN32186_c0_g1_i3	4.7616	−1.9394	0.0001	0.0286	–	–	–
TRINITY_DN37042_c3_g1_i2	4.6684	−2.2283	0.0000	0.0032	–	–	–
TRINITY_DN35392_c2_g1_i10	4.6570	−3.2271	0.0001	0.0308	Uncharacterized protein LOC107191251	8.00E-94	*Dufourea novaeangliae*
TRINITY_DN33705_c1_g1_i5	4.6475	−3.9626	0.0002	0.0392	Synaptic vesicle glycoprotein 2B-like	1.00E-112	*Amyelois transitella*
TRINITY_DN38135_c7_g3_i1	4.6201	−2.5497	0.0001	0.0266	–	–	–
TRINITY_DN33081_c0_g1_i4	4.6060	−2.4724	0.0001	0.0320	Dual specificity protein phosphatase 18	4.00E-28	*Operophtera brumata*
TRINITY_DN36290_c2_g1_i9	4.4223	−2.1098	0.0000	0.0061	Sodium/potassium-transporting ATPase subunit beta-2-like	6.00E-19	*Amyelois transitella*
TRINITY_DN38768_c0_g1_i1	4.3666	−0.2316	0.0000	0.0035	Uncharacterized protein LOC106125418	7.00E-147	*Papilio* sp.
TRINITY_DN40225_c4_g3_i4	4.3080	1.0204	0.0000	0.0024	–	–	–
TRINITY_DN35309_c0_g1_i1	4.2933	−1.4013	0.0003	0.0654	Uncharacterized protein LOC105383334	2.00E-52	*Plutella xylostella*
TRINITY_DN39696_c4_g6_i1	4.2733	−3.4922	0.0001	0.0201	–	–	–
TRINITY_DN39395_c1_g1_i5	4.1823	−3.4153	0.0000	0.0002	Serine/arginine repetitive matrix protein 1-like isoform X1	6.00E-135	*Papilio xuthus*
TRINITY_DN39527_c0_g1_i11	3.7373	6.2709	0.0000	0.0027	Z band alternatively spliced PDZ-motif protein 66	1.00E-41	*Papilio xuthus*
TRINITY_DN38817_c2_g2_i4	3.6350	−2.4653	0.0001	0.0233	Uncharacterized protein (Fragment)	1.00E-94	*Pararge aegeria*
TRINITY_DN38768_c0_g1_i3	3.4858	1.2063	0.0000	0.0023	Uncharacterized protein LOC106125418	3.00E-86	*Papilio* sp.
TRINITY_DN37183_c3_g1_i4	3.4076	3.6403	0.0001	0.0269	–	–	–

(Continued)

TABLE 2 | Continued

Transcript ID	Log_2 fold change	Ave. expression	*P*-value	FDR-adjusted *P*-value	Top UniRef90 BLASTX Hit	E-value	Organism
TRINITY_DN31771_c5_g1_i1	3.3905	−3.4420	0.0002	0.0363	–	–	–
TRINITY_DN36952_c0_g1_i7	3.3250	−1.6521	0.0001	0.0191	Cytochrome CYP341B3	0	*Spodoptera littoralis*
TRINITY_DN25843_c0_g2_i1	2.9305	−0.9304	0.0000	0.0134	–	–	–
TRINITY_DN39385_c1_g2_i2	2.8797	−1.0180	0.0001	0.0214	–	–	–
TRINITY_DN39461_c2_g2_i6	2.8066	0.9131	0.0000	0.0093	–	–	–
TRINITY_DN31963_c0_g1_i4	2.7588	−2.9390	0.0001	0.0238	–	–	–
TRINITY_DN36997_c1_g1_i5	2.6367	2.6586	0.0002	0.0402	–	–	–
TRINITY_DN39518_c1_g1_i3	2.6160	1.7318	0.0002	0.0431	ATP-binding cassette sub-family G member 8	0	*Amyelois transitella*
TRINITY_DN37039_c2_g2_i5	2.5625	0.4671	0.0002	0.0484	Phosphatidylglycero phosphatase and protein-tyrosine phosphatase 1	5.00E-123	*Amyelois transitella*
TRINITY_DN35489_c0_g1_i7	2.4620	2.2836	0.0000	0.0052	–	–	–
TRINITY_DN39905_c2_g3_i1	2.3712	−0.0139	0.0000	0.0077	–	–	–
TRINITY_DN35975_c0_g1_i7	2.3211	−0.8273	0.0000	0.0025	Protein Gawky	0	*Papilio* sp.
TRINITY_DN35944_c2_g2_i1	2.3075	−3.1464	0.0001	0.0290	Uncharacterized protein	3.00E-10	*Papilio xuthus*
TRINITY_DN40277_c8_g2_i4	2.0996	−0.4446	0.0001	0.0237	Uncharacterized protein LOC106713896 partial	2.00E-19	*Papilio machaon*
TRINITY_DN33887_c0_g2_i12	1.9515	−1.5605	0.0001	0.0237	Ubiquitin (fragment)	2.00E-57	*Protostomia* sp.
TRINITY_DN37783_c3_g1_i2	1.8835	−3.4846	0.0000	0.0061	–	–	–
TRINITY_DN30635_c2_g1_i1	1.8800	0.3746	0.0002	0.0495	REPAT30	2.00E-63	*Spodoptera* sp.
TRINITY_DN32840_c2_g1_i2	1.8271	−0.6562	0.0001	0.0248	–	–	–
TRINITY_DN39967_c1_g1_i5	1.7884	2.2680	0.0001	0.0314	Cytoplasmic dynein 1 intermediate chain isoform X8	0	*Amyelois transitella*
TRINITY_DN39493_c0_g3_i1	1.7112	−0.6333	0.0002	0.0393	Rho GTPase-activating protein 190-like	6.00E-44	*Plutella xylostella*
TRINITY_DN38296_c0_g1_i4	1.5741	0.0926	0.0001	0.0207	Uncharacterized protein	1.00E-103	*Danaus plexippus*
TRINITY_DN37877_c0_g1_i22	1.5721	−4.2336	0.2917	0.6661	–	–	–
TRINITY_DN39931_c0_g1_i9	1.5383	1.9613	0.0002	0.0415	Uncharacterized protein LOC101741686	0	*Bombyx mori*
TRINITY_DN37435_c0_g1_i4	1.3806	4.7764	0.0001	0.0298	Casein kinase I isoform gamma-3	0	*Pongo abelii*
TRINITY_DN33003_c3_g1_i2	1.2936	5.2805	0.0002	0.0438	–	–	–

synapses, respectively, and one related to neurotrophin signaling in neurons specifically (**Figure 2B**).

DISCUSSION

A Comparison of Predator-Induced Gene Expression Responses in Other Animals

Our results build on a growing body of literature detailing auditory sensory mode and predator-induced shifts in gene expression in vertebrates and invertebrates (Nanda et al., 2008; Leder et al., 2009; Preisser, 2009; Sheriff and Thaler, 2014; Takahashi, 2014; Harris and Carr, 2016; Adamo, 2017a,b). Several studies have focused on describing the gene expression dynamics of large-scale predator-induced morphological changes that occur in organisms displaying predation-related polyphenisms, including multiple species of *Daphnia* (Schwarzenberger et al., 2009; Spanier et al., 2010; Rozenberg et al., 2015) and the Hokkaido salamander (*Hynobius retardatus*; Matsunami et al., 2015). Less striking predator-induced changes also have been

TABLE 3 | List of downregulated (log_2-transformed fold change) transcripts recovered from brain tissue RNA extractions in bat call-exposed *Spodoptera frugiperda* adult male moths relative to controls, including the most significant (e-value < 1e-5) BLASTX protein annotation from the UniRef90 database and the organism from which the annotation is derived.

Transcript ID	Log_2 fold change	Ave. expression	*P*-value	FDR-adjusted *P*-value	Top UniRef90 BLASTX Hit	E-value	Organism
TRINITY_DN22838_c0_g2_i1	−10.5503	0.8597	0.0000	0.0000	–	–	–
TRINITY_DN34268_c3_g1_i3	−10.3989	−0.6024	0.0000	0.0000	27 kDa hemolymph protein	5.00E-90	*Pararge aegeria*
TRINITY_DN40225_c4_g3_i5	−10.3616	−0.3944	0.0000	0.0001	–	–	–
TRINITY_DN34268_c4_g1_i1	−10.1455	−0.6638	0.0000	0.0000	–	–	–
TRINITY_DN25356_c0_g2_i1	−9.7357	0.4281	0.0000	0.0000	–	–	–
TRINITY_DN38145_c1_g1_i1	−9.4768	0.0648	0.0000	0.0000	–	–	–
TRINITY_DN38793_c0_g2_i2	−9.3882	−0.2339	0.0002	0.0498	Equilibrative nucleoside transporter	0	*Pararge aegeria*
TRINITY_DN36116_c4_g2_i6	−9.3085	−0.4714	0.0000	0.0008	–	–	–
TRINITY_DN38739_c1_g1_i5	−9.1023	−1.2984	0.0000	0.0000	Protein polybromo-1	0	*Papilio* sp.
TRINITY_DN33671_c2_g1_i1	−9.0696	−1.3407	0.0000	0.0000	–	–	–
TRINITY_DN32305_c5_g1_i3	−8.8475	−0.6495	0.0000	0.0042	–	–	–
TRINITY_DN35489_c0_g1_i6	−8.8298	−1.4900	0.0000	0.0003	–	–	–
TRINITY_DN24438_c0_g2_i1	−8.7905	1.2303	0.0000	0.0003	–	–	–
TRINITY_DN33318_c7_g1_i4	−8.7839	−1.2434	0.0000	0.0001	–	–	–
TRINITY_DN37153_c0_g3_i7	−8.6013	−1.5878	0.0000	0.0001	Voltage-dependent T-type calcium channel subunit alpha-1G	0	*Bombyx mori*
TRINITY_DN29335_c0_g1_i1	−8.5909	−1.4573	0.0000	0.0000	Uncharacterized protein LOC106129727	4.00E-36	*Amyelois transitella*
TRINITY_DN33003_c2_g1_i2	−8.5809	−0.4078	0.0000	0.0000	–	–	–
TRINITY_DN38739_c1_g1_i1	−8.5236	−1.5621	0.0000	0.0001	Protein polybromo-1	0	*Papilio* sp.
TRINITY_DN37612_c0_g1_i1	−8.4772	−1.6945	0.0001	0.0233	Peripheral-type benzodiazepine receptor isoform X1	4.00E-83	*Bombyx mori*
TRINITY_DN32142_c0_g2_i1	−8.4481	−1.5247	0.0000	0.0000	–	–	–
TRINITY_DN36507_c0_g1_i5	−8.4379	−0.1039	0.0001	0.0313	–	–	–
TRINITY_DN38898_c0_g1_i4	−8.3471	−0.2149	0.0000	0.0001	ADP ribosylation factor	1.00E-107	*Oryctes borbonicus*
TRINITY_DN37203_c0_g1_i2	−8.3042	−1.6302	0.0000	0.0000	Integrin beta pat-3	1.00E-100	*Danaus plexippus*
TRINITY_DN30280_c6_g1_i2	−8.1934	1.1569	0.0000	0.0047	–	–	–
TRINITY_DN35996_c6_g2_i7	−8.1751	−0.4905	0.0000	0.0002	Uncharacterized protein	2.00E-120	*Operophtera brumata*
TRINITY_DN33703_c0_g1_i10	−7.8473	−0.8351	0.0000	0.0000	FH1/FH2 domain-containing protein 3	0	*Bombyx mori*
TRINITY_DN32368_c2_g1_i1	−7.8392	−1.7751	0.0000	0.0000	–	–	–
TRINITY_DN33037_c7_g1_i1	−7.7746	−0.7471	0.0000	0.0013	–	–	–
TRINITY_DN32675_c1_g1_i2	−7.7181	−0.5445	0.0000	0.0061	–	–	–
TRINITY_DN35308_c0_g7_i2	−7.6312	−1.8407	0.0000	0.0000	Uncharacterized protein LOC106143546	0	*Amyelois transitella*
TRINITY_DN32480_c1_g1_i2	−7.6141	−1.7956	0.0000	0.0003	–	–	–
TRINITY_DN38739_c1_g1_i4	−7.5996	−1.8992	0.0000	0.0003	Protein polybromo-1	0	*Papilio* sp.
TRINITY_DN30400_c2_g1_i3	−7.5346	−1.9257	0.0000	0.0001	–	–	–

(Continued)

TABLE 3 | Continued

Transcript ID	Log_2 fold change	Ave. expression	*P*-value	FDR-adjusted *P*-value	Top UniRef90 BLASTX Hit	UniRef90 Match E-value	Organism
TRINITY_DN32071_c5_g2_i3	−7.5332	−0.9221	0.0000	0.0124	–	–	–
TRINITY_DN35282_c3_g2_i3	−7.5319	−1.5419	0.0001	0.0313	2-Methylene-furan-3-one reductase-like	0	*Bombyx mori*
TRINITY_DN39284_c17_g3_i1	−7.5016	−1.1912	0.0000	0.0001	Uncharacterized protein	4.00E-19	*Danaus plexippus*
TRINITY_DN36116_c4_g2_i3	−7.4942	−0.4979	0.0001	0.0332	–	–	–
TRINITY_DN37745_c1_g3_i1	−7.4529	−2.0874	0.0000	0.0000	–	–	–
TRINITY_DN38739_c1_g1_i12	−7.3904	−2.1667	0.0000	0.0014	Protein polybromo-1	0	*Papilio* sp.
TRINITY_DN32480_c1_g1_i1	−7.3853	−2.0826	0.0000	0.0000	–	–	–
TRINITY_DN35996_c6_g3_i1	−7.3769	−1.0123	0.0000	0.0009	–	–	–
TRINITY_DN37270_c2_g1_i3	−7.3114	−2.1964	0.0000	0.0001	–	–	–
TRINITY_DN37446_c0_g1_i5	−7.2694	−0.5738	0.0002	0.0435	–	–	–
TRINITY_DN34464_c1_g2_i7	−7.2006	−1.3360	0.0000	0.0008	Kv channel-interacting protein 4-like	2.00E-112	*Amyelois transitella*
TRINITY_DN34160_c1_g2_i1	−7.1791	−1.1829	0.0000	0.0000	DNA N^6-methyl adenine demethylase-like isoform X1	2.00E-48	*Amyelois transitella*
TRINITY_DN35932_c1_g2_i2	−7.1349	−2.5094	0.0001	0.0308	Uncharacterized protein LOC106133073 isoform X1	8.00E-133	*Amyelois transitella*
TRINITY_DN32911_c0_g2_i1	−7.0475	−1.7120	0.0000	0.0001	–	–	–
TRINITY_DN38739_c1_g1_i14	−6.9385	−1.9898	0.0000	0.0000	Protein polybromo-1	0	*Papilio* sp.
TRINITY_DN39310_c1_g2_i4	−6.9376	−0.1062	0.0000	0.0024	Ankyrin repeat domain-containing protein 17-like	1.00E-121	*Papilio xuthus*
TRINITY_DN36781_c3_g1_i6	−6.9246	−1.4879	0.0002	0.0448	Cytochrome P450	0	*Spodoptera litura*
TRINITY_DN38815_c3_g4_i6	−6.8984	0.0770	0.0000	0.0000	–	–	–
TRINITY_DN32730_c0_g1_i3	−6.8032	−1.5119	0.0000	0.0000	Decaprenyl-diphosphate synthase subunit 2	0	*Amyelois transitella*
TRINITY_DN37877_c0_g1_i17	−6.7731	−1.4192	0.0000	0.0057	–	–	–
TRINITY_DN38024_c0_g2_i11	−6.7309	−1.2620	0.0000	0.0000	–	–	–
TRINITY_DN34405_c8_g4_i1	−6.7285	−1.5021	0.0000	0.0043	–	–	–
TRINITY_DN38296_c0_g1_i8	−6.7146	−1.4859	0.0000	0.0002	Uncharacterized protein	7.00E-104	*Danaus plexippus*
TRINITY_DN19414_c1_g1_i1	−6.6813	−1.5167	0.0000	0.0000	Glutamate synthase	3.00E-50	*Bombyx mori*
TRINITY_DN38328_c0_g1_i4	−6.6761	−2.2826	0.0000	0.0012	Uncharacterized protein	0	*Danaus plexippus*
TRINITY_DN38884_c1_g1_i14	−6.6106	−1.1688	0.0000	0.0022	–	–	–
TRINITY_DN35288_c0_g5_i3	−6.5728	−1.2283	0.0001	0.0234	–	–	–
TRINITY_DN37832_c2_g1_i1	−6.5413	−1.5530	0.0000	0.0000	–	–	–
TRINITY_DN38898_c0_g1_i3	−6.5141	−1.6907	0.0000	0.0008	–	–	–
TRINITY_DN37781_c1_g1_i6	−6.4907	−2.0293	0.0000	0.0010	Maltase 2-like isoform X1	4.00E-85	*Amyelois transitella*
TRINITY_DN32376_c4_g1_i4	−6.4609	−1.2338	0.0000	0.0009	–	–	–
TRINITY_DN33252_c1_g1_i3	−6.4412	−2.4937	0.0000	0.0000	–	–	–

(Continued)

TABLE 3 | Continued

Transcript ID	Log_2 fold change	Ave. expression	*P*-value	FDR-adjusted *P*-value	Top UniRef90 BLASTX Hit	UniRef90 Match E-value	Organism
TRINITY_DN36153_c0_g1_i3	−6.4325	−0.4561	0.0001	0.0254	Guanine nucleotide-binding protein-like 3 homolog	1.00E-92	*Papilio* sp.
TRINITY_DN30786_c0_g1_i4	−6.4324	−1.8255	0.0000	0.0000	–	–	–
TRINITY_DN34405_c8_g1_i1	−6.3667	−2.5342	0.0000	0.0002	–	–	–
TRINITY_DN38604_c4_g5_i2	−6.3565	−2.5853	0.0000	0.0000	–	–	–
TRINITY_DN34116_c1_g2_i1	−6.3322	−2.1255	0.0001	0.0167	Uncharacterized protein	2.00E-118	*Acyrthosiphon pisum*
TRINITY_DN19813_c0_g1_i1	−6.2812	−2.6099	0.0000	0.0000	–	–	–
TRINITY_DN19414_c0_g1_i1	−6.2801	−1.5813	0.0000	0.0000	Glutamate synthase NADH amyloplastic	6.00E-39	*Amyelois transitella*
TRINITY_DN32420_c1_g1_i2	−6.2359	−1.7600	0.0000	0.0001	–	–	–
TRINITY_DN34560_c0_g1_i3	−6.2113	−1.4242	0.0000	0.0009	Integrin beta	0	*Spodoptera frugiperda*
TRINITY_DN39620_c1_g1_i1	−6.1933	−2.6925	0.0000	0.0000	–	–	–
TRINITY_DN39051_c0_g2_i4	−6.1856	−1.5023	0.0000	0.0003	Uncharacterized protein	0	*Bombyx mori*
TRINITY_DN38145_c3_g1_i7	−6.1258	4.6107	0.0000	0.0007	Uncharacterized protein	4.00E-64	*Bombyx mori*
TRINITY_DN39075_c0_g1_i3	−6.1197	−2.0518	0.0000	0.0057	Uncharacterized protein	1.00E-82	*Bombyx mori*
TRINITY_DN37832_c3_g1_i1	−6.0722	−1.6664	0.0000	0.0036	–	–	–
TRINITY_DN32193_c2_g1_i4	−6.0557	−1.2068	0.0001	0.0284	Mitoferrin-1-like	9.00E-74	*Plutella xylostella*
TRINITY_DN32223_c5_g5_i2	−5.9885	−2.7475	0.0000	0.0000	–	–	–
TRINITY_DN39620_c1_g1_i3	−5.9855	−2.7377	0.0000	0.0000	–	–	–
TRINITY_DN13201_c0_g2_i1	−5.9681	−2.8018	0.0000	0.0000	Uncharacterized protein (fragment)	7.00E-06	*Piscirickettsia salmonis*
TRINITY_DN32859_c0_g1_i5	−5.9676	−2.3071	0.0000	0.0021	Putative pigeon protein	8.00E-97	*Danaus plexippus*
TRINITY_DN38145_c2_g1_i1	−5.9460	1.8448	0.0000	0.0001	–	–	–
TRINITY_DN40054_c3_g2_i4	−5.9348	−2.0780	0.0000	0.0001	WD repeat-containing protein 7 isoform X4	0	*Papilio* sp.
TRINITY_DN32169_c0_g1_i1	−5.9273	−2.4522	0.0000	0.0007	Muscle segmentation homeobox-like	2.00E-125	*Amyelois transitella*
TRINITY_DN28597_c2_g1_i2	−5.8573	−2.7815	0.0000	0.0001	–	–	–
TRINITY_DN38225_c0_g1_i1	−5.7856	6.9465	0.0001	0.0174	–	–	–
TRINITY_DN36707_c1_g1_i9	−5.7281	2.6878	0.0000	0.0001	Small conductance calcium-activated potassium channel protein	0	*Papilio polytes*
TRINITY_DN38163_c1_g2_i3	−5.7064	−0.9392	0.0000	0.0000	Catenin alpha	0	*Papilio polytes*
TRINITY_DN32901_c1_g5_i6	−5.6814	−2.8263	0.0000	0.0001	–	–	–
TRINITY_DN36307_c4_g1_i1	−5.6794	−2.9278	0.0000	0.0000	–	–	–
TRINITY_DN33558_c0_g2_i2	−5.6784	−1.7908	0.0000	0.0045	–	–	–
TRINITY_DN30964_c1_g2_i11	−5.6598	−2.8307	0.0000	0.0013	Ester hydrolase C11orf54 homolog	4.00E-136	*Amyelois transitella*
TRINITY_DN29565_c0_g1_i2	−5.6537	−2.9354	0.0000	0.0007	–	–	–
TRINITY_DN29467_c1_g1_i2	−5.5721	−0.9144	0.0001	0.0233	–	–	–
TRINITY_DN32901_c1_g5_i5	−5.5703	−2.7745	0.0000	0.0054	–	–	–
TRINITY_DN39896_c1_g2_i9	−5.5700	−2.0328	0.0000	0.0036	–	–	–

(Continued)

TABLE 3 | Continued

Transcript ID	Log_2 fold change	Ave. expression	*P*-value	FDR-adjusted *P*-value	Top UniRef90 BLASTX Hit	UniRef90 Match E-value	Organism
TRINITY_DN34685_c1_g1_i7	−5.4612	−3.0575	0.0000	0.0001	Laminin subunit alpha-1-like	2.00E-20	*Papilio machaon*
TRINITY_DN39620_c0_g1_i1	−5.4020	−3.0114	0.0000	0.0013	–	–	–
TRINITY_DN36528_c1_g3_i2	−5.3538	−0.7063	0.0001	0.0281	–	–	–
TRINITY_DN35630_c2_g1_i1	−5.3404	−2.3969	0.0002	0.0364	Retrovirus-related Pol polyprotein from type-2 retrotransposable element R2DM	0	*Ceratitis capitata*
TRINITY_DN39032_c0_g1_i9	−5.3317	−0.2183	0.0000	0.0016	Bromodomain-containing protein DDB_G0270170-like isoform X2	4.00E-133	*Papilio machaon*
TRINITY_DN38390_c0_g1_i4	−5.3096	−0.0925	0.0001	0.0209	Phosphatidylinositol 5-phosphate 4-kinase type-2 beta	0	*Ditrysia* sp.
TRINITY_DN37365_c2_g1_i1	−5.2580	−2.0441	0.0001	0.0248	Uncharacterized protein (Fragment)	1.00E-10	*Lottia gigantea*
TRINITY_DN32376_c4_g1_i2	−5.2177	−0.9262	0.0003	0.0586	–	–	–
TRINITY_DN39073_c3_g2_i13	−5.1049	−0.1081	0.0000	0.0141	ATP-citrate synthase	0	*Amyelois transitella*
TRINITY_DN37823_c2_g1_i8	−5.1015	−2.4653	0.0000	0.0001	Omega-amidase NIT2-A isoform X1	2.00E-145	*Amyelois transitella*
TRINITY_DN32098_c6_g2_i5	−5.0548	−1.6991	0.0001	0.0308	–	–	–
TRINITY_DN37877_c0_g1_i9	−4.9263	−1.0798	0.0003	0.0551	–	–	–
TRINITY_DN37877_c0_g1_i3	−4.9010	−1.0945	0.0001	0.0264	–	–	–
TRINITY_DN28575_c0_g1_i3	−4.8579	−2.8302	0.0000	0.0150	Solute carrier family 12 member 4 isoform X3	2.00E-22	*Papilio* sp.
TRINITY_DN28328_c0_g1_i3	−4.8107	−0.5775	0.0001	0.0237	–	–	–
TRINITY_DN29816_c0_g1_i2	−4.7990	4.0335	0.0000	0.0030	–	–	–
TRINITY_DN33031_c2_g2_i1	−4.7372	−2.7559	0.0000	0.0036	–	–	–
TRINITY_DN39545_c3_g1_i15	−4.6986	−2.8005	0.0001	0.0308	Endonuclease-reverse transcriptase	5.00E-21	*Bombyx mori*
TRINITY_DN31477_c1_g1_i4	−4.5937	−2.5111	0.0000	0.0149	Formin-like protein 15	2.00E-07	*Papilio machaon*
TRINITY_DN31395_c1_g1_i7	−4.5928	−3.1908	0.0003	0.0561	Arrestin homolog	0	*Obtectomera* sp.
TRINITY_DN34685_c1_g2_i2	−4.5483	−2.1143	0.0001	0.0309	Zinc finger MYM-type protein 1-like	6.00E-40	*Hydra vulgaris*
TRINITY_DN40097_c0_g1_i1	−4.4741	0.6703	0.0000	0.0009	c-Myc promoter-binding protein	0	*Homo sapiens*
TRINITY_DN38137_c0_g1_i4	−4.4587	−1.6514	0.0001	0.0360	Atrial natriuretic peptide-converting enzyme	0	*Bombyx mori*
TRINITY_DN36274_c0_g1_i2	−4.4428	−0.1042	0.0000	0.0001	Peptidyl-prolyl *cis–trans* isomerase FKBP65-like	5.00E-132	*Amyelois transitella*
TRINITY_DN37566_c0_g1_i2	−4.3099	−1.7781	0.0002	0.0444	–	–	–
TRINITY_DN35090_c0_g1_i3	−4.2946	−0.3012	0.0000	0.0043	Uncharacterized protein	3.00E-165	*Bombyx mori*

(Continued)

TABLE 3 | Continued

Transcript ID	Log_2 fold change	Ave. expression	*P*-value	FDR-adjusted *P*-value	Top UniRef90 BLASTX Hit	UniRef90 Match E-value	Organism
TRINITY_DN34211_c0_g1_i3	−4.2249	1.0640	0.0001	0.0178	Nuclear distribution protein NUDC	2.00E-161	*Biston betularia*
TRINITY_DN15012_c0_g2_i1	−4.1508	−1.2234	0.0001	0.0339	C-Cbl-associated protein isoform A	3.00E-10	*Operophtera brumata*
TRINITY_DN37270_c2_g1_i1	−4.1292	−3.6822	0.0002	0.0477	–	–	–
TRINITY_DN28005_c0_g1_i2	−4.1255	0.4174	0.0000	0.0017	39S ribosomal protein L34 mitochondrial	2.00E-36	*Papilio machaon*
TRINITY_DN28021_c0_g1_i1	−4.0897	−1.1177	0.0001	0.0233	Uncharacterized protein	3.00E-47	*Helobdella robusta*
TRINITY_DN40141_c1_g2_i1	−4.0186	−2.8432	0.0000	0.0010	Glutamate synthase (Fragment)	5.00E-38	*Pararge aegeria*
TRINITY_DN39099_c2_g1_i1	−3.9941	2.8840	0.0000	0.0061	–	–	–
TRINITY_DN36043_c0_g5_i1	−3.8734	−1.9678	0.0000	0.0091	–	–	–
TRINITY_DN37203_c0_g1_i3	−3.8576	3.1474	0.0000	0.0025	Integrin beta pat-3	8.00E-94	*Danaus plexippus*
TRINITY_DN37133_c4_g2_i5	−3.8209	−1.1337	0.0002	0.0402	–	–	–
TRINITY_DN31324_c0_g1_i3	−3.7809	−2.0764	0.0001	0.0207	Ubiquitin carboxyl-terminal hydrolase 34-like	2.00E-116	*Papilio machaon*
TRINITY_DN37153_c0_g3_i6	−3.6908	4.6006	0.0001	0.0207	Voltage-dependent T-type calcium channel subunit alpha-1G	0	*Bombyx mori*
TRINITY_DN39970_c7_g3_i1	−3.6002	−2.2744	0.0000	0.0036	–	–	–
TRINITY_DN36698_c2_g1_i3	−3.5166	−2.5650	0.0002	0.0369	Uncharacterized protein LOC106113347	3.00E-40	*Obtectomera* sp.
TRINITY_DN38059_c0_g1_i1	−3.4333	2.4478	0.0000	0.0053	Putative acetyltransferase ACT11	8.00E-102	*Spodoptera litura*
TRINITY_DN40211_c8_g13_i2	−3.3949	−2.6487	0.0000	0.0012	Uncharacterized protein	1.00E-17	*Piscirickettsia salmonis*
TRINITY_DN31644_c0_g1_i4	−3.2356	−1.6085	0.0000	0.0117	–	–	–
TRINITY_DN34933_c1_g1_i6	−3.1838	−1.7262	0.0000	0.0028	Collagen alpha-1(XXV) chain-like isoform X8	6.00E-69	*Bombyx mori*
TRINITY_DN39085_c0_g1_i6	−3.1011	4.5699	0.0000	0.0034	Uncharacterized protein LOC101738244	3.00E-153	*Bombyx mori*
TRINITY_DN33252_c1_g1_i1	−3.0698	2.3138	0.0000	0.0045	–	–	–
TRINITY_DN35322_c1_g2_i2	−2.9511	−1.3865	0.0003	0.0550	Putative zinc finger protein 91 (Fragment)	2.00E-92	*Operophtera brumata*
TRINITY_DN30567_c6_g2_i1	−2.9059	−1.5217	0.0000	0.0093	–	–	–
TRINITY_DN34764_c2_g2_i9	−2.8990	−2.6731	0.0001	0.0237	–	–	–
TRINITY_DN39515_c0_g1_i5	−2.7516	1.2549	0.0001	0.0286	Lachesin-like	0	*Bombyx mori*
TRINITY_DN33240_c0_g1_i6	−2.7220	−3.2631	0.0000	0.0017	Uncharacterized protein	2.00E-120	*Bombyx mori*
TRINITY_DN29565_c0_g2_i1	−2.6506	−1.7899	0.0000	0.0045	–	–	–
TRINITY_DN35544_c0_g3_i2	−2.6469	−0.4176	0.0000	0.0098	UPF0528 protein CG10038	6.00E-55	*Amyelois transitella*
TRINITY_DN38353_c3_g1_i8	−2.6247	−3.0481	0.0001	0.0232	–	–	–

(Continued)

TABLE 3 | Continued

Transcript ID	Log_2 fold change	Ave. expression	*P*-value	FDR-adjusted *P*-value	Top UniRef90 BLASTX Hit	UniRef90 Match E-value	Organism
TRINITY_DN38108_c0_g2_i6	−2.6245	2.6610	0.0000	0.0100	Uncharacterized protein LOC106136039	2.00E-152	*Amyelois transitella*
TRINITY_DN31477_c2_g1_i3	−2.5817	−0.6315	0.0000	0.0034	–	–	–
TRINITY_DN30567_c9_g1_i1	−2.5463	−1.0038	0.0000	0.0030	–	–	–
TRINITY_DN39985_c0_g1_i3	−2.5356	5.1760	0.0000	0.0027	Histone-lysine *N*-methyltransferase ash1	0	*Papilio* sp.
TRINITY_DN31213_c0_g1_i2	−2.4078	0.6190	0.0002	0.0472	GDNF-inducible zinc finger protein 1-like	9.00E-157	*Papilio* sp.
TRINITY_DN38047_c1_g1_i5	−2.2382	1.4642	0.0000	0.0068	Uncharacterized protein	6.00E-112	*Obtectomera* sp.
TRINITY_DN37035_c0_g10_i2	−2.1924	3.4328	0.0001	0.0339	–	–	–
TRINITY_DN37004_c7_g1_i3	−2.1562	−2.1811	0.0000	0.0133	–	–	–
TRINITY_DN39427_c3_g1_i2	−2.0552	−0.3606	0.0002	0.0488	Zinc finger protein 62 homolog isoform X2	2.00E-81	*Amyelois transitella*
TRINITY_DN31830_c4_g3_i2	−1.7960	−3.7434	0.0000	0.0043	Mucin-2-like	1.00E-59	*Amyelois transitella*
TRINITY_DN39449_c0_g1_i14	−1.7253	−3.5318	0.0001	0.0207	Uncharacterized protein	0	*Obtectomera* sp.
TRINITY_DN36559_c0_g2_i7	−1.7199	−0.8964	0.0001	0.0327	Uncharacterized protein	2.00E-44	*Danaus plexippus*
TRINITY_DN38544_c0_g3_i1	−1.7008	−1.8335	0.0002	0.0488	–	–	–
TRINITY_DN38707_c1_g2_i9	−1.6822	2.9542	0.0001	0.0237	Cytochrome P450 9A58	1.00E-166	*Spodoptera frugiperda*
TRINITY_DN37901_c0_g1_i4	−1.6242	1.0172	0.0001	0.0207	Uncharacterized protein LOC106132143	0	*Amyelois transitella*
TRINITY_DN37610_c5_g1_i3	−1.6189	0.6339	0.0001	0.0332	Uncharacterized protein LOC105397907	4.00E-92	*Plutella xylostella*
TRINITY_DN37496_c0_g2_i3	−1.5968	1.2808	0.0001	0.0178	Uncharacterized protein	5.00E-97	*Danaus plexippus*
TRINITY_DN32710_c0_g1_i1	−1.5769	2.2427	0.0001	0.0264	Uncharacterized protein	0	*Danaus plexippus*
TRINITY_DN39385_c2_g1_i5	−1.5003	5.7753	0.0001	0.0202	–	–	–
TRINITY_DN22676_c0_g1_i1	−1.4828	−0.6327	0.0001	0.0251	–	–	–

studied in diverse taxa, including stickleback fish (Sanogo et al., 2011) and an intertidal snail (Chu et al., 2014). Exposure to auditory cues of aerial hawking bats for 8 h resulted in significant transcriptomic responses, as evidenced by the wide-ranging fold-changes (log_2FC) in transcript expression reported here. In the brains of predator-stressed sticklebacks, low-to-moderate fold-changes ranged from 2 to 6 (log_2FC; Sanogo et al., 2011), while predator-induced polyphenic *Daphnia* displayed changes ranging from 2 to 10 (log_2FC; Rozenberg et al., 2015). Furthermore, the number of DE transcripts found here is comparable to that found in other RNA-seq studies on predator-induced gene expression among invertebrates. For instance, *Daphnia pulex* exposed to kairomones of predatory phantom midge (*Chaoborus*) larvae displayed 256 DE transcripts (Rozenberg et al., 2015), while only three transcripts were differentially regulated in the intertidal snail *Nucella lapillus* when exposed to seawater that flowed first through a chamber holding a predatory crab (*Carcinus maenas*) feeding on *N. lapillus* (Chu et al., 2014). Further, the number of DE transcripts from brain tissue after predator exposure can vary strongly based on predator identity, as shown by Matsunami et al. (2015) who found that Hokkaido salamander larvae exposed to predatory dragonfly naiads displayed 605 DE transcripts, while only 103 DE transcripts were found after exposure to predatory tadpoles. One primary difference between past studies of predator-induced transcriptional changes that must be considered when interpreting the results presented here is the time scale at which cues of predation are presented. In the case of predator-induced polyphenisms, exposure length depends highly on organism life history but ranges generally

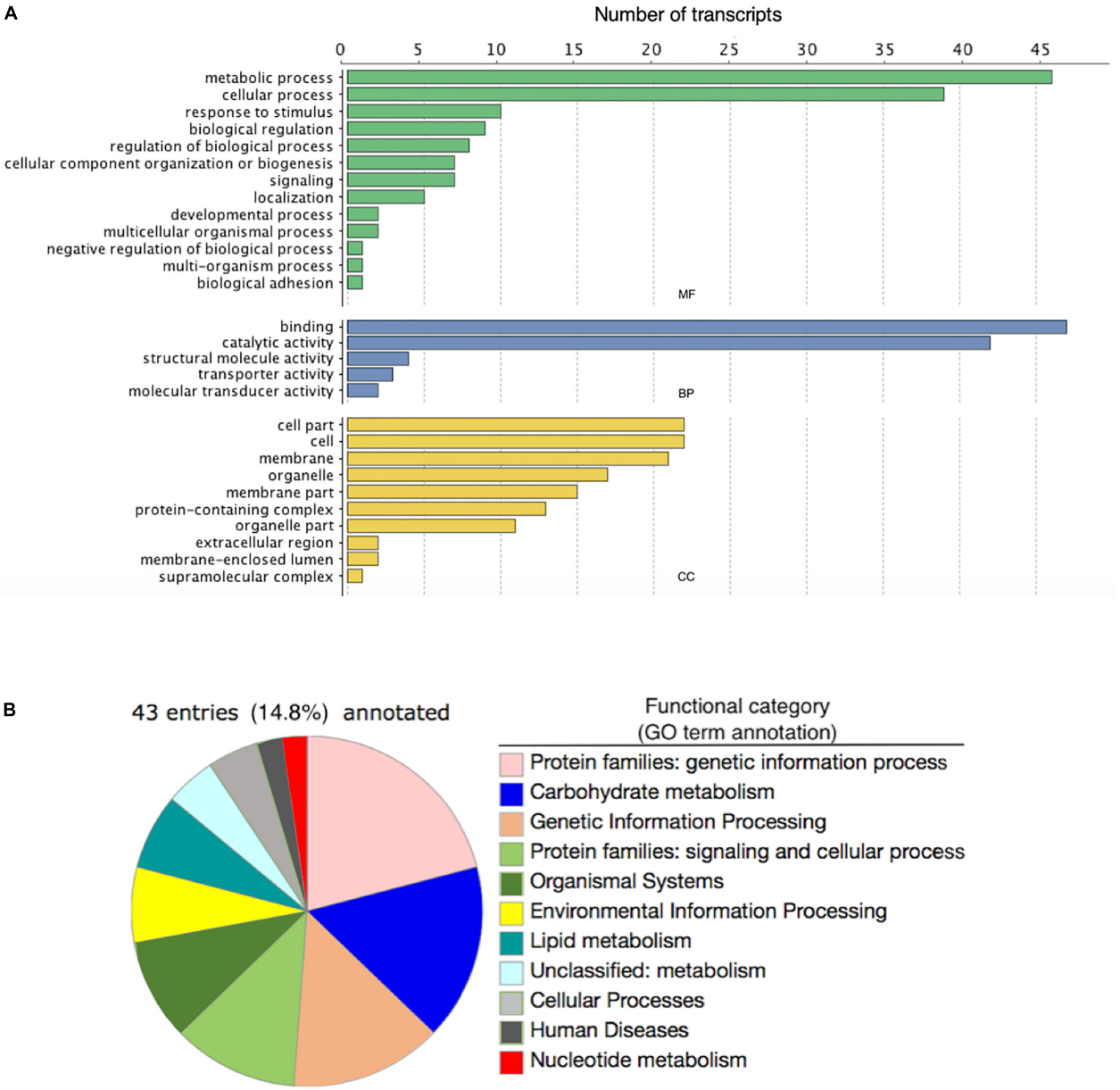

FIGURE 2 | (A) The number of differentially expressed transcripts annotated with Gene Ontology terms corresponding to either a molecular function (MF; green bars), biological process (BP; blue bars), or cellular component (CC; yellow bars) in the brains of four adult male fall armyworm (*Spodoptera frugiperda*) moths exposed to recorded bat foraging and attack calls for 8 h. **(B)** Pie graph detailing the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology annotations associated with differentially expressed transcripts in the male brains of adult fall armyworm moths exposed for 8 h to recorded bat foraging and attack calls.

from a few to several days. Though our study assesses the effects of prolonged, frequent exposure to an auditory cue of predation over a single night, it should be noted that this time scale is much shorter than used in most other studies of predator-induced transcription. Clearly, the degree to which prey respond transcriptionally to cues of predation risk can vary broadly across taxa and no clear pattern has yet emerged. However, the ubiquity with which metazoan life responds transcriptionally to these cues of predation begs the detailed description of these gene pathways, their relevance to physiology and life history, and their evolution throughout the tree of life.

Functional Relevance of Differentially Regulated Genes

Furthermore, our results indicate a broad range of functional annotations related to our DE transcripts. For instance, upregulated transcripts coded for proteins related to cellular signaling, Hsp synthesis, antioxidant metabolism, mitochondrial

TABLE 4 | List of statistically over-represented (hypergeometric test, FDR-adj. $p < 0.05$) Gene Ontology (GO) term annotations associated with the 290 differentially expressed (DE) transcripts identified after frequent, prolonged bat-ultrasound exposure in brain tissue of adult male *Spodoptera frugiperda* moths.

GO category	GO ID	Description	DE cluster frequency	GO-annotated transcriptome frequency	FDR-adjusted *P*-value
Biological process	6536	Glutamate metabolic process	3/102 (2.9%)	10/40511 (0.1%)	1.84E-06
	6537	Glutamate biosynthetic process	3/102 (2.9%)	10/40511 (0.1%)	1.84E-06
	43650	Dicarboxylic acid biosynthetic process	3/102 (2.9%)	21/40511 (0.1%)	1.99E-05
	7229	Integrin-mediated signaling pathway	4/102 (3.9%)	89/40511 (0.1%)	7.85E-05
	43648	Dicarboxylic acid metabolic process	3/102 (2.9%)	53/40511 (0.1%)	3.31E-04
	9084	Glutamine family amino acid biosynthetic process	3/102 (2.9%)	64/40511 (0.1%)	5.77E-04
Molecular function	3682	Chromatin binding	7/102 (6.8%)	77/40511 (0.1%)	1.08E-09
	44877	Macromolecular complex binding	7/102 (6.8%)	135/40511 (0.1%)	5.54E-08
	15930	Glutamate synthase activity	3/102 (2.9%)	5/40511 (0.1%)	1.54E-07
	45181	Glutamate synthase activity, NAD(P)H as acceptor	2/102 (1.9%)	4/40511 (0.1%)	3.75E-05
	16040	Glutamate synthase (NADH) activity	2/102 (1.9%)	4/40511 (0.1%)	3.75E-05
	10181	FMN binding	3/102 (2.9%)	49/40511 (0.1%)	2.62E-04
	16639	Oxidoreductase activity, acting on the CH-NH2 group of donors, NAD or NADP as acceptor	2/102 (1.9%)	11/40511 (0.1%)	3.40E-04
	16638	Oxidoreductase activity, acting on the CH-NH2 group of donors	3/102 (2.9%)	55/40511 (0.1%)	3.70E-04
	4046	Aminoacylase activity	2/102 (1.9%)	12/40511 (0.1%)	4.08E-04

metabolism, oxidoreductase activity, glutamate synthesis, ionotropic receptor activity, gene regulation, ion transport, and cilium assembly. Downregulated transcript annotations also displayed a large degree of functional variability relating to G-coupled protein signaling, cytochrome P450 activity, chromatin-mediated gene regulation, integrin signaling, glutamate biosynthesis, and voltage-dependent ion channels, among others. Several notable transcript upregulations corresponded to unexpected protein annotations, including a mitochondrial calcium uniporter protein ($\log_2$FC = 9.66), an RPGR homolog ($\log_2$FC = 9.86), mutant cadherin ($\log_2$FC = 5.60), mitochondrial choline dehydrogenase ($\log_2$FC = 6.78), and acyl-coenzyme A synthetase short-chain family member 3 ($\log_2$FC = 6.22). The mitochondrial calcium uniporter protein acts as a transmembrane transporter for uptake of calcium ions into mitochondria for use during respiration (Marchi and Pinton, 2014) after these ions are mobilized from intracellular stores by inositol triphosphate. Notably, another significantly upregulated gene among exposed individuals was type 2 inositol 1,4,5-triphosphate 5-phosphatase ($\log_2$FC = 6.06). In humans, this phosphatase hydrolyzes inositol triphosphate and functions as a signal-terminating enzyme, preventing further calcium release (Ross et al., 1991; Contreras et al., 2010).

The second most upregulated transcript codes for a RPGR homolog, a protein usually associated with cilia development in the photoreceptors of vertebrate eyes (Gakovic et al., 2011), although it localizes to other tissues and cell types as well (Khanna et al., 2005). We suggest that RPGR upregulation may be related to increased cilia development and neuronal connections but since its expression has not been studied in insect eyes or other tissues, further conclusions about the function of this protein under predator-stressed conditions in *S. frugiperda* cannot be made. Because *S. frugiperda* brains were excised without compromising pigment-storing ommatidial cells, the RPGR expression pattern observed here likely is intrinsic to brain tissue and may be related to neural tissues extending from innervations of the eye. Notably, the entire suite of phototransduction proteins found in the *Drosophila* visual system is also found to act in the fly's auditory transduction system, with visual rhodopsins serving mechanical transduction and amplification roles in auditory neurons of the Johnston's organ (Pumphrey, 1940).

Another upregulated transcript that may be related to neuronal development encoded a mutant cadherin protein found in humans. Cadherins are calcium-dependent cell-cell adhesion proteins that are integral in nearly every step of neural development in larval *Drosophila* (Fung et al., 2009),

have been implicated in guiding new neuron development contributing to neural plasticity (Edsbagge et al., 2004), and are even involved in hair bundle development in vertebrate ears (Hirano and Takeichi, 2012). As expression of cadherins is usually repressed and localized only to synaptic areas in mature brain tissues (Hirano and Takeichi, 2012), the fact that it is highly upregulated in predator-cue exposed *S. frugiperda* coupled with RPGR upregulation suggests that neural plasticity and development of new neural connections upon exposure to novel environmental cues may play key roles in functionally responding to auditory predator cues.

Several strongly downregulated transcripts also mapped to unexpected protein annotations, including a 27 kDa hemolymph protein ($\log_2$FC = −10.40), DNA 6mA demethylase-like isoform X1 ($\log_2$FC = −7.18), decaprenyl-diphosphate synthase subunit 2 (DDSS2; $\log_2$FC = −6.80), FH1/FH2 domain-containing protein 3 (FHOD3; $\log_2$FC = −7.85), and Pb-1 (isoform $\log_2$FC = −9.10, −8.52, −7.60, −7.39, −6.94, 8.54). The 27 kDa hemolymph protein family consists of proteins found in diverse insect taxa but their function remains unknown. DNA 6mA demethylase is another enzyme correlated with a highly downregulated transcript. Methylation of 6mA has been studied primarily in prokaryotes, where it serves as the primary mechanism for epigenetic signaling via DNA methylation—as opposed to the primary mechanism found in eukaryotes, 5-methylcytosine methylation (Vanyushin et al., 1968). Demethylases associated with 6mA and 5-methylcytosine serve to remove methyl groups from DNA and RNA, affecting the transcription and translation of affected nucleic acid chains. In plants and vertebrates, 6mA methylation both increases and decreases transcription factor binding (Luo et al., 2015), while in *Drosophila melanogaster* loss of a putative 6mA demethylase resulted in increased transposon expression (Zhang et al., 2013). Notably, a transcript annotated with histone-lysine *N*-methyltransferase ($\log_2$FC = −2.54) and five transcript isoforms annotated with Pb-1 were downregulated after predator-cue exposure, although another Pb-1 isoform was also upregulated. These proteins are involved in histone H3 remodeling and binding, respectively (Chandrasekaran and Thompson, 2007; An et al., 2011). Although the functional significance of these downregulated genes in the brain of predator-exposed *S. frugiperda* is unclear, epigenetic mechanisms appear to be induced in some manner.

The enzyme DDSS2 catalyzes a reaction to supply decaprenyl diphosphate for use in ubiquinone-10 biosynthesis. Ubiquinone-10 is concentrated in mitochondria, where it acts as a component of the electron transport chain during aerobic cellular respiration (Ernster and Dallner, 1995), although it also is found in many diverse organelles at lower concentrations. In this context, ubiquinone-10 acts as an electron transport enzyme moving electrons from enzyme complexes I and II to III in the electron transport chain, a function only it and vitamin K_2 are able to perform (Bhalerao and Clandinin, 2012). Ubiquinone-10 also serves as an antioxidant due to its weak electron affinity when reduced. In this state, electrons are held so loosely that the molecule readily gives up electrons to oxidized substrates. For instance, within mitochondria, ubiquinone-10 prevents the oxidation of DNA nucleotides during interactions between peroxidase and DNA-bound metal ions (López et al., 2010; Miyamae et al., 2013). Although the down-regulation of DDSS2 does not directly imply that lower levels of ubiquinone-10 were present in predator-cue exposed *S. frugiperda*, further studies should examine ubiquinone-10 responses to predator exposure. With knowledge of the increased mitochondrial metabolic activity suggested by several upregulated transcripts discussed previously, it is surprising that DDSS2 is downregulated, as a greater need for electron transport substrates and antioxidants with enhanced energy production might be expected. Clearly, there is still much to learn in elucidating the role of DDSS2, and mitochondrial metabolism in general, in the context of predator-induced stress responses.

Formin homology 1/formin homology 2 domain-containing protein 3 (FHOD3), another protein that mapped to a highly downregulated transcript in the predator-exposed *S. frugiperda* brain, acts as an actin regulator with a scaffolding function and has been found, in humans, to affect organogenesis, tissue homeostasis, and cancer-cell invasion (Katoh and Katoh, 2004). Actin, a protein that forms microfilaments and constitutes the actin cytoskeleton in all eukaryotic cells, plays a key role in cellular locomotion and shape (Lodish et al., 2000). FHOD family proteins are thought to bind to the growing barbed-end of actin polymers and serve both to deliver new actin monomers and promote actin polymerization, effectively mediating the growth of the actin cytoskeleton (Bechtold et al., 2014). FHOD family proteins are regulated by rho-GTPases, a member of which was downregulated after predator-exposure. Furthermore, actin-binding Lin11, Isl-1, Mec-3 protein 3 and alpha catenin were also down-regulated and act as a scaffold protein (Barrientos et al., 2007) and a cellular linking protein between cadherins and actin-containing filaments (Geoffrey and Robert, 2000; Drees et al., 2005; Yamada et al., 2005), respectively. Considering that a transcript encoding a mutant cadherin was upregulated in predator-exposed brains as well, these patterns suggest that the actin cytoskeleton is affected by predator-exposure and that changes in cellular morphology and motility may be involved.

Overrepresented Gene Ontology Terms and KEGG Pathway Reconstruction in Predator-Stressed Brain Tissue

Relative to the list of DE transcript annotations in this study, the overrepresented GO terms enriched in the brains of *S. frugiperda* after predator exposure were generally restricted to three biochemical pathways: (1) chromatin and macromolecule binding, (2) glutamate synthesis and metabolism, and (3) aminoacylase activity, although terms related to oxidoreductase activity, flavin mononucleotide binding, and integrin signaling also were overrepresented. To the best of our knowledge, these GO terms have not been implicated in any other study of predator-induced transcription. The small set of GO-annotated DE transcripts identified here limit the statistical detection of subtly over- and under-represented terms; regardless, we found 15 GO terms to be highly significantly overrepresented in our set of annotated DE transcripts relative to the frequency at which these terms were found in our GO-annotated

transcriptome ($p < 0.0004$). Chromatin binding ($p < 0.0000$) and macromolecular complex binding ($p < 0.0000$) were the most highly overrepresented GO terms identified both with 7 out of 102 GO-annotated DE transcripts mapped to these terms. The binding of cellular proteins to chromatin can elicit varied cellular responses, such as transcriptional regulation, DNA replication, and chromatin remodeling (Ricke and Bielinsky, 2005). Considering that transcripts mapping to ash1 and Pb-1 protein annotations were also differentially regulated, the presence of these GO terms again implies that epigenetic modifications seem to be induced upon exposure to predator cues.

The set of GO terms pertaining to glutamate synthesis and metabolism included glutamate synthase activity, as well as glutamate biosynthesis and metabolism, glutamine family amino acid biosynthesis, and dicarboxylic acid biosynthesis and metabolism. Glutamate, an amino acid anion derived from its dicarboxylic state, glutamic acid, is used during protein synthesis, but is the most abundant excitatory neurotransmitter in the vertebrate brain (Locatelli, 2005). Although acetylcholine is the primary excitatory neurotransmitter in the insect nervous system (Wnuk et al., 2014), glutamate also plays an excitatory role (Leboulle, 2012) as glutamate immunoreactivity (Sinakevitch et al., 2001) and glutamate-induced ion currents (Cayre et al., 1999) have been observed in insect neurons. Intriguingly, application of glutamate to the mushroom body brain regions of the honeybee, *Apis mellifera*, facilitates glutamatergic neurotransmission and olfactory learning (Locatelli, 2005), and glutamate-mediated neurotransmission has also been implicated in the visual and tactile (Liang et al., 2012) sensory systems. Notably, one of the strongly upregulated ($\log_2$FC = 5.70) transcripts we found mapped to a fragment of the ionotropic receptor 75d (IR75d). Benton et al. (2009) found that IR75d and 69 other IR-family proteins carry ionotropic glutamate receptor-like amino acid positions and surmised that IRs may similarly act in chemosensory neuron signaling. Although 21 of these 69 novel IRs showed transcriptional responses to chemical signals in the *Drosophila* antenna, including IR75d, the remaining 46 displayed no chemosensory-related expression (Benton et al., 2009). Further, the presence of different IR subtypes on a given neuron also influences synaptogenesis, synaptic activity, and experience-dependent neural plasticity in *Drosophila* (Thomas and Sigrist, 2012). Knowing that biochemical pathways pertaining to glutamate production were altered in the brains of predator-cue exposed *S. frugiperda* coupled with evidence that IR75d was upregulated post-exposure, we suggest that IR75d and its relatives may be involved in the development and function of auditory mechanosensory neurons.

Manual analysis of the KEGG pathway reconstruction of DE transcripts revealed a variety of interconnected neuron-specific metabolic and signaling cascades that were affected by bat ultrasound exposure, including the mechanistic target of rapamycin (mTOR)/Akt, MAPK, Wnt, prolactin, Hippo, and calcium signaling systems, and associated regulatory responses, such as p53, renin-angiotensin, and NF-κB transcript expression. Notably, recent research on the conserved function of these biochemical pathways in the nervous systems of metazoan taxa across phyla describes the function and biological relevance of these pathways on an organismal scale (Mattson and Camandola, 2001; Lilienbaum and Israe, 2003; Pan, 2007; Lau and Bading, 2009; Tedeschi and Di Giovanni, 2009; Brown et al., 2012; Lin et al., 2012; Graber et al., 2013; Flentke et al., 2014; Mao et al., 2014; Patil et al., 2014; Layden et al., 2016; Guo et al., 2017; Haspula and Clark, 2018). For instance, synaptic glutamate (Sinakevitch et al., 2010; Thomas and Sigrist, 2012; Li et al., 2016), mTOR/Akt (Guo et al., 2017), intracellular calcium (Kaltschmidt et al., 2005; Lau and Bading, 2009), and prolactin signaling (Brown et al., 2012; Belugin et al., 2013), followed by differential p53 and NF-κB transcription (Kaltschmidt et al., 2005; Lau and Bading, 2009) are each implicated in the apoptotic and synaptic-activity mediated induction of neural plasticity, learning, and memory from diverse taxa spanning arthropods to chordates. Clearly, much work remains to divulge how the vast evolutionary divergences inherent between the conserved physiological cellular signaling and gene networks of most, if not all, metazoan taxa correlate with lineage and ecology-specific organismal responses to diverse stressors, including predation risk.

CONCLUSION AND FUTURE DIRECTIONS

This study demonstrates that exposure to ecologically relevant auditory cues of predation risk in *S. frugiperda* results in varied but strong patterns of up- and down-regulation of a broad range of protein products within the moth brain. The most strongly up- and down-regulated transcripts found in this study correspond to many cellular functions which include mitochondrial metabolism, glutamate synthesis and metabolism, actin cytoskeleton morphology and motion, axon guidance, neural structure, and epigenetic modifications. This is a promising first step in developing a model for the transcriptional impacts of frequent and repeated exposure to bat predation cues in *S. frugiperda,* which may represent acute and chronic responses of cells to predator-induced stress. Several novel predator-cue induced transcriptional pathways are implicated in these results and present promising opportunities for future research. These broad predator-induced transcriptional responses are characteristic of those found in previous studies, such as in predator-stressed stickleback fish (Sanogo et al., 2011), *Daphnia* (Rozenberg et al., 2015), and the Hokkaido salamander (Matsunami et al., 2015). Contrary to our expectations, there is little overlap between previously reported responses to predator-induced stress, such as neuropeptide production and increased antioxidant activity, and the novel predator-induced functional annotations reported here. However, mitoferrin, a solute carrier responsible for iron uptake by red blood cells in vertebrates, was significantly upregulated in the brains of stickleback fish repeatedly exposed to a chemical cue of predation (Sanogo et al., 2011), although it was downregulated ($\log_2$FC = −6.06) in *S. frugiperda* post-exposure. In insects, the function of mitoferrin is less well understood, though *D. melanogaster* with mitoferrin mutations experienced problems with spermatogenesis and

development to adulthood (Metzendorf and Lind, 2010). Apart from this similarity, the novel transcriptional responses to predation in *S. frugiperda* observed here may be specialized to auditory perception or found only in Lepidoptera. Furthermore, although efforts were made to avoid auditory habituation in this study, expression profiles described here bear similarities to past studies of bird-song habituation in the brains of zebra finches (*Taeniopygia guttata*), with both resulting in the downregulation of genes pertaining to cytoskeletal dynamics and mitochondrial metabolism (Dong et al., 2009).

One primary limitation of our study is a lack of time-series expression data that would have bolstered our ability to infer the functional relevance of specific transcripts for both short- and long-term physiological acclimations to auditory cues of predation. Further work, such as comparing expression profiles through time and between frequent and infrequent cue exposures, would aid in parsing the effects due to neural habituation/auditory stimulation, *per se*, and those related specifically to predator cue exposure. Specifically, producing a detailed time-course transcriptional profile of tissue-specific prey physiology beginning after the first moments of predator-cue exposure and proceeding over the course of hours to days in cue-exposed *S. frugiperda* or other predator–prey systems would provide comparative insights into the temporal dynamics of stress-induced transcription during acute relative to prolonged exposure to predation risk. Another limitation of this study is a lack of transcript validation via quantitative reverse-transcriptase polymerase chain reaction assays, yet we argue the novelty of the system and the foundational datasets we have produced that can inform future hypotheses warrant their use by the scientific community. Another confounding factor that may have contributed to the relatively noisy patterns of expression in exposed *S. frugiperda* brains shown here is the type of auditory stimulus we used. For instance, by using three bat calls from three different species, we have endeavored to replicate an ecologically relevant cue of predation risk, yet the nightly soundscape a moth is exposed to *in situ* varies hour-to-hour and night-to-night in sound intensity, conspecific and interspecific composition, and many other attributes that we did not incorporate into our experiments. We encourage future investigators to develop high quality, ultrasonic soundscape recordings in relevant field settings ahead of time, when possible, and replicate these via nightly broadcasts of each night's recording. In conclusion, as more diverse, annotated insect genomes become available and the function of more genes are elucidated by experimental and comparative evidence, studies that assess the physiological effects of prolonged predation risk on prey across the tree of life will continue to divulge remarkably conserved patterns of stress-induced molecular mechanisms between lineages.

AUTHOR CONTRIBUTIONS

SC conducted the experiments and developed this report. ST contributed to the conceptual development, logistical support, and proofreading of this work.

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Testosterone Reduces Fear and Causes Drastic Hypomethylation of Arginine Vasopressin Promoter in Medial Extended Amygdala of Male Mice

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Testosterone reduces anxiety-like behaviors in rodents and increases exploration of anxiogenic parts of the environment. Effects of testosterone on innate defensive behaviors remain understudied. Here, we demonstrate that exogenous testosterone reduces aversion to cat odor in male mice. This is reflected as increased exploration of area containing cat urine when castrated male mice are supplied with exogenous testosterone. We also report that exogenous testosterone leads to DNA hypomethylation of arginine vasopressin (AVP) promoter in posterodorsal medial amygdala (MePD) and medial bed nucleus of stria terminalis (BNST). Our observations suggest that testosterone acting on AVP system within extended medial amygdala might regulate defensive behaviors in mice.

Keywords: androgen, testosterone, defensive behaviors, semiochemicals, AVP methylation, predator cues

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HIGHLIGHTS

- Exogenous testosterone reduces innate fear to cat odor in male mice.
- Testosterone reduces AVP methylation in extended medial amygdala.

INTRODUCTION

Approach-avoidance conflict has been routinely used to model anxiety in laboratory tests. This includes elevated plus-maze where approach to a novel area and simultaneous avoidance of open spaces is used to create a test with remarkable construct validity. Testosterone induces anxiolysis in this test as manifested by greater time spent on the open arms in both male mice (Aikey et al., 2002) and male rats (Bitran et al., 1993; Frye and Seliga, 2001). Anxiolytic effect of testosterone can also be observed in tests that do not use exploration to construct approach-avoidance continuum, e.g., defensive burying in rats (Fernández-Guasti and Martínez-Mota, 2005). In contrast to anxiety, innate or unconditioned fear is often modeled in laboratory using tests that do not use approach-avoidance conflict as a building block. Instead innate fear is often measured as uni-dimensional avoidance of a predator, or more often an olfactory cue of the predator (Wallace and Rosen, 2000; Dielenberg and McGregor, 2001; King et al., 2005).

Behavioral ecology of innate defensive behaviors, on the other hand, is intertwined with that of reproductive behaviors. The investment in reproductive behaviors often parallels with taking risks and with disengagement from cues of threats within the environment. Thus, imperatives of sexual behaviors are often in conflict with those of protection from predation (Magnhagen, 1991). Pertinent examples include increased parasitism of male crickets caused by eavesdropping of mating calls by parasitoids (Mangold, 1978; Fowler, 1987; Walker and Wineriter, 1991; Walker, 1993). Similarly, sexual investment in acoustic signals enhances predation by bats in bushcrickets and frogs (Zuk and Kolluru, 1998). *Toxoplasma gondii* infection of rats provides another example of the relationship between defensive behaviors and sexual investment. This protozoan parasite is both venereally transmitted from infected males to females and trophically transmitted from infected rats to cats. Rats infected with *Toxoplasma gondii* produce more sexual pheromones that increase their perceived attractiveness to uninfected females (Kumar et al., 2014). The infection also reduces innate aversion to cat odors, suggesting a parallel between sexual investment and reduction in innate fear (Vyas, 2015).

The inverse relationship between defensive behaviors and reproductive investment suggests that these behaviors converge on a similar set of regulatory neuroendocrine mechanisms. It is also likely that testosterone is a crucial part of mechanisms underlying the balance between fear and reproduction. The role for testosterone is supported by its anxiolytic activity, albeit in behavioral experiments that do not directly test aversion to predator cues (Albert et al., 1986; Aikey et al., 2002). Moreover, testosterone is required for reproductive behavior in males (Bialy and Sachs, 2002). For example, production of male pheromones in rats and mice requires testosterone (Kumar et al., 2014; Vasudevan et al., 2015). Thus, it is possible that testosterone can be used as a reliable neuroendocrine signal of ongoing reproductive investment and can lead to reduced defensive behaviors that usually accompany such episodes. This possibility remains hitherto understudied.

Interestingly, both aversion to predator odors and approach to sexual signals require medial extended amygdala within rodent brain (Meredith, 1998; Newman, 1999; Dielenberg et al., 2001; Gross and Canteras, 2012; Bowen et al., 2014). Medial amygdala contains copious amounts of receptors for testosterone and its metabolites (Commins and Yahr, 1985; Simerly et al., 1990; Hines et al., 1992). Posterodorsal part of the medial amygdala is enriched in neurons containing arginine vasopressin (AVP). This neuronal population is part of extra-hypothalamic AVP system and is characterized by its testosterone-dependence (Mizukami et al., 1983; Wang et al., 1993; Wang and De Vries, 1995; Bialy and Sachs, 2002; Cooke, 2006; Auger et al., 2011; Rood et al., 2013). In rats, testosterone causes DNA hypomethylation within the AVP promoter in this brain region (Auger et al., 2011). AVP neurons within extended medial amygdala are also activated during copulation in male mice (Ho et al., 2010) and appetitive approach of male rats to female pheromones in rats (Hari Dass and Vyas, 2014a). In short, role of medial amygdala in both fear and reproduction, and testosterone-responsive nature of AVP neurons within medial amygdala, suggest that these neurons can reduce defensive behaviors during episodes of reproductive preparedness and high testosterone. A testosterone effect on medial amygdala neurons has been shown in rats. Yet, it remains unclear if testosterone causes hypomethylation of AVP promoter in medial amygdala of mice; if similar effects of testosterone are also seen in homologous bed nucleus of stria terminalis (BNST); and if testosterone reduces defensive behaviors.

In this backdrop, we studied effects of testosterone on innate fear by castrating male mice and supplementing them with either placebo or exogenous testosterone over a chronic time window. We also studied ability of testosterone to induce DNA hypomethylation in promoter of AVP gene in two homologous nuclei of medial extended amygdala, posterodorsal medial amygdala (MePD) and posteromedial BNST.

MATERIALS AND METHODS

Animals

Adult male C57BL/6 mice (7–8 weeks old at start of experiment) were procured from InVivos Pte Ltd. Animals were acclimatized for at least 1 week before start of experiments (12:12 light-dark cycle; lights on at 07:00 h; *ad libitum* food and water). This study was carried out in accordance with the recommendations of Institutional Animal Care and Use Committee of Nanyang Technological University. The protocol was approved by the Institutional Animal Care and Use Committee of Nanyang Technological University.

Castration and Testosterone Supplementation Experiment

All animals were castrated at the start of the experiment. Surgery was conducted in an aseptic environment under deep anesthesia (2% gaseous isoflurane with pure O_2). Castration was performed by a medial scrotal incision and bilateral removal of testes and vas deferens. Castrated mice were randomly assigned in two experimental groups: those supplemented with placebo (14 mice) and supplemented with exogenous testosterone (12 mice). A silastic tubing (outer diameter = 0.5 cm; inner diameter = 0.3 cm; length = 1 cm; sealed with wood) containing either testosterone or sham was implanted in each animal subcutaneously at the level of the scapula through a small incision at the nape. Testosterone supplementation group received silastic tubing filled with 1.5 mg of testosterone propionate (Sigma-Aldrich). This treatment is reported to result in circulating testosterone concentration similar to that found in uncastrated males (Nyby, 2008). Placebo mice were implanted with empty silastic tubing.

Cat Odor Aversion Assay

Aversion to bobcat urine was quantified 14 days after implantation of silastic tubing. Experiment was conducted in a rectangular arena (46 × 9 cm) which was divided into two opposing and identical sections. Male mice were first individually habituated in the testing maze for two consecutive days for 1,200 s in the absence of odor. This was followed by another day

of habituation in the presence of a novel odor at one terminus (2 ml of 1:4 dilution of vanilla essence). On the following day, animals were exposed to 1 ml of bobcat urine placed at the same terminus where novel odor was placed on the preceding day (Predator Pee, USA). Bobcat urine was replenished after every trial which lasted for 1,200 s. Aversion to cat odor was quantified by the percentage of time spent in bisect containing bobcat urine, relative to total time spent in vicinity of vanilla odor and cat odor.

Tissue Processing

Animals were restrained and sacrificed immediately after cat odor aversion behavioral assay. Brains were removed and snap frozen in liquid nitrogen. Brains from a randomly chosen sub-sample of placebo and testosterone group were further probed during subsequent assay (n = 4 for placebo and 5 for testosterone). The brains were sectioned at 100 μm using a cryotome (−20°C) and mounted on glass slides. Glass-mounted sections were then micro-dissected to harvest paraventricular nucleus (PVN) of hypothalamus, posterodorsal medial amygdala and BNST. Genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen).

Methylation Sensitive Restriction Enzyme Assay

The extent of AVP promoter methylation was quantified using the methylation-sensitive restriction enzyme (MSRE) digestion assay coupled with quantitative polymerase chain reaction (qPCR). Equal amount of DNA from each mouse was divided into two tubes: restriction enzyme-treated and sans-enzyme. Methylation-sensitive endonucleases, Hpall (New England Biolabs, Ipswich, MA, USA) and BstUl (Fermentas Inc., Glen Burnie, MD, USA), were used to specifically bind and cleave un-methylated DNA at any CCGG and CGCG sequence respectively. Subsequently, primers flanking AVP promoter regions were used to estimate abundance of un-cleaved DNA using qPCR. Hypermethylation in this assay manifests as lower divergence DNA abundance estimates between enzyme-treated and sans-enzyme samples.

Statistics

Data was analyzed with GraphPad Prism 7.0 software. Inter-group differences were analyzed using independent sample Student's t-test ($^{*}p < 0.050$, $^{**}p < 0.010$, $^{***}p < 0.001$, $^{****}p < 0.000$; n is reported in corresponding figure legends).

RESULTS

Exogenous Testosterone Reduced Innate Aversion to Cat Odor

Castrated male mice were implanted subcutaneously with testosterone-filled or empty silastic tubing for 14 successive days. Control animals exhibited robust aversion of bobcat urine, as evidenced by lower occupancy in arena bisect containing the predator odor (**Figure 1A**, placebo group; one-sample t-test against chance expectation of 50%; $t_{(13)} = -18.044$, $p < 0.0001$). Control animals did not exhibit aversion to a new non-predator odor on the preceding day (**Figure 1B**, placebo group; one-sample t-test against chance expectation of 50%; $t_{(13)} = -1.734$, $p = 0.107$). Time spent in cat bisect relative to sum of time spent in cat and vanilla bisects also showed statistical significance and robust departure from chance estimate of 50% (**Figure 1C**, placebo group; one-sample t-test against chance expectation of 50%; $t_{(13)} = -10.487$, $p < 0.0001$).

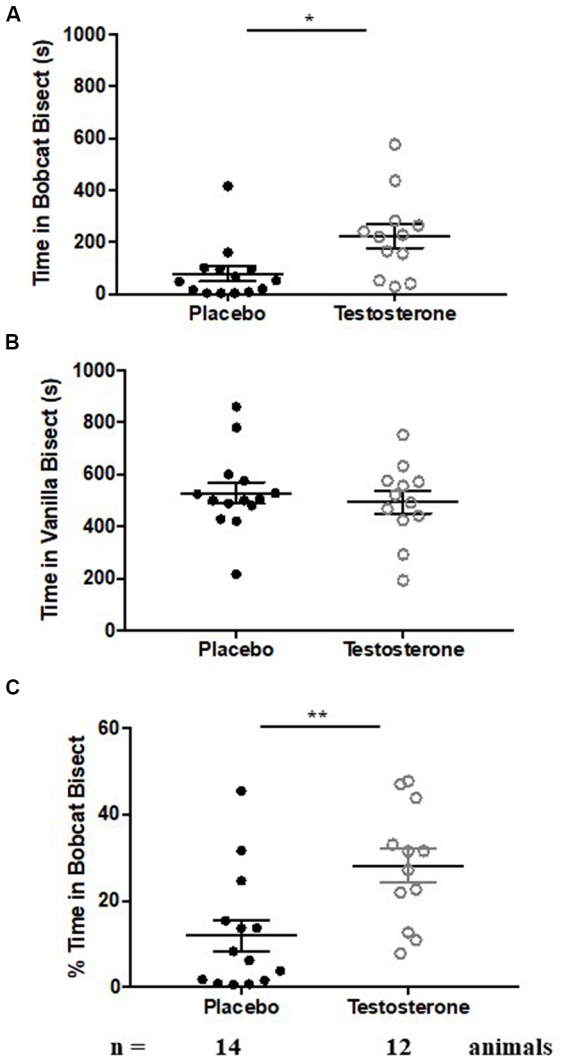

FIGURE 1 | Testosterone supplementation results in reduction in fear to predator odor. Aversion to cat odor in animals chronically supplemented with testosterone (*white circles*) or placebo (*black circles*). Panel **(A)** depicts time spent exploring the bisect containing cat odor. Panel **(B)** depicts time spent exploring the bisect containing vanilla odor. Panel **(C)** depicts percentage time spent exploring cat odor relative to sum of time spent exploring cat and vanilla odor. Bars depict median and inter-quartile range for placebo (*black circles*) and testosterone supplemented (*white circles*) male mice. $^{*}p < 0.05$, $^{**}p < 0.01$, independent sample Student's t-test.

Testosterone supplemented mice retained significant aversion to bobcat odor, shown by reduced occupancy in cat odor bisect

FIGURE 2 | Promoter region of mouse arginine vasopressin (AVP) gene with possible CpG sites, along with primers used to assess methylation levels. Forward and reverse primers for all CpG sites are underlined and CpG sites capitalized and in bold. DNA base sequences and numbering were obtained from Genbank accession no. M88354.1 (1–1,440 base pairs).

(**Figure 1A**, testosterone group; one-sample *t*-test against chance expectation of 50%; $t_{(11)} = -8.107$, $p < 0.0001$). This aversion was independent of neophobia to non-predator novel odor (**Figure 1B**, testosterone group; one-sample *t*-test against chance expectation of 50%; $t_{(11)} = -2.492$, $p = 0.03$). Testosterone mice also exhibited significant aversion to cat odor when time spent in cat bisect was normalized to sum of occupancy in cat and vanilla bisects (**Figure 1C**, testosterone group; one-sample *t*-test against chance expectation of 50%; $t_{(11)} = -5.552$, $p < 0.0001$).

Testosterone supplemented mice exhibited a significant reduction in innate fear during inter-group comparisons (**Figure 1A**, time spent in cat odor bisect; independent sample-*t*-test: $t_{(24)} = -2.749$, $p = 0.011$; Cohen's $d = 1.064$). Occupancy in cat bisect for 75% of testosterone supplemented animals was observed to be above the 75th percentile of the control group. The reduction of fear response was present when time spent in cat odor bisect was normalized against the sum of occupancy in vanilla and cat bisects (**Figure 1C**; independent sample-*t*-test: $t_{(24)} = -3.009$, $p = 0.006$; Cohen's $d = 1.134$). Inter-group comparison demonstrated no significant difference in time spent exploring the vicinity of vanilla odors (**Figure 1B**; independent sample-*t*-test: $t_{(24)} = 0.602$, $p = 0.553$; Cohen's $d = 0.237$; observed power = 0.093 at $\beta = 0.05$).

Testosterone Supplementation Decreased DNA Methylation at Arginine Vasopressin Promoter in Extended Medial Amygdala

NCBI's Basic Local Alignment Search Tool (BLAST) was used to obtain the mouse AVP promoter region sequence of accession number: M88354.1 (1–1,440 base pairs). Four individual CpG sites were then identified in this DNA sequence using the bioinformatics software EMBOSS Cpgplot[1], based on abundance of CG content. DNA methylation was experimentally quantified on these CpG sites. **Figure 2** depicts targeted CpG sites along with primers used in the DNA methylation assay.

We quantified methylation status using methylation-specific endonuclease treatment (HpaII and BstUl) and subsequent qPCR. The HpaII enzyme was used to bind three specific CCGG sequences at CpG site 1, 3 and 4. The BstU1 enzyme was used to bind CGCG sequence at CpG site 2. Inter-group comparison revealed that only one CCGG site, located at base 1250 (CpG site 4), displayed significant hypomethylation with the testosterone supplementation treatment in both posterodorsal medial amygdala (MePD) (**Figure 3**, **Tables 1**, **2**; independent sample-*t*-test: $t_{(7)} = 15.163$, $p < 0.0001$; Cohen's $d = 9.945$) and bed nucleus of stria terminalis (BNST) ($t_{(7)} = 11.908$, $p < 0.0001$; Cohen's $d = 7.606$). Testosterone-treated animals exhibited reduced DNA methylation on this site compared to placebo treatment.

We also examined DNA methylation at the corresponding CpG sites in tissues taken from PVN of hypothalamus. Testosterone did not alter methylation status of DNA on the CpG site 4 (**Figure 3**, **Tables 1**, **2**; $t_{(7)} = 0.018$, $p = 0.986$; Cohen's $d = 0.012$; probability of type 2 error = 0.05). Similarly, testosterone did not alter DNA methylation status on CpG sites 1 through 3 ($p > 0.4$). Thus, effects of testosterone on DNA methylation of AVP promoter CpG site 4 were specific to extended medial amygdala.

[1]http://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/

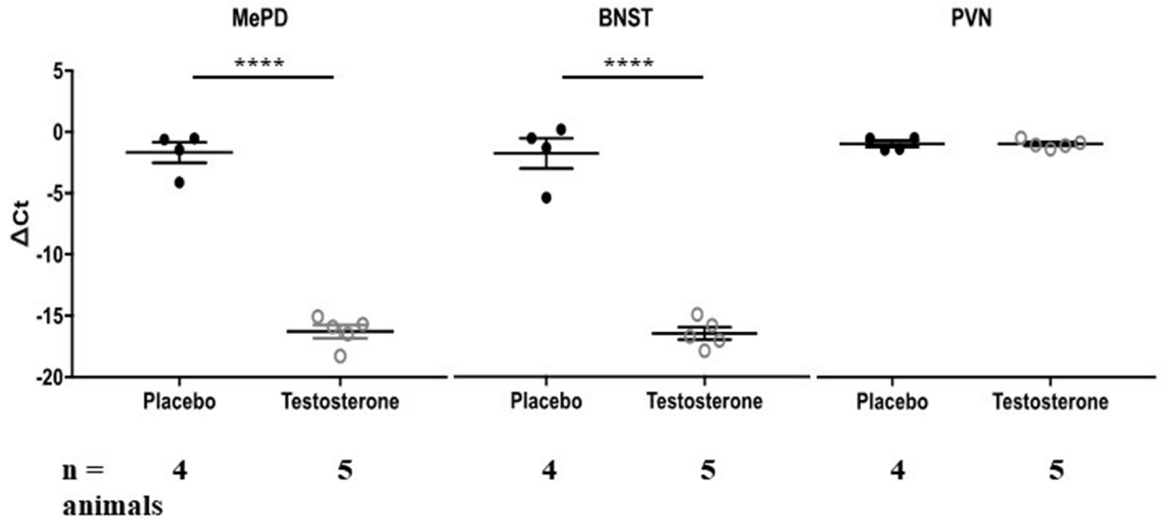

FIGURE 3 | Testosterone supplementation results in hypomethylation of arginine vasopressin (AVP) promoter in posterodorsal medial amygdala (MePD) and bed nucleus of stria terminalis (BNST). DNA methylation status at AVP promoter CpG site 4 was quantified in the MePD, BNST and PVN of all animals chronically supplemented with testosterone (*white circles*) or placebo (*black circles*). Methylation was quantified as ΔCt value (difference in threshold cycle value between samples with or without methylation-specific restriction enzyme treatment). Values that are more negative represent lesser methylation. ****$p < 0.000$, independent sample Student's *t*-test.

TABLE 1 | Raw data of MSRE assay.

	Testosterone	ΔCt	Placebo	ΔCt
MePD	1A	−16.497	2A	−0.530
	1B	−15.679	2B	−1.440
	1C	−18.275	2C	−4.118
	1D	−15.075	2D	−0.609
	1E	−15.910		
BNST	1A	−17.863	2A	−0.509
	1B	−15.812	2B	−1.310
	1C	−16.696	2C	−5.363
	1D	−17.028	2D	0.196
	1E	−14.907		
PVN	1A	−0.459	2A	−0.540
	1B	−1.094	2B	−1.453
	1C	−0.866	2C	−1.390
	1D	−1.376	2D	−0.471
	1E	−1.047		

Testosterone supplementation results in hypomethylation of arginine vasopressin (AVP) in posterodorsal medial amygdala (MePD) and bed nucleus of stria terminalis (BNST) at AVP promoter CpG site 4. This is observed by the raw ΔCt values between testosterone and placebo groups. There is no difference in ΔCt values in paraventricular nucleus (PVN) which serves as a control to our Methylation Sensitive Restriction Enzyme (MSRE) assay.

TABLE 2 | Statistical analysis of methylation at every CpG site for each brain region.

	CpG	*Z*	*P*-value	Cohen's *d*	Type II error
MePD	Region 1	−0.490	0.624	0.684	0.162
	Region 2	−0.735	0.462	0.052	0.051
	Region 3	NA	NA	NA	NA
	Region 4	−2.449	0.014	9.945	1.000
BNST	Region 1	−0.289	0.773	0.578	0.129
	Region 2	−0.245	0.806	0.066	0.051
	Region 3	−2.309	0.021	5.524	1.000
	Region 4	−2.449	0.014	7.606	1.000
PVN	Region 1	−1.715	0.086	1.199	0.448
	Region 2	−0.490	0.624	0.352	0.080
	Region 3	0.624	0.221	0.844	0.232
	Region 4	−0.490	0.624	0.012	0.050

Differences in methylation between placebo-treated and testosterone supplemented male mice for all identified CpG sites. Z-value, p value, effect size and probability of type 2 error for all CpG sites for each brain region.

DISCUSSION

The observations presented here demonstrate that testosterone reduced defensive behavior in male mice, similar to previously reported effects on anxiety (Bitran et al., 1993; Frye and Seliga, 2001; Aikey et al., 2002). We also show that testosterone leads to epigenetic change in BNST and medial amygdala of male mice in the form of DNA hypomethylation in AVP promoter. This agrees with testosterone-mediated epigenetic changes in the rat's medial amygdala (Auger et al., 2011).

A variety of animals exhibit greater circulating testosterone when reproductive opportunities are available or when males engage in reproductive behaviors (Harding, 1981; Wood and Newman, 1995; Wood and Coolen, 1997). Supplementation of testosterone-filled cannulas in the medial amygdala of male Syrian hamsters stimulates male sexual and reproductive behaviors (Harding, 1981; Wood and Newman, 1995; Wood and Coolen, 1997). Previous reports have also suggested that increase in plasma testosterone results in increase in aggression (Harding, 1981; Albert et al., 1986). Specifically, plasma testosterone rises in male birds during aggression that is related to reproduction such as mate guarding and territory formation (Wingfield and Ramenofsky, 1985; Wingfield et al., 1990). This suggests that an up-regulation of testosterone increases

the propensity of the individual to invest in reproduction. Reproductive episodes are often accompanied by reduced defensive behaviors. Observations in this report suggest that testosterone can mediate reduction in defense in addition to mediating reproductive behaviors. In agreement with our observations, reduced testosterone leads to increased analgesia in rats after exposure to a synthetic chemical reminiscent of fox odor (King et al., 2005). These observations suggest that innate fear and reproductive investment are continually mediated by testosterone and form two ends of an approach-avoidance continuum.

We also show testosterone-mediated epigenetic modification of AVP in posterodorsal medial amygdala (MePD) and BNST. These two nuclei are homologous components of extended medial amygdala (Newman, 1999). AVP produced within the medial extended amygdala is positioned as a prime target to mediate inverse relationship between defense and reproduction. Exposing male rats to estrus females or to copulation increases number of medial amygdala AVP neurons colabeled with Fos, an immediate early gene marker of recent neuronal activity (Hari Dass and Vyas, 2014a). Similarly, the same neuronal population shows increased Fos colabeling during experimental treatment that reduces defensive behaviors, i.e., *Toxoplasma gondii* infection in rats (Hari Dass and Vyas, 2014b). Similarly, inter-individual variation in defensive behavior of male rats can be related to medial amygdala AVP (Bowen et al., 2014). Additionally, both the BNST and MePD contain steroid hormone receptors and thus respond to gonadal steroid hormones and their metabolites (Zhou et al., 1994; Tsukahara et al., 2011). Our data is consistent with these aforementioned studies and suggest a role for the medial extended amygdala in regulation of defensive behaviors during reproductive episodes in male rodents.

Testosterone has been demonstrated to act on steroid receptors and then alter the expression of AVP in MePD (Wang et al., 1993; Wang and Vries, 1995) and BNST of the male rat brain (Wang et al., 1993; Zhou et al., 1994; Gabor et al., 2012). AVP neurons within MePD (MePD-AVP neurons) are responsive to testosterone and its metabolites (Mizukami et al., 1983; Wang et al., 1993; Wang and De Vries, 1995; Bialy and Sachs, 2002; Cooke, 2006; Auger et al., 2011; Rood et al., 2013). This responsiveness takes form of DNA hypomethylation in the AVP promoter (Auger et al., 2011). This epigenetic change provides medial amygdala further information about internal metabolic status and resources needed for investment in sexual pursuits. Thus, testosterone may possibly act as an arbitrator for the trade-off between current and residual reproduction in male mice via the MePD-AVP system. It should nonetheless be noted that the present study does not explicitly quantify the trade-off between reproduction and defense, with this being a difficult task in a laboratory environment. The trade-off between defensive and reproductive behaviors might be mediated by extrinsic ecological factors. For example, testosterone is an immune-suppressant and might explain higher parasite burden of males in natural conditions (Roberts et al., 2004; Muehlenbein and Watts, 2010; Martínez-Guijosa et al., 2015). This can reduce lifespan and thus create a pressure on life history for earlier reproduction. Similarly, reproductive behaviors entail reducing fear and taking risks when males create sexual advertisements or engage in mate searching (Stearns, 1989; Magnhagen, 1991; Grostal and Dicke, 1999). This can increase mortality through predation. Both possibilities require extrinsic ecological factors like parasitism or predation to materialize the trade-off. We have been unable to quantify these in the laboratory model.

Observations in this report present interesting parallels with a naturally occurring perturbation models for diminished defensive behaviors. Male rats chronically infected with the parasitic protozoan *Toxoplasma gondii* display an atypical reduction in innate fear to cat odors (Vyas et al., 2007). This infection creates AVP hypomethylation within medial amygdala while not affecting the PVN (Hari Dass and Vyas, 2014b). The reduction in fear by *Toxoplasma gondii* can be blocked by raising animals on a methyl-donor diet, which also creates AVP hyper-methylation in medial amygdala. This suggests that DNA methylation during the infection is causal to loss of fear. This systemic experiment is supported by a region-specific perturbation. The enzyme DNA methyltransferase is required for methylation of DNA bases. This enzyme can be blocked by RG-108. Prior experiments show that when RG-108 is specifically administered within medial amygdala through a cannula, it results in loss of fear and hypomethylation of AVP (Hari Dass and Vyas, 2014b). Thus, molecular events in *Toxoplasma gondii* perturbation model show remarkable congruence with experimental effects on fear and medial extended amygdala AVP obtained through exogenous testosterone. This further supports the possibility that testosterone, acting through medial amygdala AVP, is a likely mediator of reduced defense.

In conclusion, our data shows that testosterone supplementation leads to reduction of fear response to predator odor. We also provide data that implicates testosterone or its metabolites in altering the methylation state of AVP promoter within the medial extended amygdala. We argue that testosterone increase indicates an intention to mate and this shifts the balance between reproduction and defensive behaviors.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript.

AUTHOR CONTRIBUTIONS

WT carried out the experiments, performed the data analysis and prepared the manuscript. SA-S carried out the experiments. AV performed the data analysis, conceived the idea, supervised the research and prepared the manuscript. All authors approved the final manuscript.

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Ecological Stoichiometry: A Link Between Developmental Speed and Physiological Stress in an Omnivorous Insect

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The elemental composition of organisms belongs to a suite of functional traits that may adaptively respond to fluctuating selection pressures. Life history theory predicts that predation risk and resource limitations impose selection pressures on organisms' developmental time and are further associated with variability in energetic and behavioral traits. Individual differences in developmental speed, behaviors and physiology have been explained using the pace-of-life syndrome (POLS) hypothesis. However, how an organism's developmental speed is linked with elemental body composition, metabolism and behavior is not well understood. We compared elemental body composition, latency to resume activity and resting metabolic rate (RMR) of western stutter-trilling crickets (*Gryllus integer*) in three selection lines that differ in developmental speed. We found that slowly developing crickets had significantly higher body carbon, lower body nitrogen and higher carbon-to-nitrogen ratio than rapidly developing crickets. Slowly developing crickets had significantly higher RMR than rapidly developing crickets. Male crickets had higher RMR than females. Slowly developing crickets resumed activity faster in an unfamiliar relative to a familiar environment. The rapidly developing crickets did the opposite. The results highlight the tight association between life history, physiology and behavior. This study indicates that traditional methods used in POLS research should be complemented by those used in ecological stoichiometry, resulting in a synthetic approach that potentially advances the whole field of behavioral and physiological ecology.

Keywords: carbon-to-nitrogen ratio, developmental speed, ecological stoichiometry, elemental body composition, trait-based ecology, *Gryllus integer*, pace-of-life syndrome, physiological stress

INTRODUCTION

Ecological communities consist of a variety of species and are shaped by a complex array of intra- and interspecific interactions that maintain nutrient and energy flows through ecosystems (Meunier et al., 2017; Sperfeld et al., 2017). Importantly, for any given individual, the availability of resources in any particular environment is limited; time, effort and energy used for one purpose diminish those available for another (Stearns, 1992; Sperfeld et al., 2017). This often causes trade-offs in allocations of an individual's resources to such competing life functions as immunity, reproduction, self-maintenance, development and growth (Roff, 1992; Krams et al., 2013a; Luoto, 2019).

Biotic and abiotic environmental stressors (e.g., predation, food limitation, extreme temperatures, drought, competition, growth in stressful conditions) may challenge organismal homeostasis (Boonstra, 2013; Wingfield, 2013; Ferguson et al., 2018). The stressed individual may then alter its behavior and functional traits to accommodate to the challenge, resulting in implications to their dietary choices and the elemental composition of their bodies and waste materials (Christianson and Creel, 2010; Hawlena and Schmitz, 2010a,b). This suggests that investments in stress tolerance and biochemical and behavioral adaptations to environmental stress may further affect the amount of energy available to each individual (Hochachka and Somero, 2002; Ellis and Del Giudice, 2014; Luoto, 2019). Ecological stoichiometry, a framework based on energetics, links the study of these trade-offs with the relative supply of elements in the environment and the metabolic demands and physiological traits of organisms (Meunier et al., 2017; Sperfeld et al., 2017).

Differences in behavioral and physiological responses to stress can be explained using the pace-of-life syndrome (POLS) hypothesis (Réale et al., 2010; Debecker and Stoks, 2018; Mathot and Frankenhuis, 2018; Royauté et al., 2018). This hypothesis originated from the classic concept of *r*- and *K*-selection (MacArthur and Wilson, 1967; Pianka, 1970) and the more recent idea of fast-slow life history continuum (e.g., Promislow and Harvey, 1990; Bielby et al., 2007). It suggests that differences in life history strategies among species or populations are associated with physiological (e.g., metabolic rate; Ricklefs and Wikelski, 2002; Wikelski et al., 2003; Wiersma et al., 2007) and behavioral differences (Wolf et al., 2007; Biro and Stamps, 2008; Réale et al., 2009; Luoto, 2019). The POLS hypothesis predicts that rapidly developing individuals with high activity and boldness have faster life histories (e.g., faster development and reproduction) and higher metabolic rate, which reduces life span through increased oxidative damage (Janssens and Stoks, 2018). This prediction is based on the assumption that high activity and boldness increase resource acquisition. Passive and shy individuals, in contrast, are expected to show the opposite features (Réale et al., 2010).

However, evidence suggests that slower development of prey individuals may also incur stress as it is associated with upregulation of stress-related genes (Gutiérrez-Adán et al., 2004). Wings of late-hatched female damselflies *Lestes viridis* were found to be more asymmetrical than those of early-hatched ones (De Block et al., 2008). Importantly, selection for slower development confers higher levels of anxiety/neuroticism along the stress reactivity axis in crickets (Krams et al., 2017). The observed anxiety in behavior and resting metabolic rate (RMR) in slowly developing crickets under stressful conditions decreased after a selective serotonin reuptake inhibitor (SSRI) treatment (Krams et al., 2018). On the other hand, slower development may be associated with longer lifespan (Brooks and Garratt, 2017; Kecko et al., 2017) which may require an improved immune system (Niemelä et al., 2013; Krams et al., 2015, 2016a). In some species where females have longer lifespan than males, the strength of immune responses and inflammatory immune responses are generally higher in females than in males (Klein, 2012; Klein and Flanagan, 2016; Kecko et al., 2017). However, females often suffer a higher propensity to many autoimmune diseases such as rheumatoid arthritis, fibromyalgia, anxiety and depression (e.g., Dumont-Lagacé et al., 2015), suggesting associations between slower development, longer lifespan and stress resistance (Brooks and Garratt, 2017).

It is considered that rapidly developing individuals are bolder and more stress resistant than slowly developing shy individuals (e.g., Steimer et al., 1997). Nevertheless, how an organism's developmental speed is linked with elemental body composition, metabolism and behavior is not well understood, and a comprehensive approach that combines stoichiometry with behaviors has been lacking in prior research. Selective lines is an effective method to produce comparable individuals of varying developmental times (Krams et al., 2017). Here, we tested behavioral responses to handling (as a proxy of stress resistance; Adamo et al., 2013), RMR and elemental body composition of three selected lines of western stutter-trilling crickets (*Gryllus integer*) that differ in developmental speed.

Based on existing findings on the effects of stress on organismal stoichiometry (Hawlena and Schmitz, 2010a,b; Krams et al., 2016b), we predicted higher concentrations of carbon (C), lower nitrogen (N), a greater C/N ratio and greater RMR as indicators of physiological stress in the slow developmental line compared with the rapid line and possibly also with the control line because of the higher sensitivity to antidepressants (selective serotonin reuptake inhibitor, SSRI) found in the slow and control developmental lines (Krams et al., 2018). We predicted shorter latency of resuming activity among startled slowly developing individuals (as opposed to rapidly developing crickets) in an unfamiliar environment as an indicator of physiological stress. It has been shown that SSRI treatment increased the time to resume movements (i.e., decreased anxiety) of slowly developing crickets in an unfamiliar environment (Krams et al., 2018). Since males and females may differ in their stress responses, we tested for possible sexual dimorphism in concentrations of C, N and the C/N ratio (Bayer and Hobert, 2018).

MATERIALS AND METHODS

Insects and Selection Lines

The laboratory stock originated from a wild population (Davis, CA, USA). This stock was first maintained at the University of

Oulu and the University of Eastern Finland, and then moved to the University of Tartu in Estonia, where the present data were collected. In this study, we tested crickets that had been selected for five generations for their developmental speed. *G. integer* nymphs were reared individually in plastic containers (28 × 98 × 73 mm: length, width, height, respectively) with a hole of 30 mm in diameter covered with plastic netting for ventilation. Each container was equipped with a shelter made of cardboard. The individual crickets were kept under a constant 12:12 h light–dark cycle, at 26 ± 1°C with *ad libitum* food consisting of fish flakes (Eheim) and reindeer pellets (Rehuraisio Oy, Poron herkku) and *ad libitum* water. Although nymphal density does not affect adult behavior, it does increase life history investments in immune function and maturation (Niemelä et al., 2012b).

The selection design consisted of three main selection lines (rapid development, slow development and control). In each generation, offspring were obtained from ∼20 families within each main line. For rapid and slow developmental lines, mated males and females were selected according to their maturation time, and only the most rapid or slowest maturing individuals were used for matings in each main line (for more details on selection, see Krams et al., 2017). In the control line, matings were randomized over the whole natural maturation time range. Two months after hatching, random samples of offspring from the rapid developmental line were placed into individual containers in a random order. The same procedure was performed 3 months after hatching in the control line and 4 months after hatching in the slow line in each generation.

After five generations of selection for developmental speed, developmental time (the average maturation time ± SD) for rapidly developing individuals was 91.03 ± 6.06 days (n = 29 crickets), 117.33 ± 7.53 days (n = 30) for the control individuals and 136.17 ± 8.28 days (n = 24) for slowly developing crickets. All groups differed significantly in their developmental time [one-way analyses of variance (ANOVA): $F_{(2,77)} = 257.89$, $P < 0.0001$].

Body C and N Content

Following food deprivation of 15 h and water *ad libitum*, all crickets were immediately frozen at −80°C (Angelantoni Lifescience, Italy). Before elemental analysis, we dried bodies of 29 rapidly developing crickets, 30 control crickets and 24 slowly developing crickets at 60°C for 48 h. Each individual was ground to a homogenous powder and measured for C and N content using a C-N combustion auto-analyzer (Hawlena and Schmitz, 2010a,b; Krams et al., 2016b).

Behavioral Trials: Resuming Activity in a Familiar Environment

We started behavioral trials on day 10 after crickets reached maturity. Before behavioral trials and measuring RMR, we weighed each individual using a Kern analytical balance (ABT 120-4M; Kern and Sohn, Balingen, Germany). Behavioral trials were conducted under constant temperature (25 ± 1°C) and sound-proof conditions. We used dim red light (25 W red incandescent bulb) since *Gryllus* spp. cannot see long (red) wavelengths properly (Briscoe and Chittka, 2001), which allowed us to observe these nocturnal insects without disturbing them. The crickets were provided with drinking water before the onset of the trials, while food was removed 5 h before the beginning of experimental trials.

We captured the focal cricket in its housing-box and handled it by holding the insect in the hand for 1 min. After the handling procedure, the cricket was placed back in its burrow-like triangular cardboard shelter (5 cm long, with a 1 × 1 × 1 cm entrance). We recorded the latency to resume activity when the insect started to move inside the cardboard shelter. We waited for all crickets to resume activity (max. 908 s). The same procedure was repeated for each individual 4 days later. Latency to resume activity indicates the duration of freezing or immobile state, a widespread anti-predator response occurring in many taxa (Chelini et al., 2009; Krams et al., 2013a,b).

Behavioral Trials: Resuming Activity in an Unfamiliar Environment

Two days after the first test conducted in an environment familiar to the crickets, we handled them for 1 min and then gave them the opportunity to escape into a burrow-like conical plastic Eppendorf test tube (volume 5 mL, Sigma-Aldrich), which was used as an insect chamber. The insect chamber was connected to the respirometer with rubber tubing (Lighton, 2008). When reaching the insect chamber, each of the crickets immediately became completely immobile, as if it was hiding in a burrow. We waited for all crickets to resume active struggling movements (max. 1850 s).

Metabolic Rate Measurements

We measured cricket RMR as the rate of carbon dioxide emission in an incurrent flow-through system. The LI-7000 differential CO_2/H_2O analyzer (LiCor, Lincoln, NE, USA) was calibrated at different flow rates by means of calibration gases (Trägergase, VEB, Saxon Junkalor GmbH, Dessau; Quinlan and Lighton, 1999; Lighton, 2008) with gas injection (see also Kuusik et al., 2002; Mänd et al., 2006). While measuring CO_2 emissions, the insect chamber was perfused with dry (5–7%RH) CO_2-free air, produced by passing air over Drierite (W. A. Hammond Drierite Co. Ltd., Xenia, OH, USA) and soda-lime granules at an airflow rate of 60 ml min^{-1}. Average ambient temperature within the respirometry chamber was 23.60 ± 0.30°C. Baseline drift of the analyzer was corrected during analysis from the measurements at the beginning and end of each trial with the respirometer chamber empty (Duncan, 2003; Duncan and Byrne, 2005; Gray and Bradley, 2006). The respirometric device was combined with an infrared optical system using IR emitting diodes (TSA6203) and IR-sensor diodes (BP104) that were placed on the sides of the insect chamber. IR-diodes made it possible to record CO_2 production and to follow movements of each cricket simultaneously. The insects remained in their chambers for 4 h, and we recorded their minimum rates of metabolism at moments when crickets were immobile. As soon as the measurements were over, we returned the crickets back to their plastic housing-boxes.

We repeated the trials 5 days later. Since insects differed in their body mass, we used body mass-specific RMR values in this study.

Statistical Analyses

We used two-way ANOVAs with developmental line (slow, rapid, control) and sex as fixed factors to assess differences in elemental composition (C, N and C/N ratio). We considered elemental composition analyses to belong to the same families of tests (Rubin, 2017). We thus controlled for multiple testing using Holm-Bonferroni procedure to adjust *P*-values. An adjusted *P*-value < 0.05 was considered to be statistically significant. Assumptions of homogeneity of variances were met (Levene's test, $P > 0.05$). We report only the main effects when no significant interactions between fixed factors were found; otherwise, Tukey's HSDs are also reported. To test for the effects of developmental line and sex on RMR, linear mixed effects model (LMM) was used. Another LMM was fitted to test for the effects of developmental line, sex and environment (familiar, unfamiliar) on differences in latency to resume activity (log-transformed). In both LMMs, individual cricket ID was included as a random factor to account for the possible correlation of repeated measurements of the same individual. Analyses were performed using IBM SPSS 22 for Windows and the program R, version 3.3.2 (R Development Core Team, 2016).

RESULTS

Carbon

Rapidly developing crickets had less body C (%; 50.5 ± 3.5, mean ± SD) than slowly developing (52.7 ± 3.0, mean ± SD) and control (53.0 ± 3.5, mean ± SD) crickets (Tukey's tests, $P = 0.047$ and $P = 0.02$, respectively), while slowly developing and control crickets did not differ in body C (Tukey's test, $P = 0.96$; **Figure 1A**). The main effect of developmental line to body C was significant (two-way ANOVA: $F_{(2,77)} = 4.562$, $P = 0.039$). Body C of females and males did not show significant differences (two-way ANOVA: $F_{(1,77)} = 0.962$, $P = 0.33$). There was no significant interaction between developmental line and sex to body C (two-way ANOVA: $F_{(2,77)} = 0.484$, $P = 0.618$).

Nitrogen

In contrast, rapidly developing crickets had higher body N (%; 11.5 ± 1.4, mean ± SD) than slowly developing (10.6 ± 1.2, mean ± SD) and control (10.5 ± 1.5, mean ± SD) crickets (Tukey's tests, $P = 0.031$ and $P = 0.022$, respectively). Slowly developing and control crickets did not differ in body N (Tukey's test, $P = 1.0$; **Figure 1B**). The main effect of developmental line to body N was significant (two-way ANOVA: $F_{(2,77)} = 4.452$, $P = 0.03$), while sex had no effect on it (two-way ANOVA: $F_{(1,77)} = 1.062$, $P = 0.306$). There was no significant interaction between developmental line and sex to body N (two-way ANOVA: $F_{(2,77)} = 0.368$, $P = 0.693$).

C/N Ratio

Rapidly developing crickets had a lower C/N ratio (4.48 ± 0.91, mean ± SD) than control crickets (5.18 ± 1.10, mean ± SD; Tukey's test, $P = 0.028$). A marginally non-significant difference was found when comparing rapidly developing crickets with slowly developing crickets (5.1 ± 0.94, mean ± SD; Tukey's test, $P = 0.068$). Slowly developing and control crickets did not differ in their C/N ratio (Tukey's test, $P = 0.98$; **Figure 1C**). The main effect of developmental line was significant (two-way ANOVA: developmental line:

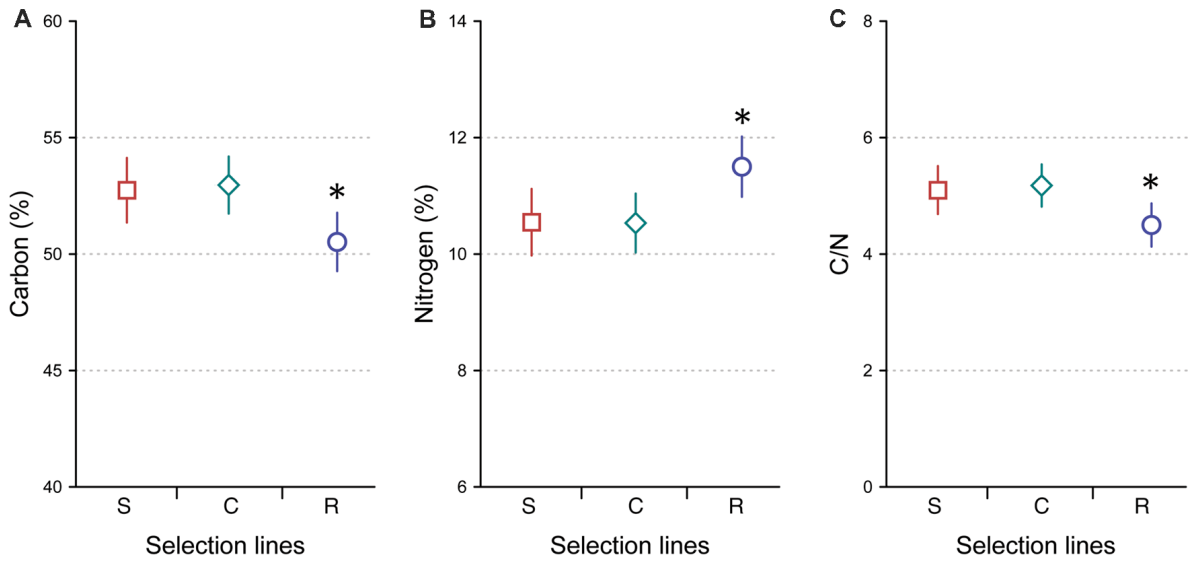

FIGURE 1 | Average carbon percentage **(A)**, nitrogen percentage **(B)** and carbon-to-nitrogen ratio **(C)** in *Gryllus integer* crickets selected for slow development (S, squares, $n = 24$), rapid development (R, circles, $n = 29$) and control developmental (C, diamonds, $n = 30$) lines. Error bars represent 95% confidence intervals. Asterisks indicate significant differences between the lines (*$P < 0.05$).

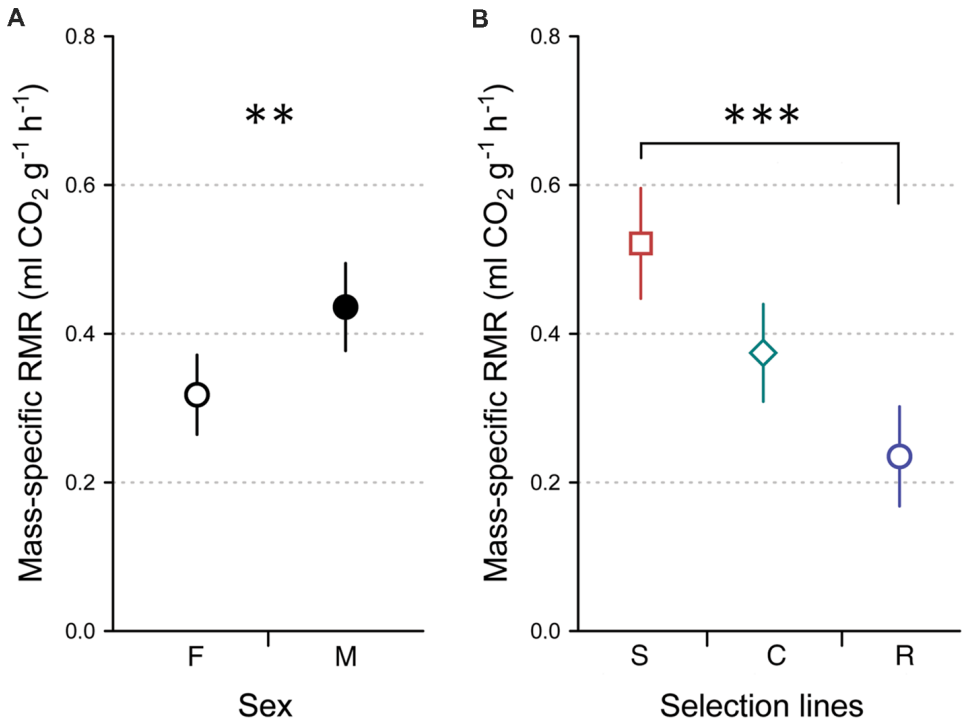

FIGURE 2 | Mass-specific resting metabolic rate (RMR) of females (F, open circle, $n = 45$) and males (M, closed circle, $n = 38$; **A**), selected for slow development (squares, $n = 24$), rapid development (circles, $n = 29$) and control developmental (diamonds, $n = 30$; **B**) lines. Symbols and error bars show averages and 95% CIs, respectively. Asterisks indicate significant differences (**$P < 0.01$; ***$P < 0.001$).

$F_{(2,77)} = 3.904$, $P = 0.024$), while C/N ratios of females and males were not significantly different (two-way ANOVA: $F_{(1,77)} = 1.144$, $P = 0.288$). There was no significant interaction between developmental line and sex to C/N (two-way ANOVA: $F_{(2,77)} = 0.229$, $P = 0.796$).

Resting Metabolic Rate

Body mass-specific RMR differed significantly between females and males (LMM, $F_{(1,77)} = 8.704$, $P = 0.004$), with male crickets having higher RMR than females (**Figure 2A**), and between developmental lines (LMM, $F_{(2,77)} = 16.329$, $P < 0.0001$). The highest mean RMR was recorded in crickets from the slow developmental line and the lowest RMR was recorded in crickets from the rapid developmental line (**Figure 2B**). We found no significant interaction between developmental line and sex (LMM, $F_{(2,77)} = 0.430$, $P = 0.652$).

Resuming Activity in Familiar and Unfamiliar Environments

Selection line and environment had significant effects on crickets' behaviors (LMM, $F_{(2,332)} = 44.663$, $P < 0.0001$, and $F_{(1,332)} = 5.319$, $P = 0.022$, respectively), while sex had no effect (LMM, $F_{(1,332)} = 0.778$, $P = 0.378$; **Figure 3**). There was also a significant interaction between selection line and environment (LMM, $F_{(2,332)} = 452.9$, $P < 0.0001$). Thus, slowly developing crickets resumed activity faster in an unfamiliar but more slowly in a familiar environment on average, while rapidly developing crickets did the opposite. Other interactions were respectively non-significant and marginally non-significant: sex*environment (LMM, $F_{(1,332)} = 0.486$, $P < 0.486$) line*sex (LMM, $F_{(2,332)} = 3.01$, $P = 0.051$).

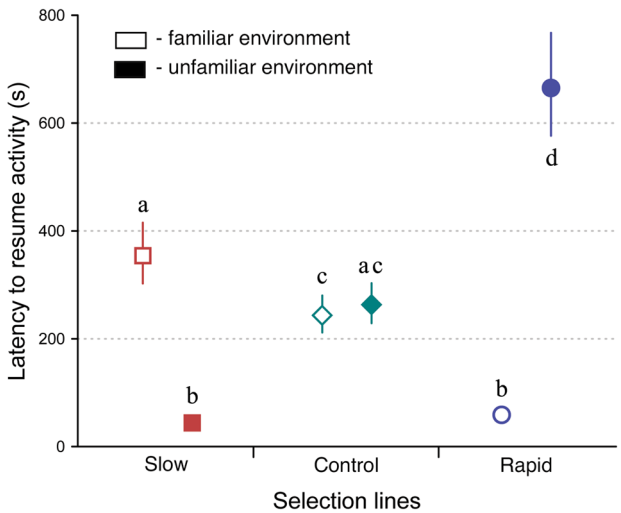

FIGURE 3 | Latency to resume activity (back-transformed means ± 95% CIs) of selected slow (squares), rapid (circles), and control (diamonds) developing crickets in familiar (open symbols) and unfamiliar environments (closed symbols). Different letters denote significant differences at $P < 0.05$.

DISCUSSION

Our results show that differences in POLS (developmental speed) alter insect body stoichiometry and that those changes were associated with different behavioral and physiological stress responses. Thus, developmental speed should be considered as an important factor in research on ecological stoichiometry as it may influence the need for specific nutrients (see Snell-Rood et al., 2015; Camus et al., 2017) and POLS trait interactions more generally.

Surprisingly, slowly developing crickets did not differ significantly from the control line on any of the three stoichiometric parameters while rapidly developing crickets did on all three of them. Rapidly developing crickets had the lowest C, the highest N concentrations and the lowest C/N ratio. This suggests that rapidly developing crickets probably experienced the lowest levels of stress during their nymphal development or were more resilient to stress because of genetic or epigenetic factors. Rapidly developing individuals should benefit from faster growth because this provides a higher chance of surviving to reproduction at low cost (Roff, 1992; Stearns, 1992). This finding is contradictory with predictions arising from life history theory because it has been traditionally assumed that juvenile growth rates operate near their physiological maximum (Stearns and Koella, 1986; Roff, 1992; Stearns, 1992) where tight energy budgets may bring oxidative stress and other costs (Fischer et al., 2004; Slos and Stoks, 2008; Krams et al., 2017). A number of studies confirm that rapid growth is costly, which suggests that growth rates are generally optimized rather than maximized in many species (Gotthard et al., 1994; Nylin et al., 1996; Lankford et al., 2001; Arendt, 2003; Fischer et al., 2004). However, we show that slowly developing and control crickets may be under higher levels of stress as indicated by their higher body C concentration, lower body N and higher C/N ratio. This may be especially true in an unfamiliar environment where slowly developing and control crickets were found to have higher RMR than rapidly developing crickets.

These results can be framed in a larger context of ecological stoichiometry by considering an interesting set of results reported in another species. Grasshoppers (*Melanoplus sanguinipes*) from a subarctic region have about 80 days shorter growing (rapid growth) season compared to those from temperate areas (Fielding and Defoliart, 2007). This shorter growth period is associated with decreased body mass of the subarctic grasshoppers but improved post-ingestive efficiencies and N assimilations on low- and high-quality foods. The temperate grasshoppers, in contrast, are less efficient in digesting low-quality food. This shows that faster development may not generally affect digestive abilities of orthopterans and are unlikely to be associated with elevated levels of physiological stress.

The current results on behavioral traits support an earlier study which showed that slowly developing crickets (compared with rapidly developing ones) resume their daily activities more slowly after being handled in a familiar (less stressful) environment and faster when being handled in a novel (more stressful) environment (Krams et al., 2017). This suggests that slowly developing, often shy crickets are more stressed than rapidly developing, often bold ones. Previous studies show that slowly and rapidly developing crickets markedly differ in behavioral and physiological traits: slowly developing individuals are shy, generally larger, more stressed under unfamiliar conditions, they show stronger encapsulation responses and have higher RMR and lower maximum metabolic rate (MMR) compared with rapidly developing crickets (Niemelä et al., 2012a, 2013; Krams et al., 2017).

Although field cricket females are bigger in body size than males and can occasionally kill them (Kortet and Hedrick, 2005), we did not find any signs of higher stress in males. It is likely that the higher dominance position of female crickets is outweighed by their higher somatic and reproductive costs because a significant correlation between female body size and fecundity is often observed in *G. integer* and other insects (Blanckenhorn, 2000; Hedrick and Kortet, 2012; but see Tammaru et al., 2002). It is important to note that crickets lived in individual cages in this study so that males and females could not interact with each other. We hypothesize that living in groups would increase stress in males, which needs to be tested by measuring behavioral reactions, concentrations of hormones and metabolic rate coupled with research methods used in ecological stoichiometry.

While it is possible that hormone concentrations in the brains of slowly developing crickets serves as a proximate mechanism underlying their higher stress levels and differences in body elemental composition (Stevenson et al., 2005; Zhou et al., 2008; Adamo and McKee, 2017; Krams et al., 2018), it is not clear what is the ultimate reason for becoming shy when developing slowly. Intuitively, shy individuals may benefit from limited activities and greater suspiciousness under higher predator risk. However, it is not clear why this adaptation should be reached *via* a stressed phenotype (e.g., higher C, higher RMR, lower N, slower development). Acquiring a more holistic understanding of these questions would be facilitated by studying crickets' physiological condition, body elemental composition, neurotransmitter concentrations, antipredator responses, sexual selection and survival under natural conditions to test whether shy personality always co-occurs with elevated anxiety and heightened physiological markers of stress. Future studies should include an assessment of phosphorus concentration which is important for RNA production and serves (quantified as the RNA:DNA ratio) as a proxy for protein synthesis (Janssens et al., 2017). These approaches are needed to further develop the general stress paradigm (Hawlena and Schmitz, 2010a).

CONCLUSION

The results of this study show that slow development is associated with a stressed phenotype. This phenotype is characterized by shorter behavioral latencies in a novel environment and higher stress levels associated with higher body C and lower N concentrations. This study shows that ecological stoichiometry is a tool that needs to be used alongside

other traditional methods to study animal stress. Explicit focus on ecological stoichiometry has the potential to explain contradictory results, to sharpen predictions and to move the general stress research paradigm forward through a more holistic understanding of organismal responses to fluctuating selection pressures.

AUTHOR CONTRIBUTIONS

GT, IK and RKo: conceptualization. IK, TK, RKr, SH, RKo and MR: designed the methodology. DE, GT, JC-G and IK: performed the formal analysis. SEI, DB, TK, RKr, IK, PJ and MR: performed the experiments. IK, GT, SH and SL: wrote the original draft. TK, RKo, RKr, SEI, DB, DH, PJ, MR, JC-G and SL: wrote, reviewed and edited the submitted version. GT designed the figures. IK and TK: responsible for funding acquisitions, supervision and administration of the project.

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Impacts of Global Warming and Elevated CO_2 on Sensory Behavior in Predator-Prey Interactions: A Review and Synthesis

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Ecosystems are shaped by complex interactions between species and their environment. However, humans are rapidly changing the environment through increased carbon dioxide (CO_2) emissions, creating global warming and elevated CO_2 levels that affect ecological communities through multiple processes. Understanding community responses to climate change requires examining the consequences of changing behavioral interactions between species, such as those affecting predator and prey. Understanding the underlying sensory process that govern these interactions and how they may be affected by climate change provides a predictive framework, but many studies examine behavioral outcomes only. This review summarizes the current knowledge of global warming and elevated CO_2 impacts on predator-prey interactions with respect to the relevant aspects of sensory ecology, and we discuss the potential consequences of these effects. Our specific questions concern how climate change affects the ability of predators and prey to collect information and how this affects predator-prey interactions. We develop a framework for understanding how warming and elevated CO_2 can alter behavioral interactions by examining how the processes (steps) of sensory cue (or signal) production, transmission and reception may change. This includes both direct effects on cue production and reception resulting from changes in organismal physiology, but also effects on cue transmission resulting from modulation of the physical environment via physical and biotic changes. We suggest that some modalities may be particularly prone to disruption, and that aquatic environments may suffer more serious disruptions as a result of elevated CO_2 and warming that collectively affect all steps of the signaling process. Temperature by itself may primarily operate on aspects of cue generation and transmission, implying that sensory-mediated disruptions in terrestrial environments may be less severe. However, significant biases in the literature in terms of modalities (chemosensation), taxa (fish), and stressors (elevated CO_2) examined currently prevents accurate generalizations. Significant issues such as multimodal compensation and altered transmission or other environmental effects remain largely unaddressed. Future studies should strive to fill these knowledge gaps in order to better understand and predict shifts in predator-prey interactions in a changing climate.

Keywords: climate change, global warming, carbon dioxide, sensory ecology, behavior, predator-prey interactions

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INTRODUCTION

Ecological communities are regulated by key biotic interactions (e.g., predation, competition, mutualism, and parasitism) that are affected by information obtained from and affected by the physical and biological environment (Dusenberry, 1992). In particular, predator-prey interactions are powerful regulators of community dynamics (Paine, 1966; Ripple et al., 2001; Schmitz, 2008) and these interactions can depend on behaviors mediated by sensory cues passing between predators and prey. Thus, processes governing the production, transmission, and detection of sensory cues from other organisms strongly affect predator-prey dynamics. Animals use these cues to respond in the appropriate way; i.e., a predator that successfully detects a prey species must be able to pursue and capture it, while prey detecting a predator must be able to employ the appropriate anti-predator defense. Therefore, sensory processes govern, at least partially, the density mediated effects that depend on predators consuming prey (consumptive effects) as well as those that depend on prey responses to predator risk such as flight or induced morphological defenses (non-consumptive effects) (Werner and Peacor, 2003; Weissburg et al., 2014).

The properties of both senders and receivers affect information transfer between predators and prey, but the environment also has an important role. Environmental context affects predator-prey dynamics by modulating the ability of predator and prey to detect one another. For example, in marine systems the fluid environmental context (e.g., bulk flow and turbulence) can reduce consumptive effects by interfering with the ability of predators to find prey using chemical signals (Weissburg and Zimmerfaust, 1994). Fluid motion also can increase non-consumptive effects by enhancing the ability of prey to remotely detect predators via predator scent (Leonard et al., 1998; Shears et al., 2008; Smee et al., 2010; Pruett and Weissburg, 2018). The nature of these effects depends on the intensity and characteristics of the flow environment. Generally speaking, community interactions must be placed in an environmental context to fully understand ecosystem dynamics.

Anthropogenic impacts will strongly affect the environmental context in which predator-prey sensory processes operate. Increased carbon dioxide (CO_2) emissions from human activities (e.g., burning of fossil fuels) have caused global atmospheric temperatures to increase over the recent years in both terrestrial and marine systems. Mean temperature increases likely will exceed an additional 2°C by year 2100 (IPCC, 2014). Additionally, oceans and coastal systems (and some freshwater systems) are experiencing direct changes in water chemistry known as ocean acidification (Doney et al., 2009), where increased dissolved CO_2 shifts the balance of carbonates to produce more bicarbonate and less carbonate ions, while also reducing pH of seawater by 0.3–0.4 units by 2100 (Orr et al., 2005; IPCC, 2014) from the current value of roughly 8.1.

The changing environmental conditions associated with elevated CO_2 and increased temperature can affect the processes of cue generation, propagation, and reception underlying predator prey dynamics (i.e., the *sensory transduction cascade*), or place limits on the subsequent behavioral responses. Although there has been increasing attention to alterations in predator foraging rates and behavioral responses to climate change across taxa (Briffa et al., 2012; Rosenblatt and Schmitz, 2016; Beever et al., 2017), these studies often fail to indicate clearly the mechanism. We are just beginning to understand how climate change alters sensory processes and the consequences of such alterations for predator-prey interactions. But this research agenda is especially important given the potential of climate-induced change in species interactions to scale up to community effects (e.g., Goldenberg et al., 2018). It also may inform species and environmental conservation efforts by identifying which sensory modalities drive predator-prey behaviors in a changing climate and potential mitigation strategies.

This review is an initial step in developing a framework for studying impacts of climate change (i.e., warming and elevated CO_2) on sensory and behavioral mechanisms altering predator-prey interactions. We review the current research on climate change effects on predator prey interactions in light of the general processes governing cue production, transmission, reception, and response. Our goals are to suggest which elements in this sequence are affected, how they may be affected, and whether there are modality-specific patterns in climate change effects. We also attempt to distinguish between effects due to increased temperature and elevated CO_2.

METHODS

We conducted comprehensive literature searches in Web of Science and Google Scholar for publications within the last 20 years using the keywords "climate change," "carbon dioxide/CO2" "warming," "acidification," "sensory/visual/auditory/chemosensory/olfactory/ mechanosensory," "predator/prey," and "behavior." We sought studies from terrestrial, freshwater, and marine environments that tested some component of sensory ecology in any taxon (i.e., fish, insects, other invertebrates), which resulted in ∼60 relevant studies. We then supplemented our search to include relevant publications from the references sections of papers from the initial literature search, but that did not have these keywords explicitly listed in the title, keywords, or abstract (∼10 additional studies). We also included relevant papers that have cited studies from the initial search (i.e., forward citation search; ∼15 additional studies), and several topical reviews on behavioral responses to climate change stressors. We then read the abstracts to exclude studies based on the following criteria: (1) while we recognize the large body of knowledge documenting physiological responses to climate change, studies that did not include sensory-based behavioral metrics relevant to predator-prey responses were excluded; (2) we focused on studies examining how cues from predators or prey affected interactions and we excluded any study where the cues eliciting behavior were not obviously connected to prey finding or predator sensing; (3) freshwater studies that did not explicitly test CO_2-induced acidification (i.e., conditions in naturally acidic streams) were excluded (∼5 studies), as acidification in freshwater systems can

also occur from acid rain and thus possibly confound analyses in this review; (4) terrestrial studies involving herbivore-plant interactions were not explicitly considered as this is a specific subfield and signaling aspects of climate change effects have been reviewed recently (Peñuelas and Staudt, 2010; Ode et al., 2014).

We identified 57 studies that matched our criteria and which document an effect of climate change (i.e., warming and/or elevated CO_2) on a component of the sensory transduction cascade governing predator-prey interactions. We categorized these studies by sensory modality and sensory step affected, species tested, and relevant habitat, and climate change stressor; and we summarized the results of each study (**Table 1**). We synthesize emerging global patterns from these studies below, and we recognize that it is possible that we did not include every study that fit our criteria due to synonymous terminology used to describe these studies. We also included in our discussion papers on general sensory biology relevant to our review that gave insight to sensory processes impacted by climate change.

One emerging pattern identified from our analysis is that warming and elevated CO_2 affect interactions targeting different components of the sensory transduction pathway: warming largely will affect consumptive effects by physiological changes in activity and metabolic rates (with some effects on cue production, transmission, and reception), while elevated CO_2 largely can affect non-consumptive effects by behavioral changes in the sensory transduction pathway (with some effects on metabolism), changing various aspects of predator-prey information exchange (**Figure 1**). However, many investigations only examine behavioral endpoints and not specific mechanisms, and literature is biased toward a few environments and taxonomic groups, preventing robust generalizations. Further, the ecological consequences often will depend on how both predator and prey are affected, since both use information about the presence of the other to regulate their behavior. Almost no studies examine how the sensory ecology of predator-prey interactions may or may not change when predators and prey both are challenged with warming and/or elevated CO_2. We discuss these issues below and how climate change stressors affect information exchange and interactions governed by specific modalities.

DIRECT VS. INDIRECT SENSORY EFFECTS

On a basic level we may distinguish effects of climate change that depend on general changes to metabolic processes vs. those that operate by changing the production, transmission, and reception of sensory cues (**Figure 1**). Increased temperatures may change predator-prey interactions by increasing metabolic rates of predators, therefore increasing foraging demand in order to meet the same requirements for a given growth rate. This has been demonstrated in multiple species of fish and crustacean consumers (Wu et al., 2017; Goldenberg et al., 2018) and sharks (Pistevos et al., 2015). A similar phenomenon occurs in prey species, although the effect is reversed; warming has been shown to reduce prey activity (Kidawa et al., 2010) and suppress prey metabolism (Paganini et al., 2014), potentially reducing escape responses from predators. Since predator consumptive effects are based on activity levels of both predator and prey, warming may increase predator consumptive effects via changes to metabolic processes (but see Miller et al., 2014 for warming increasing non-consumptive effects). However, activity level also may affect the production of sensory cues that mediate predator-prey interactions. Movement, for instance, produces mechanical and visual cues that both predators and prey may use. Thus, warming may indirectly affect cue production via changes in metabolism or activity and has some direct effects on cue transmission (see below).

Elevated CO_2 can affect directly all elements in the transduction cascade and may strongly impact sensory processes in receivers that determine the ability of animals to detect and process cues, although this conclusion is based on limited studies focused on chemosensation (**Table 1**). Both predators and prey may be affected. Smooth dogfish shark predators *(Mustelus canis*) in high CO_2 conditions avoid food chemical cues while their general activity levels remain unaffected, suggesting a deficit in processing (Dixson et al., 2015). Elevated CO_2 also can decrease foraging success of Port Jackson shark predators *(Heterodontus portusjacksoni)* by dramatically increasing time to chemically locate prey (although it is unclear if this is a result of changes to cue production, detection or processing), thus leading to reduced growth rates overall (Pistevos et al., 2015). Prey species also may show reduced ability to identify predators, leading to behaviors that increase their susceptibility. Pea aphids under increased CO_2 produce less alarm pheromone and respond less when exposed to alarm pheromones (Boullis et al., 2017). Simpson et al. (2011) report that high CO_2 reduces the response of clown fish to predator sounds, and degraded reefs are known to have significantly different sound scales than healthy ones (Gordon et al., 2018). Elevated CO_2 also decreases the intensity of anti-predator behaviors in juvenile damselfish in response to visual predation risk (Ferrari et al., 2012).

Elevated CO_2 may cause some indirect physiological effects with behavioral consequences for predator-prey interactions. High CO_2 levels can indirectly decrease foraging success of brown crab predators (*Cancer pagurus*) through increased resting metabolic rates and thus less energy allocated to foraging behavior (Wang et al., 2018). Thick shell mussels (*Mytilus coruscus*) also experience reduced excretion rates in elevated CO_2 conditions, which may indirectly affect chemical cues used by predators although this has not been tested (Wang et al., 2015). Despite substantial studies on physiological changes in response to climate change stressors (Pörtner and Farrell, 2008; Kroeker et al., 2013), it is not yet clear how often these indirect effects occur or their importance in the sensory transduction pathway.

SENSORY PROPERTIES AND MODALITIES

Sensory behavior involves information transfer via a series of steps from the sender to receiver: production and release of cue, transmission through the environment, detection and processing of cue (collectively known as cue reception), and behavioral response (**Figure 1**, **Table 1**). These steps are all important in

TABLE 1 | Summary of studies on impacts of warming and elevated CO_2 on sensory behavior in predator-prey interactions.

Study	Modality	Sensory step affected	Focal species (common name)	Organism type	Life stage	Predator/ Prey	Metric variable	Ecosystem	Habitat	Stressor(s)	Response
Luo et al., 2013	Audition	Transmission	Multiple species	Bat	Adult	Predator	Prey detection volume	Terrestrial	Tropical/ temperate	Warming	↑/↓ prey detection volume
Rossi et al., 2016a	Audition	Production	*Alpheus novaezelandiae* (snapping shrimp)	Crustacean	Adult	Predator	Frequency and intensity of snaps	Marine	Tropical reef	Elevated CO_2	↓ snap frequency, ↓/= snap intensity
Simpson et al., 2011	Audition	Processing	*Amphiprion percula* (clownfish)	Fish	Juvenile	Prey	Avoidance of predator noise	Marine	Tropical reef	Elevated CO_2	↓ predator noise avoidance
Kidawa et al., 2010	Chemosensation	Detection/ Processing	*Odontaster validus* (sea star)	Echinoderm	Adult	Predator	Attraction to food cue	Marine	Antarctic benthos	Warming	↓ attraction to food cue
Cripps et al., 2011	Chemosensation	Detection/ Processing	*Pseudochromis fuscus* (dottyback)	Fish	Adult	Predator	Attraction to food cue	Marine	Tropical reef	Elevated CO_2	↓ attraction to food cue
de la Haye et al., 2012	Chemosensation	Detection/ Processing	*Pagurus bernhardus* (hermit crab)	Crustacean	Adult	Predator	Attraction to food cue	Estuarine	Temperate rocky intertidal	Elevated CO_2	↓ attraction to food cue
Majeed et al., 2014	Chemosensation	Detection/ Processing	*Aedes aegypti* (mosquito)	Insect	Adult	Predator	Take-off and location of cue source	Terrestrial	Tropical/ temperate	Elevated CO_2	↓ take-off and location of cue source
Barry et al., 2014	Chemosensation	Detection/ Processing	*Strongylocentrotus fragilis* (sea urchin)	Echinoderm	Adult	Predator	Foraging time	Marine	Temperate deep-sea benthos	Elevated CO_2	↑ foraging time
Queirós et al., 2015	Chemosensation	Detection/ Processing	*Nucella lapillus* (dogwhelk)	Gastropod	Adult	Predator	Time to locate food source	Estuarine	Temperate rocky intertidal	Elevated CO_2 + Warming	↑ time to locate food source
Dixson et al., 2015	Chemosensation	Detection/ Processing	*Mustelus canis* (smooth dogfish)	Shark	Adult	Predator	Attraction to food cue	Marine	Temperate benthos	Elevated CO_2	↓ attraction to food cue
Pistevos et al., 2015	Chemosensation	Detection/ Processing	*Heterodontus portusjacksoni* (Port Jackson shark)	Shark	Larval, Juvenile	Predator	Attraction to food cue	Marine	Temperate reef	Elevated CO_2 + Warming	↓ attraction to food cue
Natt et al., 2017	Chemosensation	Detection/ Processing	*Pseudochromis fuscus* (dottyback)	Fish	Adult	Predator	Attraction to food cue	Marine	Tropical reef	Elevated CO_2	= attraction to food cue
Jiahuan et al., 2018	Chemosensation	Detection/ Processing	*Acanthopagrus schlegelii* (sea bream)	Fish	Adult	Predator	Attraction to food cue	Estuarine	Temperate reef	Elevated CO_2	↓ attraction to food cue
Kim et al., 2016	Chemosensation	Detection/ Processing	*Pagurus tanneri* (hermit crab)	Crustacean	Adult	Predator	Time to locate food source	Marine	Deep-sea benthos	Elevated CO_2	↑ time to locate food source
Manríquez et al., 2014	Chemosensation	Detection/ Processing	*Concholepas concholepas* (muricid snail)	Gastropod	Juvenile	Predator + Prey	Prey detection, predator detection	Marine	Temperate rocky intertidal	Elevated CO_2	= prey detection, ↓ predator detection
Porteus et al., 2018	Chemosensation	Detection/ Processing	*Dicentrarchus labrax* (sea bass)	Fish	Juvenile	Predator + Prey	Prey detection, predator detection, responses to predator cue (activity, freezing)	Estuarine	Temperate estuary	Elevated CO_2	↓ prey detection, ↓ predator detection, ↓ activity, ↑ freezing
Dixson et al., 2010	Chemosensation	Detection/ Processing	*Amphiprion percula* (clownfish)	Fish	Larval, Juvenile	Prey	Avoidance of predator cues	Marine	Tropical reef	Elevated CO_2	↓ predator cue avoidance
Munday et al., 2010	Chemosensation	Detection/ Processing	*Amphiprion percula* (clownfish), *Pomacentrus wardi* (damselfish)	Fish	Larval, Juvenile	Prey	Avoidance of predator cues, avoidance of predation	Marine	Tropical reef	Elevated CO_2	↓ predator cue avoidance, ↑ mortality rates
Nilsson et al., 2012	Chemosensation	Processing	*Amphiprion percula* (clownfish), *Pomacentrus wardi* (damselfish)	Fish	Juvenile	Prey	Avoidance of predator cue, presence of lateralization	Marine	Tropical reef	Elevated CO_2	↓ predator cue avoidance, ↓ lateralization
Munday et al., 2013	Chemosensation	Detection/ Processing	*Plectropomus leopardus* (coral trout)	Fish	Juvenile	Prey	Avoidance of predator cue, activity	Marine	Tropical reef	Elevated CO_2	↓ avoidance of predator cue, ↑ activity
Jutfelt and Hedgärde, 2013	Chemosensation	Detection/ Processing	*Gadus morhua* (Atlantic cod)	Fish	Juvenile	Prey	Avoidance of predator cue, avoidance of CO2 water	Marine	Temperate benthos	Elevated CO_2	= avoidance of predator cue, = avoidance of CO2
Chivers et al., 2014	Chemosensation	Processing	*Pomacentrus amboinensis* (damselfish)	Fish	Juvenile	Prey	Antipredator responses, learning, survivorship	Marine	Tropical reef	Elevated CO_2	↓ antipredator responses, ↓ learning, ↓ survivorship

(Continued)

TABLE 1 | Continued

Study	Modality	Sensory step affected	Focal species (common name)	Organism type	Life stage	Predator/ Prey	Metric variable	Ecosystem	Habitat	Stressor(s)	Response
Ou et al., 2015	Chemosensation	Detection, Processing	*Oncorhynchus gorbuscha* (pink salmon)	Fish	Larval, Juvenile	Prey	Avoidance of chemical alarm cue, anxiety	Freshwater	Temperate stream	Elevated CO_2	↓ avoidance of chemical alarm cue, ↓ anxiety
Heuer et al., 2016	Chemosensation	Processing	*Acanthochromis polyacanthus* (damselfish)	Fish	Adult	Prey	Avoidance of chemical alarm cue	Marine	Tropical reef	Elevated CO_2	↓ avoidance of chemical alarm cue
Tix et al., 2017	Chemosensation	Detection/ Processing	*Pimephales promelas* (fathead minnow), *Hypophthalmichthys molitrix* (silver carp)	Fish	Adult	Prey	Antipredator response to chemical alarm cue	Freshwater	Temperate pond/river	Elevated CO_2	↓/= response of chemical alarm cue
Sundin et al., 2017	Chemosensation	Detection/ Processing	*Acanthochromis polyacanthus* (damselfish)	Fish	Juvenile	Prey	Reduction of activity after predator chemical cue	Marine	Tropical reef	Elevated CO_2	= reduction of activity after predator chemical cue
Boullis et al., 2017	Chemosensation	Production, Detection, Processing	*Acyrthosiphon pisum* (pea aphid)	Insect	All	Prey	Production, emission, detection, and response of chemical alarm cue	Terrestrial	Legume crops	Elevated CO_2	↓ production, ↓ emission, = detection, ↓/= response to chemical alarm cue
McCormick et al., 2017	Chemosensation	Transmission	*Pomacentrus amboinensis* (damselfish)	Fish	Juvenile	Prey	Antipredator response to chemical alarm cue	Marine	Tropical reef	Habitat degradation ("Elevated CO_2 + Warming")	↓ response to chemical alarm cue
Cattano et al., 2017	Chemosensation	Detection/ Processing	*Symphodus ocellatus* (wrasse)	Fish	Juvenile	Prey	Avoidance of predator cue	Marine	Temperate reef	Elevated CO_2	= avoidance of predator cue
Boullis et al., 2018	Chemosensation	Production	*Acyrthosiphon pisum* (pea aphid)	Insect	All	Prey	Quantity and quality of kairomones	Terrestrial	Legume crops	Elevated CO_2	↓ quantity, Δ composition of kairomones
Andrade et al., 2018	Chemosensation	Detection/ Processing	*Citharichthys stigmaeus* (flatfish)	Fish	Juvenile	Prey	Foraging response to alarm cue	Marine	Temperate benthos	Elevated CO_2	= foraging response to alarm cue
McMahon et al., 2018	Chemosensation	Detection/ Processing	*Amphiprion percula* (clownfish)	Fish	Juvenile	Prey	Reduction of foraging after predator chemical cue	Marine	Tropical reef	Elevated CO_2	↓ reduction of foraging
Williams et al., 2018	Chemosensation	Processing	*Oncorhynchus kisutch* (salmon)	Fish	Juvenile	Prey	Avoidance of alarm cue	Marine	Temperate coastal	Elevated CO_2	↓ avoidance of alarm cue
Abboud et al., 2019	Chemosensation	Detection/ Processing	*Physella columbiana* (snail), *Daphnia magna* (daphnid)	Gastropod, Crustacean	Adult	Prey	Activity, shelter use	Freshwater	Temperate pond/lake	Elevated CO_2	↓ activity, ↓ shelter use
Munday et al., 2016	Chemosensation, Mechanosensation, Neural Processing	Processing	*Amphiprion percula* (clownfish), *Pomacentrus amboinensis* (damselfish)	Fish	Larval	Prey	Avoidance of predator cue, escape response	Marine	Tropical reef	Elevated CO_2	↓/= avoidance of predator cue, ↓ escape response
Pistevos et al., 2017	Chemosensation, Vision	Detection/ Processing	*Heterodontus portusjacksoni* (Port Jackson shark)	Shark	Larval, Juvenile	Predator	Attraction to food cue	Marine	Temperate reef	Elevated CO_2 + Warming	↑/= attraction to food cue
McCormick and Lönnstedt, 2013	Chemosensation, Vision	Transmission	*Pomacentrus amboinensis* (damselfish)	Fish	Juvenile	Predator + Prey	Feeding rate, risk assessment	Marine	Tropical reef	Habitat degradation ("Elevated CO_2 + Warming")	↑ feeding rate, ↑ risk assessment
Manciocco et al., 2015	Chemosensation, Vision	Processing	*Dicentrarchus labrax* (sea bass)	Fish	Juvenile	Predator + Prey	Attraction to food cue, avoidance of visual aversive cue	Estuarine	Temperate estuary	Warming	↑ attraction to food cue, ↑ avoidance of visual aversive cue
Goldenberg et al., 2018	Chemosensation, Vision	Detection/ Processing	Multiple species	Fish, Crustacean	Adult	Predator + Prey	Attraction to food cues, avoidance of predator cues	Marine	Temperate rocky reef, seagrass, open sand	Elevated CO_2 + Warming	↓/= attraction to food cues, ↓/= avoidance of predator cues
Lönnstedt et al., 2013b	Chemosensation, Vision	Detection/ Processing	*Pomacentrus amboinensis* (damselfish)	Fish	Juvenile	Prey	Antipredator responses (foraging, activity, area use, bobbing behavior)	Marine	Tropical reef	Elevated CO_2	↓/= antipredator responses
Lönnstedt et al., 2013a	Chemosensation, Vision	Transmission	*Pomacentrus amboinensis* (damselfish)	Fish	Juvenile	Prey	Antipredator responses (reductions in feeding and activity)	Marine	Tropical reef	Habitat degradation ("Elevated CO_2 + Warming")	↓ antipredator responses

(Continued)

TABLE 1 | Continued

Study	Modality	Sensory step affected	Focal species (common name)	Organism type	Life stage	Predator/ Prey	Metric variable	Ecosystem	Habitat	Stressor(s)	Response
Charpentier and Cohen, 2016	Chemosensation, Vision	Detection/ Processing	*Hemigrapsus sanguineus* (shore crab)	Crustacean	Larval	Prey	Photosensitivity to predator kairomone	Estuarine	Temperate estuary	Elevated CO_2	↓ Photo-sensitivity to predator kairomone
Sundin and Jutfelt, 2016	Chemosensation, Vision/ Neural processing	Detection/ Processing	*Ctenolabrus rupestris* (goldsinny wrasse)	Fish	Juvenile	Predator + Prey	Attraction to food cue, avoidance of predator cue, lateralization	Marine	Temperate rocky reef	Elevated CO_2	= attraction to food cue, ↓ avoidance of predator cue, = lateralization
Welch et al., 2014	Chemosensation, Vision/ Neural processing	Detection/ Processing	*Acanthochromis polyacanthus* (damselfish)	Fish	All	Prey	Avoidance of chemical alarm cue, behavioral lateralization	Marine	Tropical reef	Elevated CO_2	↓ avoidance of chemical alarm cue, ↓ lateralization
Jarrold et al., 2017	Chemosensation, Vision/ Neural processing	Detection/ Processing	*Acanthochromis polyacanthus* (damselfish), *Amphiprion percula* (clownfish)	Fish	Juvenile	Prey	Avoidance of predator cue, behavioral lateralization	Marine	Tropical reef	Elevated CO_2	↓/= avoidance of predator cue, ↓/= lateralization
Wang et al., 2017	Mechanosensation	Detection/ Processing	*Oryzias melastigma* (medaka)	Fish	Larval	Prey	Startle response	Estuarine	Tropical estuary	Elevated CO_2	↓ startle response
Warren et al., 2017	Mechanosensation	Detection/ Processing	*Pomacentrus moluccensis, P. amboinensis* (damselfish)	Fish	Juvenile	Prey	Escape response	Marine	Tropical reef	Warming	↑/= directionality and escape response
Jarrold and Munday, 2018	Mechanosensation, Vision/ Neural Processing	Detection/ Processing	*Acanthochromis polyacanthus* (damselfish)	Fish	Juvenile	Prey	Fast starts, lateralization	Marine	Tropical reef	Elevated CO_2 + Warming	↑/= fast starts, ↓/= lateralization
Coker et al., 2009	Vision	Transmission	*Pseudochromis fuscus* (dottyback)	Fish	Adult	Predator	Predation rate	Marine	Tropical reef	Habitat degradation ("Warming")	↑ predation rate
Allan et al., 2013	Vision	Production, Processing	*Pseudochromis fuscus* (dottyback predator), *Pomacentrus amboinensis* (damselfish prey)	Fish	Adult, Juvenile	Predator + Prey	Attack distance, predation rate, reactive distance, looming threshold	Marine	Tropical reef	Elevated CO_2	= predator success, ↓ reactive distance, ↑ looming threshold
Allan et al., 2017	Vision	Production, Processing	*Pseudochromis fuscus* (dottyback predator), *Pomacentrus wardi* (damselfish prey)	Fish	Adult, Juvenile	Predator + Prey	Attack rate, predation rate, reactive distance, looming threshold	Marine	Tropical reef	Elevated CO_2 + Warming	↑ predator success, ↓ prey reactive distance, ↑ looming threshold
McCormick et al., 2018	Vision	Production, Processing	*Pseudochromis fuscus* (dottyback predator), *Pomacentrus wardi* (damselfish prey)	Fish	Adult, Juvenile	Predator + Prey	Attack distance, predation rate, reactive distance, looming threshold	Marine	Tropical reef	Elevated CO_2	= predator success, ↓ reactive distance, ↓ looming threshold
Ferrari et al., 2012	Vision	Detection/ Processing	*Pomacentrus amboinensis* (damselfish)	Fish	Juvenile	Prey	Antipredator responses (foraging, activity, area use, bobbing behavior)	Marine	Tropical reef	Elevated CO_2	↓ antipredator responses
Chung et al., 2014	Vision	Detection	*Acanthochromis polyacanthus* (damselfish)	Fish	Adult	Prey	Retinal response	Marine	Tropical reef	Elevated CO_2	↓ retinal response
Spady et al., 2014	Vision	Production, Detection/ Processing	*Idiosepius pygmaeus* (squid)	Squid	Adult	Prey	Activity, Defense posture, Escape response	Marine	Tropical	Elevated CO_2	↑ activity, ↓ defense posture, ↑ escape response
Jutfelt et al., 2013	Vision/ Neural processing	Detection/ Processing	*Gasterosteus aculeatus* (three-spined stickleback)	Fish	Adult	Prey	Behavioral lateralization, escape time	Estuarine	Temperate, multiple	Elevated CO_2	↓ lateralization, ↑ escape time
Näslund et al., 2015	Vision/ Neural processing	Detection/ Processing	*Gasterosteus aculeatus* (three-spined stickleback)	Fish	Adult	Prey	Shelter use, freezing behavior, behavioral lateralization	Estuarine	Temperate, multiple	Elevated CO_2	= shelter use, ↑ freezing behavior, ↓ lateralization

*The table highlights sensory modality, sensory step affected (see **Figure 1**), focal species with life stage and predator/prey role(s), variable measured (with emphasis on behavior), ecosystem, habitat, stressor(s), and observed responses.*

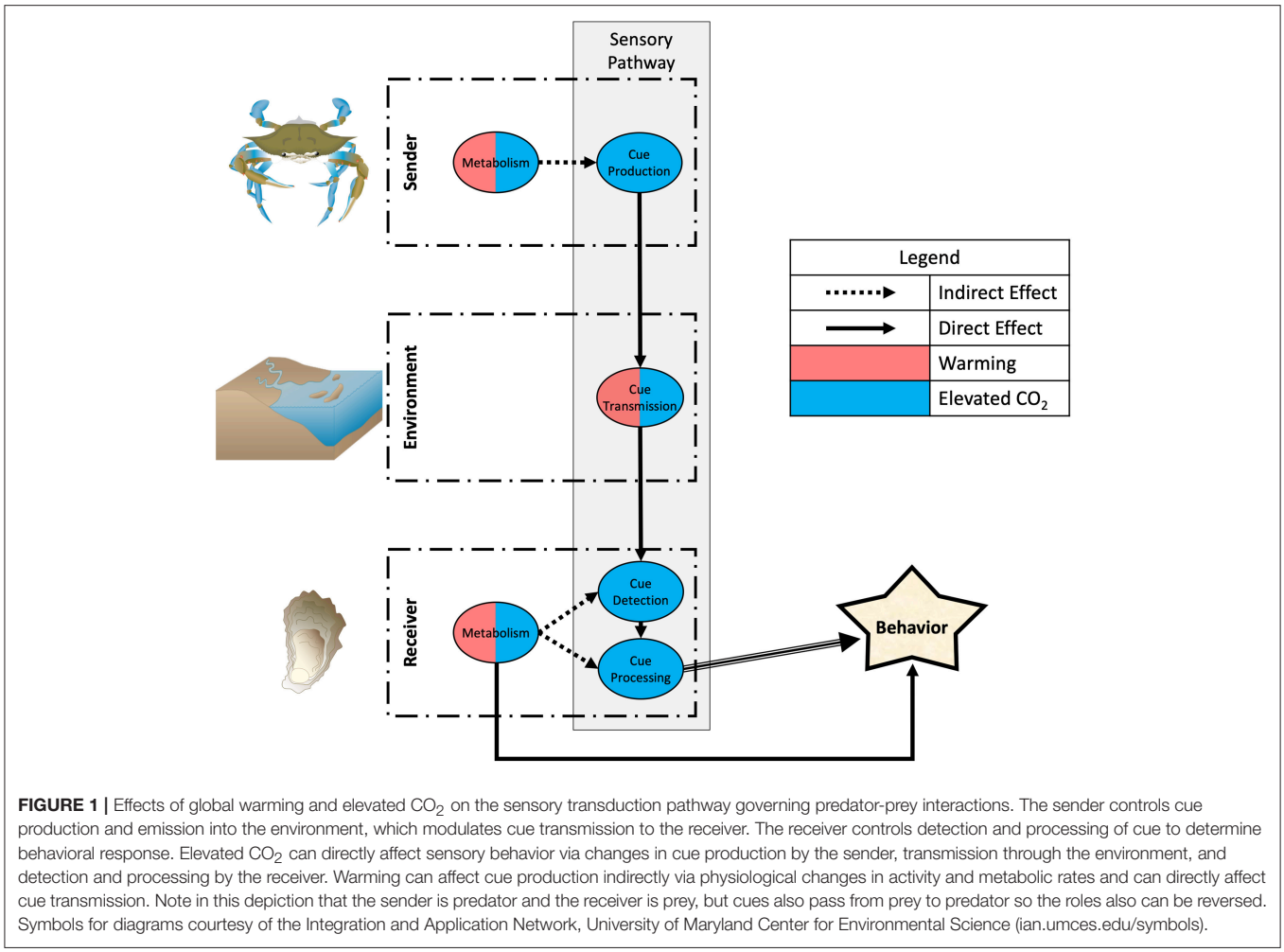

FIGURE 1 | Effects of global warming and elevated CO_2 on the sensory transduction pathway governing predator-prey interactions. The sender controls cue production and emission into the environment, which modulates cue transmission to the receiver. The receiver controls detection and processing of cue to determine behavioral response. Elevated CO_2 can directly affect sensory behavior via changes in cue production by the sender, transmission through the environment, and detection and processing by the receiver. Warming can affect cue production indirectly via physiological changes in activity and metabolic rates and can directly affect cue transmission. Note in this depiction that the sender is predator and the receiver is prey, but cues also pass from prey to predator so the roles also can be reversed. Symbols for diagrams courtesy of the Integration and Application Network, University of Maryland Center for Environmental Science (ian.umces.edu/symbols).

regulating predator-prey interactions (Weissburg et al., 2014). Cue production is dependent on characteristics of the sender; for example, acoustic or visual cues depend on movement. Cue emission is closely related to cue production, which as defined here corresponds to cue intensity, and emission duration and frequency. The environment plays a large role in transmitting sensory cues and consequently has great potential to alter cue intensity and persistence (Smee et al., 2010; Weissburg et al., 2014), which modulates how well the cue can be perceived against background "noise." Cue intensity diminishes as it propagates through the environment, and other properties also may change to affect how or whether the cue may be perceived (**Table 2**). For example, physical properties of water attenuate light, lowering light levels in deeper waters in a frequency-dependent manner to shift the color spectrum toward blues (Dusenberry, 1992). This reduces contrast and the ability to detect particular colors. The chemical, spectral and auditory environments can therefore prevent cues from being detected or discriminated from the background, a process we refer to generally as "masking" (Dusenberry, 1992). Note that masking can occur directly as a result of environmental changes or indirectly when the environment changes biotic factors that in turn affect background sensory properties. For instance, terrestrial environments differ in background spectral quality as a result of the interaction of biological features and physical properties, which makes certain colors stand out and masks others (Endler, 1993). A receiver able to detect a cue and distinguish it from the environment uses this information to detect potential prey or predation risk and respond appropriately to the sender. Climate change (i.e., warming and elevated CO_2) has the potential to directly or indirectly affect any of these sensory steps (**Figure 1**), and the sections below discuss modality-specific effects (**Table 2**).

Audition

Sound perception occurs over somewhat large spatial scales (i.e., tens of meters), and can be used to detect habitats or potential predators or prey (Weissburg et al., 2014). Sound speed and distance traveled is dependent on density of medium, nearby objects, and potential mixing forces (Dusenberry, 1992). Elevated CO_2 has been the focal stressor of the limited studies examining changes in behavior to auditory cues (**Table 2**, **Figure 2**), although there is theoretical evidence for warming effects (**Table 3**). Warming may indirectly affect auditory cue production via changes in metabolism and activity levels that

TABLE 2 | Modality-specific parameters of the sensory transduction pathway.

Sensory modality	Sender	Environment	Receiver
Audition	Frequency, intensity	Speed, sound pressure level, frequency	Intensity, frequency discrimination
Chemosensation	Concentration, molecular structure	Spatial and temporal odor plume structure, cue degradation	Intensity, spatial and temporal structure, molecular cue discrimination
Vision	Intensity, contrast, movement	Intensity, frequency	Intensity, looming, contrast detection
Mechanosensation	Frequency, intensity	Intensity, frequency	Intensity, frequency, spatial and temporal structure

The second column gives relevant properties of the stimulus produced by the sender, the third column lists properties that are changed during transmission, whereas the last column shows the cue properties used by the receiver to extract information. Masking occurs when the environment modulates the cue so that it can no longer be discerned against the background, even when it is in principle detectable.

would affect how much or how often sound is produced. These effects may occur in interactions such as bat predators detecting prey in cluttered environments where echolocation is not effective, and where detection and localization of prey is modulated by prey-generated sounds (Arlettaz et al., 2001). However, these effects of warming on auditory cue production remain unexplored. Elevated CO_2 also reduces the frequency and intensity of predatory snapping sounds (Rossi et al., 2016a). General activity levels in this case were not affected by elevated CO_2 but other potentially confounding metabolic effects were not examined (see below).

Warming has a direct effect on auditory cue transmission; sound speed in seawater increases by $\sim$4 ms^{-1} per 1°C rise in ocean temperature (Munk and Forbes, 1989), and an increased sound speed of 0.6 ms^{-1} per 1°C temperature rise in air. Since auditory location depends partially on time of arrival differences at sound-receiving organs, increasing the speed of sound may reduce the ability of animals (particularly small ones), to discern sound direction (Miles et al., 1995). Sound attenuation also is predicted to change with warming, which has consequences for animals that use sounds to detect other organisms. Global warming will change sound transmission of echolocating bats, and the direction of change depends on call frequency and local climate conditions; bats living in temperate regions and producing higher frequency calls will lose prey detection space, while tropical bats with lower frequency calls will be able to detect more prey (Luo et al., 2013). Changes in community composition are likely to result from these changes in sound transmission, with stronger impacts in temperate regions. Ambient or background noise also may directly increase as a result of ocean acidification as the environment can modulate reflection and attenuation of sounds; increased CO_2 will reduce sound absorption in the oceans, particularly for low-frequency sounds (<10 kHz), via shifts in chemical reactions of sound-absorbing compounds (e.g., magnesium sulfate and boric acid), making for a noisier environment that reduces transmission and detection of ecologically relevant sounds such as hearing and communication in cetaceans (Hester et al., 2008; Ilyina et al., 2010). In combination with warming, an ocean pH reduction of 0.3–0.6 units will reduce absorption of sounds below 1 kHz by almost 40–60%, especially in regions that receive increased amounts of low-frequency noise from human activities such as shipping (Hester et al., 2008; Ilyina et al., 2010).

Elevated CO_2 also can directly change the receptors of auditory cues such as otoliths used by fishes to detect sound waves. Bignami et al. (2013) found that ocean acidification increases otolith size and auditory sensitivity; despite the potential benefit of detecting relevant cues with larger auditory receiving structures, hypersensitivity may impair the ability to discriminate these cues from "background noise." This would make it more difficult to locate food or avoid predators using auditory cues.

In addition to affecting the sound reception organ, elevated CO_2 also can affect auditory processing. Juvenile clownfish (*Amphiprion percula*) in ambient conditions avoid daytime reef habitat noise indicative of predation risk. However, clownfish in high-CO_2 laboratory conditions showed neither avoidance of, nor attraction to, habitat noise. The lack of response was not due to changes in the sound reception organ; otolith morphology was unaffected suggesting an inability to process the auditory inputs effectively (Simpson et al., 2011). These results have important implications for habitat selection to minimize predation risk, and of course also affect responses to attractive auditory stimuli. Habitat sounds can be attractive cues used for orientation during settlement, with elevated CO_2 levels reducing these attractive behaviors as demonstrated in settlement-stage tropical and temperate fishes of several species (Rossi et al., 2015, 2016b). Reduced responses to both attractive and aversive auditory cues suggest deficits in cue detection or processing may be the sensory mechanism in these cases, although this remains to be explicitly tested using physiological investigations of sensory receptor responses or central nervous system function (e.g., Nilsson et al., 2012; Porteus et al., 2018).

Chemosensation

Chemical cues are prevalent in sensory-mediated predator-prey behaviors and may mediate detection of both prey (Weissburg et al., 2002) and predators (Kats and Dill, 1998) as these cues have the potential to persist over large distances (i.e., tens of meters) and long times (i.e., minutes) (Weissburg et al., 2014). Chemical cue production and emission is controlled by the sender; prey often determine predation risk from chemical waste products unavoidably released by predators (Scherer and Smee, 2016; Poulin et al., 2018) or by cues released by conspecific prey (e.g., alarm pheromones). The amount of cue produced can be an indicator of biomass and thus predator threat (Hill and Weissburg, 2013), and predator diet can change the identity

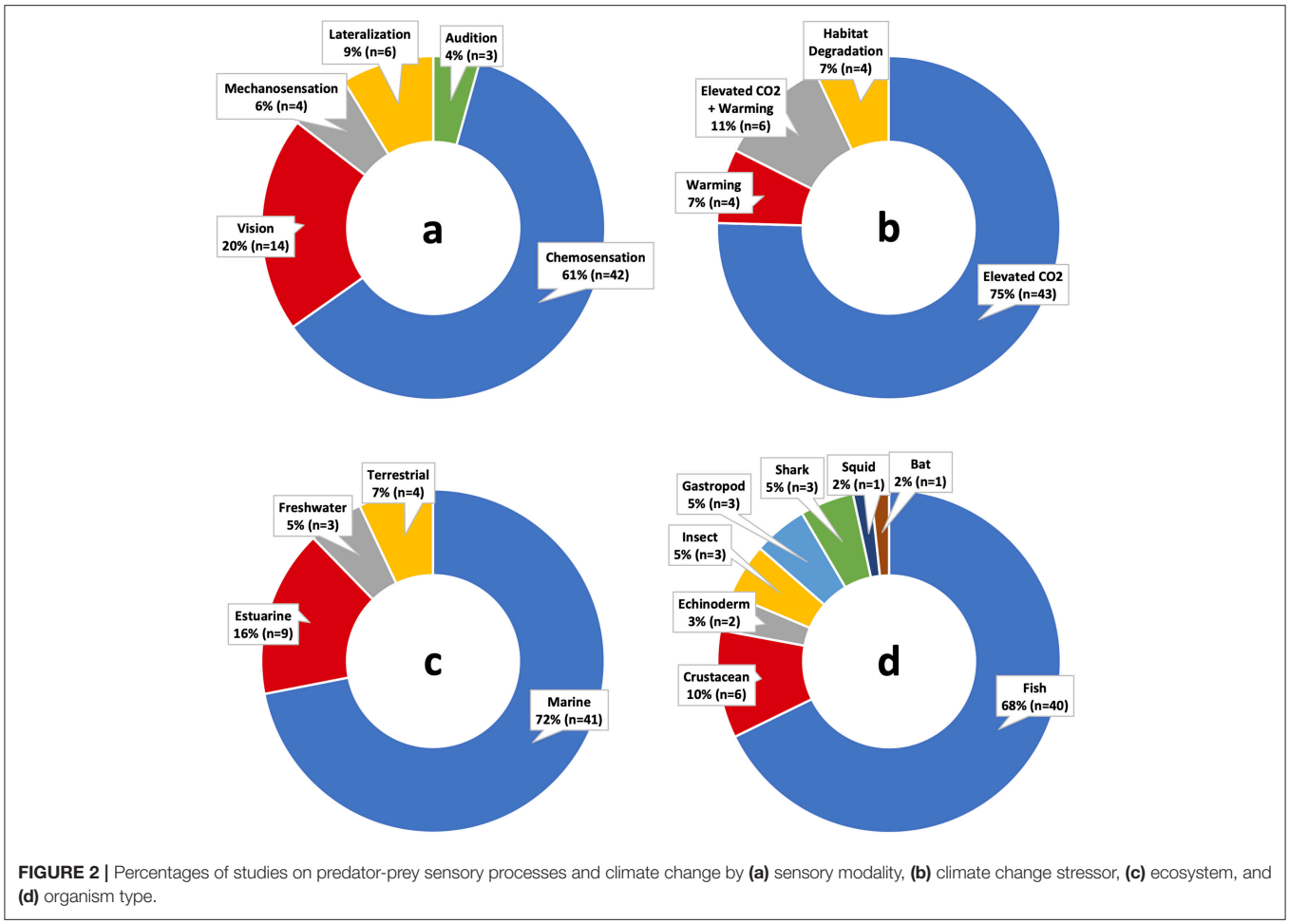

FIGURE 2 | Percentages of studies on predator-prey sensory processes and climate change by **(a)** sensory modality, **(b)** climate change stressor, **(c)** ecosystem, and **(d)** organism type.

TABLE 3 | Summary of impacts of climate change stressors on sensory systems.

	Climate change stressor					
	Warming			Elevated CO_2		
System	Terrestrial	Freshwater	Marine	Terrestrial	Freshwater	Marine
Audition	Yes	Putative	Putative	Putative	Putative	Yes
Chemosensation	Yes	Putative	Yes	Yes	Yes	Yes
Vision	Putative	Yes	Yes	No	Yes	Yes
Mechanosensation	Putative	Putative	Yes	No	Putative	Yes

Yes, published experimental evidence; Putative, theoretical or indirect evidence; No, no experimental or theoretical evidence.

of metabolites released by predators (Poulin et al., 2018) to chemically influence risk assessment by prey (Schoeppner and Relyea, 2005; Turner, 2008; Scherer et al., 2016). The ubiquity of chemical signaling processes in aquatic environments has engendered a great deal of effort investigating the consequences of climate change to predators and prey (**Table 1**, **Figure 2**).

Chemical cue production can change due to shifts in metabolic rates from increased temperatures that cause predators to consume more prey. This may be especially important in dose-dependent responses to chemical metabolites used in predator-prey interactions (e.g., Weissburg and Beauvais, 2015). Increased carbon dioxide also can directly reduce production of chemical cues, as shown in alarm signaling in aphids discussed previously (Boullis et al., 2017). Interestingly, elevated CO_2 changes the chemical composition of aphid honeydew, a metabolic byproduct containing kairomones used by aphid predators to detect prey. The qualitative changes in composition were insufficient to impact predator search behavior in this study, suggesting that this interaction is robust to climate change (Boullis et al., 2018). That cue quality may be sufficiently conserved so that behavior is not impacted may be due to the cue being composed of primary metabolites that are the product of fundamental metabolic

processes. These cues are important in other cases, such as mud crab prey detecting primary metabolites of blue crab predators (Poulin et al., 2018).

Chemical cues have been suggested to be modified directly in future environments, although the specific mechanisms have been defined poorly or not at all. Potential mechanisms of altered chemical cues include protonation of peptides from ocean acidification, which appears to alter peptide signaling molecules used in other contexts (Roggatz et al., 2016; Lecchini et al., 2017). Warming also may denature proteins or make them more subject to decomposition. Although these examples do not evaluate predator-prey cues specifically they illustrate the general possibility of acidified environments changing the composition of conspecific chemical cues, which has been suggested but not tested for chemical alarm cues in Atlantic salmon (Leduc et al., 2010), fathead minnows and finescale dace (Brown et al., 2002), and European sea bass (Porteus et al., 2018).

The mechanisms by which climate change may affect chemical cues indicate that proteins and peptides would likely be most affected by climate change; this implies broad impacts on chemically-mediated interactions, especially in marine systems. Proteins and associated amino acids are arguably the most commonly used compounds in predator-prey interactions (Rittschof, 1990; Carr et al., 1996), for they are often released as water-soluble metabolites that rapidly spread and degrade. These chemical classes are important in attracting predators to prey (Hay, 2009) and also may be important constituents of risk cues released by predators (Poulin et al., 2018). However, other molecules, such as alarm substance released by prey, may be more specialized (i.e., secondary metabolites) and less prone to environmental degradation (Chivers and Smith, 1998; Ferrari et al., 2010). Unfortunately, there are only a handful of studies that have identified water born cues used by prey to detect predators (Poulin et al., 2018).

Climate change may have indirect consequences to predator and prey information transfer by modifying biotic factors affecting perception. Lönnstedt et al. (2013a) tested antipredator responses of tropical marine damselfish (*Pomacentrus amboinensis*) to chemical cues indicative of predation risk, in degraded (i.e., bleached or dead) vs. healthy coral communities. By crossing healthy and degraded habitat types with exposure and testing, this study found that fish did not respond (i.e., no changes in feeding rate, swimming, and shelter use) to conspecific damaged-released chemical cues when in dead coral habitats. Chemical cues in degraded habitats can be masked by other compounds present in the environment, especially if the habitat has drastically changed (e.g., coral overgrown by algae). Algae and cyanobacteria that become dominant on degraded reefs produce chemicals that mask anti-predator cues released by conspecific prey (McCormick et al., 2017). In Lönnstedt et al. (2013a) study, reversibility (i.e., masking) of the chemical alarm cue was not supported; this suggests that degraded habitats can directly alter chemical structure of alarm cue and thus reduce antipredator responses, although the chemical compounds present in degraded vs. healthy habitats were not explicitly examined. As chemical alarm cues are naturally broken down throughout the day (Ferner et al., 2005; Ferrari et al., 2007; Bytheway et al., 2013; Chivers et al., 2013), warming may accelerate these processes in all ecosystems and reduce the effectiveness of chemical alarm cues as reliable indicators of predation risk. In contrast, olfactory preference of dottyback predators toward damaged-released damselfish prey cues is maintained in both healthy and degraded coral environments (Natt et al., 2017). This suggests that some predators may have a sensory advantage over prey in degraded coral habitats, potentially altering the outcome of predator-prey interactions.

Much climate change behavioral research has focused on cue detection and processing in the sensory transduction pathway. Effects of ocean acidification effects on chemosensation in aquatic systems have been especially well-examined (**Table 1**, **Figure 2**). Chemical reception (i.e., detection and processing) relies on membrane bound receptors and combinatorial responses to diverse and novel combinations of chemicals (Bargmann, 2006; Symonds and Elgar, 2008). Changes to the structure of the membrane-bound chemosensory receptors could conceivably alter detection of chemical cues. Changes in central nervous system processing that underlies recognition also can affect the ability of predators or prey to respond to each other (Nilsson et al., 2012).

Elevated CO_2 effects on chemosensory reception mostly examine only behavioral endpoints and do not directly reveal the underlying mechanism. Some studies show changes in detection and response to chemical food cues as a result of elevated CO_2. This includes reduced attraction to prey chemical cues, as shown in brown dottyback predators (Cripps et al., 2011). Some shark predators in high CO_2 conditions have been shown to fail to track, or even avoid, prey odors (Dixson et al., 2015; Pistevos et al., 2015), suggesting both cue detection and processing are being affected. Hermit crabs in high CO_2 conditions take longer to locate chemical food cues and spend less time in contact with cue, suggesting deficits in cue detection as there were no effects of cue pH on these behaviors (de la Haye et al., 2012); however, small changes in general motility also occurred from reduced pH and cannot be excluded completely from this analysis. Importantly, all of these studies ruled out changes to cue structure by testing cues in both elevated and ambient CO_2 conditions. Studies show both reductions in activity levels related to chemosensory-mediated foraging (e.g., handling time, time to respond) in crab predators (Dodd et al., 2015; Glaspie et al., 2017) and increases in activity of predatory reef fish (Cripps et al., 2011). These imply that elevated CO_2 levels may cause some underlying physiological changes in addition to behavioral changes, but there currently is not enough evidence to support this hypothesis strongly within the scope of sensory ecology.

Prey chemosensory abilities also are affected. Manríquez et al. (2014) tested the effects of ocean acidification on chemical detection of predators and food by a muricid snail using y-maze experiments. Orientation of juvenile snails to food cues in elevated CO_2 conditions was not suppressed by the presence of predator odor; orientation behavior in attractive + aversive cues was similar to that in response to only attractive cues. The inability to detect aversive cues or discriminate them from attractive cues could increase predation risk. Aversive predator cues often inhibit or eliminate attraction to food in untreated

animals (Moir and Weissburg, 2009; Weissburg et al., 2012). These studies, like most others, do not suggest a mechanism. It could be that detection of specific predator cues were affected whereas food cues were not, as they are typically composed of a number of different molecules (Hay, 2009). Elevated CO_2 has been demonstrated to reduce olfactory sensitivity in juvenile European sea bass (*Dicentrarchus labrax*), possibly by changing the affinity the receptors have for odorants (Porteus et al., 2018), although this could involve either changes to the receptor or changes to the chemical itself (e.g., Roggatz et al., 2016). Alternately, as discussed below, elevated CO_2 levels can interfere with central nervous system processes that allow animals to discriminate attractive from aversive cues.

Some studies show sharp changes in behavioral responses to chemical risk that arise clearly from deficits in sensory processing by the central nervous system. For example, tropical larval clownfish (*A. percula*) are attracted to predator chemical cues rather than avoiding them and do not distinguish predator from non-predator cues, which translates to higher mortality rates on natural marine reefs (Dixson et al., 2010; Munday et al., 2010). These studies rule out changes in cue production or changes during transmission; predators were held in ambient conditions and prey tested in normal seawater, thus implicating changes in the receiver (prey). High CO_2 conditions do not change olfactory receptor morphology (Munday et al., 2009), suggesting some central neural process is responsible. Predator cue avoidance can be restored by using the GABA-A antagonist gabazine to rescue neurotransmitter function in animals exposed to elevated CO_2 conditions (Nilsson et al., 2012; but see Abboud et al., 2019). Lack of ability to identify, distinguish, and avoid chemical cues indicative of predation risk also has been shown in damselfish (Munday et al., 2010; Chivers et al., 2014), snails (Manríquez et al., 2014; Abboud et al., 2019), trout (Munday et al., 2013), and salmon (Ou et al., 2015). Future atmospheric conditions have also been shown to mask carbon dioxide cues used by mosquitos to find their hosts (Majeed et al., 2014), with elevated CO_2 background levels causing a reduction in olfactory receptor sensitivity and potentially habituation in higher-order neurons (i.e., cue processing). Disruptions of processing in the olfactory bulb receptor pathway also has been demonstrated in ocean-phase coho salmon that show reduced avoidance of chemical alarm cues as a result of elevated CO_2 (Williams et al., 2018).

However, other studies have shown no changes in chemosensory reception of predators and prey in response to elevated CO_2. Goldsinny wrasse predators (*Ctenolabrus rupestris*) acclimated and tested in ambient or elevated CO_2 conditions maintain olfactory-mediated behaviors to food cues (Sundin and Jutfelt, 2016). In a similar fashion, Atlantic cod (*Gadus morhua)* avoid predator chemical cues regardless of CO_2 level that they were acclimated and tested in Jutfelt and Hedgärde (2013). Likewise, damselfish (*Acanthochromis polyacanthus)* continue to respond to predator chemical cues by reducing activity in both ambient and elevated CO_2 treatments, especially when exposure to high CO_2 is longer-term and the predator is also exposed to high CO_2 conditions (Sundin et al., 2017). Interestingly, increased foraging behavior of speckled sanddab in response to damaged-released chemical cues from conspecifics is maintained in elevated CO_2 (Andrade et al., 2018). Other temperate fish from naturally high CO_2 environments (i.e., CO_2 seeps) also do not show disruption of GABA-mediated olfactory predator recognition (Cattano et al., 2017). These studies suggest that variation in behavioral responses to elevated CO_2 may arise from predisposed adaptations to local habitat conditions, allowing for chemically-mediated behaviors in some species to remain robust in a variable environment. This is supported by Jarrold et al. (2017), which suggests that fluctuating elevated CO_2 environments that reflect diel CO_2 cycles can reduce the negative effects of stable elevated CO_2 on antipredator behavior (e.g., lateralization and predator cue avoidance) (but see Jellison et al., 2016). Given that these studies rule out effects on cue production, transmission or detection/processing, the robustness of chemical information transfer must reflect properties of the receiver.

Warming has only been directly tested on isolated chemosensory reception in the Antarctic sea star *Odontaster validus* (Kidawa et al., 2010); in addition to reducing ability for sea stars to right after being flipped and reducing locomotor activity, this study reported that warming weakened attraction to food odor cues. This suggests that warming can indirectly reduce chemosensory detection and processing by reducing overall activity.

Vision

Vision is important for detecting predation risk as well as potential prey, especially in well-lit systems such as coral reefs with predators that possess good visual systems. The success of visual cue information transfer is highly dependent on degree of movement, light availability and contrast against an environmental background to transmit the cue, as well as physiological eye function (**Table 2**). Consequently, visual information transfer is highly variable across spatial (i.e., within one meter or over tens of meters) and temporal (i.e., seconds or minutes) scales (Weissburg et al., 2014). All of these factors have been shown to be affected by climate change stressors, although there are limited studies (**Table 1**; **Figure 2**). Increased predator movement from warming has been shown in European sea bass (*D. labrax*) with higher levels of swimming and other activity (Manciocco et al., 2015), which may indicate higher predation risk to potential prey. In a similar fashion, increased prey metabolism from warming can increase production of prey visual cues via increased activity levels, causing prey to become more susceptible to predation. This has been suggested with increased activity levels (and consequently encounters) between spider predators and fly prey from increased temperatures (Kruse et al., 2008), but the sensory ecology of these interactions remains to be tested. Elevated CO_2 can increase activity levels in juvenile coral trout (*Plectropomus leopardus*), and produces bolder behavior and less time spent in shelter (Munday et al., 2013). Pygmy squid also are more active and use less cryptic behaviors in high CO_2 conditions (Spady et al., 2014). These changes increase production of visual cues by prey and potentially increase predation risk.

Warming is predicted to have a strong impact on transmission of visual cues. Coker et al. (2009) demonstrated

that piscivorous dottybacks (*Pseudochromis fuscus*) initially avoid bleached corals as a degraded habitat but increase strike rates at prey positioned against the white background of bleached corals. This indicates a mismatch between camouflaged prey and background, which can lead to increased predation as a consequence of increased temperature. Degraded habitats have also been shown to maintain transmission of visual predation risk via reduced habitat complexity (McCormick and Lönnstedt, 2013), strengthening the visual transduction pathway between predator and prey.

Warming also is predicted to have indirect effects on visually mediated predator-prey dynamics. Climate change conditions favor algal blooms (Paerl and Huisman, 2009) that can increase turbidity and reduce light availability. Turbidity has been shown to directly interfere with visually-mediated foraging success of pinfish predators on crustacean prey (Lunt and Smee, 2015) by reducing reactive distance (Sweka and Hartman, 2003), although other fish predators with well-developed visual systems (e.g., Atlantic cod) may not experience as dramatic effects (Meager et al., 2005). Turbidity also drastically reduces the distance at which cormorant (*Phalacrocorax carbo sinensis*) predators can visually detect fish prey (Strod et al., 2008). Increased turbidity also has been demonstrated to decrease transmission of visual cues that are imperative for avoiding predators (Swanbrow Becker and Gabor, 2012). This has been tested in freshwater systems, where perception of predation risk is reduced in turbid waters (Abrahams and Kattenfeld, 1997; Hartman and Abrahams, 2000). Together, these studies suggest that turbidity can change trophic interactions by reducing indirect effects of predators (i.e., predation risk assessment) and shifting top-down control to only direct (i.e., consumptive) effects as encounters become random (Van de Meutter et al., 2005).

Elevated CO_2 effects on visual detection and processing have been tested directly in a few cases. Larval temperate gobies (*Gobiusculus flavescens*) in elevated CO_2 conditions are more attracted to light (i.e., increased phototaxis), which suggests that visual hypersensitivity may be costly by attracting to weak or fluctuating light stimuli (Forsgren et al., 2013); the specific fitness effects of this behavioral change remain unknown, but may include attraction to new habitats with higher or lower predation rates. Retinal responses of a marine tropical damselfish species (*A. polyacanthus*) are slowed after exposure to high CO_2 waters, which suggests impaired visual assessment of fast events such as predator or prey movement via disruption of cue detection (Chung et al., 2014). Antipredator responses (e.g., reductions in foraging and activity, bobbing behavior) of another juvenile tropical damselfish (*P. amboinensis*) to the sight of a predator are reduced or lost (i.e., no antipredator response) in high CO_2 conditions, suggesting that elevated CO_2 can reduce visual risk assessment and promote bolder prey behavior via disruption of cue processing (Ferrari et al., 2012). Prey reactivity (i.e., reactive distance, looming threshold) to visual predation risk also has been shown to be reduced by warming and elevated CO_2, but the outcome of predation depends on stressor effects on the predator as well as prey (Allan et al., 2013, 2017).

Mechanosensation

Mechanosensation, such as by lateral lines in fishes and mechanoreceptors in arthropods, is used to detect changes in pressure or fluid motion around the organism (Dusenberry, 1992). Detecting nearby (i.e., within 1 m–Weissburg et al., 2014) disturbances such as movement can be useful in foraging or avoiding predators and other mortality risks, but there are very limited studies on effects of climate change on mechanosensory information transfer (**Table 1**; **Figure 2**).

As noted previously, changes in movement via changes in metabolism from warming may cause mechanosensory cues to be altered in both terrestrial and aquatic systems. Specifically, increased movement or activity would increase mechanosensory cue production, strengthening the specific location of predator or prey. Directionality and escape responses of damselfish to a startle stimulus increase after short-term exposure to elevated temperatures (Warren et al., 2017). However, longer-term exposure to warming allows escape responses to return to control levels, demonstrating that physiological plasticity is dependent on length of exposure. Damselfish acclimated to elevated CO_2 conditions are less responsive and they take longer to respond to a stimulus (i.e., a weight dropped on the surface of the water) while locomotor activities are maintained, suggesting a deficit in processing these mechanosensory cues (Munday et al., 2016). Likewise, the probability of a startle response from marine medaka to a mechanosensory stimulus is decreased in elevated CO_2 conditions due a deficit in processing (Wang et al., 2017).

Multimodal Cues

Although studying responses to individual cues can help explain mechanisms or drivers of behavior, it is important to note that multiple senses often are utilized by organisms in natural ecosystems, especially when one sensory modality is compromised by an environmental stressor. Therefore, studies incorporating multisensory responses to warming and elevated CO_2 provide a more realistic prediction of changes in overall behavior. These studies are limited, however, and mostly explore the interaction and possible "sensory redundancy" (Lönnstedt et al., 2013b) between chemical and visual cues (**Table 1**). Manciocco et al. (2015) studied sea bass from temperate estuaries in warmed conditions and found that attraction to food chemical cues and avoidance of visual aversive cue (i.e., mirror test) both increased in intensity and occurred at faster rates (i.e., shorter behavioral response time to cues). This emphasizes how warming changes the speed of sensory information transfer in multiple modalities and consequent behavioral responses. Elevated CO_2 also has been shown to affect visual and chemical cues in damselfish found in tropical reefs (Lönnstedt et al., 2013b): while elevated CO_2 caused fish to fail to identify (i.e., no response) chemical alarm cues completely, antipredator responses to visual predator cues were reduced but not completely lost. When visual and chemical cues were combined, responses to visual cues were able to partially (but not fully) compensate for lack of responses to chemical cues. Goldenberg et al. (2018) used mesocosm experiments with multiple species of fish and crustacean consumers found in temperate marine systems, testing effects of warming and elevated CO_2 on the

efficacy of visual and chemical cues in isolation and together. Elevated CO_2 but not warming reduced attraction to food chemical and visual cues, but the consumers restored their attraction to food when chemical and visual cues were combined regardless of temperature or CO_2 level. These studies suggest that sensory redundancy can mitigate the effects of climate change on individual sensory modalities, and some ecological interactions and processes may remain relatively resilient to these stressors. Consequently, certain community dynamics may be relatively robust to climate change when multiple sensory cues are employed.

However, multimodal cues may not be as reliable in degraded habitats as in normal conditions because degraded habitats may create environments where one cue is strongly favored or disfavored. As previously mentioned, McCormick et al. explored the effects of habitat degradation on predation risk assessment by marine damselfish *P. amboinensis* using visual and chemical cues. Visual predation risk cues become more important in degraded habitats with less structure as prey are more easily able to detect predators (Lönnstedt et al., 2013a); chemical predation risk cues become more important in topographically complex (i.e., healthy reef) habitats when visual risk cues are restricted, and prey are more vigilant (i.e., reduced feeding and activity, increased shelter use) (McCormick and Lönnstedt, 2013). Habitat degradation may increase the availability of visual cues, but this may come at a cost of degraded chemical cues (Lönnstedt et al., 2013a). Damselfish are less likely to use degraded coral (i.e., loss of live coral tissue) as shelter in response to predation risk, potentially due to altered visual or chemical cues (Boström-Einarsson et al., 2018). Together, these findings suggest that fishes in degraded habitats may experience higher mortality rates from predators if habitat changes alter the availability of one type of cue relative to one another, especially when chemical cues are important sources of information regarding predation risk.

Sensory behaviors stemming from different modalities may share universal disruptions in central nervous system processing, exemplified in changes to behavioral lateralization of marine fish challenged with high CO_2 conditions (Domenici et al., 2012; Jutfelt et al., 2013; Welch et al., 2014; Näslund et al., 2015). These studies have attempted to show how elevated CO_2 disrupts neural processing by assuming lateralization requires minimal visual input from the environment and is driven by brain functional asymmetries that create a right- or left-side preference. In these studies using T-mazes to measure turning preference in damselfish, elevated CO_2 has been shown to reduce lateralization (i.e., no turning preference). Nilsson et al. (2012) further demonstrated that the addition of an antagonist of the GABA-A receptor (i.e., gabazine) reversed elevated CO_2 effects in both lateralization and chemical cue preference, suggesting high CO_2 affects neural processing of multiple sensory systems by interfering with neurotransmitter function. This is supported by Heuer et al. (2016), which demonstrates that elevated CO_2 causes altered ion gradients in the GABA-A receptor of fish as a means of CO_2 compensation. Impaired neurotransmitter function from elevated CO_2 also has been demonstrated to decrease learning of predator identity in juvenile damselfish prey, consequently reducing survivorship in the field after being treated in the laboratory (Chivers et al., 2014). The availability of multiple sensory cues may be insufficient to allow predators or prey to regain normal sensory function in the presence of such fundamental disruptions of neural processing.

CONCLUSION AND PROSPECTUS

Global warming and elevated CO_2 will have extensive impacts on sensory behavior in predator-prey interactions. However, lack of a framework for identifying the underlying mechanisms makes it difficult to establish the generality of effects or their magnitude. Using a behavioral endpoint to examine stressor effects on predation (e.g., Li et al., 2015; Sui et al., 2015) often fails to identify whether effects stem from cue production, transmission, or reception (**Figure 1**) unless the study is properly constructed. Studies should examine stressor effects on predator and prey separately as well as together, as well as isolating the effect of differential transmission from changes to sender and receiver properties.

Understanding which process is affected is essential to fully understand subsequent effects as changes to particular processes have different consequences. For instance, transmission may alter the effective distance of predator-prey signaling, whereas deficits in reception by predators or prey have global effects. Since warming and elevated CO_2 seem to affect different steps in the transduction cascade (**Figure 1**), clearly identifying the nature of the disruption also can predict synergistic or antagonistic responses. In particular, warming seems to affect metabolism and activity (in addition to transmission in some cases), whereas elevated CO_2 often impacts the ability to receive or process cues. The effects of warming on metabolism, and in turn, the impacts on foraging ability, suggests warming may primarily alter consumptive effects of predators despite some changes to the signaling process. Elevated CO_2 clearly affects all different steps in the predator-prey signaling cascade, and may therefore change both consumptive and non-consumptive effects depending on which organism is most compromised in a given interaction. If sensing by predators is more affected then non-consumptive effects may increase, whereas consumptive effects will increase when sensing by prey is strongly affected.

Future studies should address both behavioral endpoints and identify in so far as possible the step (production, transmission, or reception) that is affected. Although this can be cumbersome, a substitutive design where acclimated animals are placed in normal environments and vice versa, can at least identify environmental effects on transmission. Independent measures of cue strength also can be helpful, particularly when movement related cues are responsible for predator-prey information transfer and stressors are shown to affect activity. Chemical cues that are metabolic by-products may be difficult to quantify, but metabolomics approaches offer promise (e.g., Poulin et al., 2018) and can at least establish quantitative differences in production even when the cue remains unidentified. Deficits in neural processing can be difficult to document without examining physiology of sensory receptor cells or central nervous system properties, which has been done only in some cases (e.g., Munday

et al., 2009; Boullis et al., 2017; Porteus et al., 2018). Careful consideration is also needed when determining measurements of sensory responses that distinguish these from changes in general motility or activity (e.g., lacking in Kidawa et al., 2010). General observations on movement in response to a stimulus can be confounded by changes in metabolism and/or motor function, potentially misidentifying sensory effects (e.g., de la Haye et al., 2012). Possible sensory tests should include at least a choice between stimulus and blank control (e.g., Dixson et al., 2010).

A second major challenge going forward is to integrate both predator and prey responses into studies examining the effect of climate change stressors. Predators and prey participate in a duet where both parties can gather information about the presence of the other, and the effects of climate change on predator-prey dynamics will depend strongly on which participant is more compromised. For example, ocean acidification has a greater negative effect on mud crab foraging behaviors (i.e., consumption rate, handling time, duration of unsuccessful predation attempts) than calcification rates of oyster prey, resulting in overall reduced prey consumption (Dodd et al., 2015). Moreover, the extent to which predators vs. prey are affected may change the nature of predator control as well as its magnitude (e.g., Goldenberg et al., 2018). We predict a shift to consumptive effects if climate change stressors more strongly influence prey, whereas the importance of NCEs will increase if predator abilities are more impacted.

Knowledge gaps remain due to biases in the literature. Approximately 56% of climate change studies focused on sensory cues governing predator-prey interactions use marine reef fish as model organisms, especially for elevated CO_2 effects (**Figure 2**). Invertebrate predators have also been shown to be important community regulators such as blue crabs in estuarine habitats (Silliman and Bertness, 2002; O'Connor et al., 2008; Hill and Weissburg, 2013), which often rely on chemical cues to locate prey. Invertebrates as model organisms should be tested more frequently in future studies given their ecological importance.

Studies of sensory modalities affected by climate change have emphasized chemosensation over others (e.g., vision, audition, mechanosensation), with ∼61% of climate change sensory studies using chemosensation as the primary modality (**Figure 2**; **Table 3**). Cue detection/processing deficits have been shown in chemosensory, visual, and possibly mechanosensory interactions, but few studies to date have tested auditory processing. Some animals utilize specialized senses, such as electrosensation in sharks and other elasmobranchs. Currently, there are no known studies that examine how responses to these cues might change in the face of climate change. Changes in neurotransmitter function induced by climate change (Nilsson et al., 2012) suggest that processing deficits may be occurring in multiple sensory systems.

Environmental effects are important in changing sound propagation and altering the visual and chemical environment so as to mask incoming cues from predators and prey, or enhance the utility of one modality vs. another. These effects also might be important in terrestrial systems despite the lack of studies (**Figure 2**; **Table 3**). Vegetation affects both spectral and acoustic properties and can alter both cue transmission and ambient sound and light levels, and change airflow patterns affecting mechanosensory signaling (Dusenberry, 1992; Endler, 1993; Ladich, 2000; Slabbekoorn and Smith, 2002; Brumm and Slabbekoorn, 2005; Casas and Dangles, 2010). Changes to vegetative structure produced by changing climate (e.g. Cramer et al., 2001) therefore may alter predator-prey information exchange in terrestrial habitats. The environment may also modify cues directly as occurs with waterborne or airborne chemicals. Most studies to date that manipulate only the predator or prey do not capture these effects, and so further studies should attempt to incorporate the sensory environment (**Figure 1**) to correctly capture future interactions. It also is critical to keep in mind that these potential changes in the environment are independent of changes to the receiver or sender, but may interact with these changes in both opposing or complementary fashion.

This field has been disproportionally represented by elevated CO_2 studies rather than warming, promoting a consequent bias in marine systems and very few terrestrial examples (**Figure 2**; **Table 3**). Temperature is the primary global stressor affecting sensory-mediated interactions in terrestrial systems, and so the current state of knowledge implies that these interactions may be less severely affected. However, warming needs to be studied in animals whose metabolic processes and consequent behavior is dependent on temperature, such as invertebrates. Metabolic changes from temperature can also change cue production and processing speed, and these effects should be more explicitly tested.

It is important to note that while warming and elevated CO_2 can have independent effects on sensory systems, there is potential for these global stressors to interact as both are predicted to occur in the future (IPCC, 2014). There are several studies examining multiple stressors on behavioral interactions that have potential sensory mechanisms, which were not explicitly tested. For example, warming and elevated CO_2 act synergistically to increase overall predation rate of dottyback predators (*P. fuscus*) on multiple species of damselfish prey (*P. amboinensis and P. nagasakiensis*), whereas each of these stressors independently have no effect on predation rate (Ferrari et al., 2015). This effect could not be predicted from general changes in metabolism alone, which is argued to demonstrate that trophic outcomes are not driven solely by physiological tolerances to these climate change stressors. Also shown in this study were antagonistic interactions between warming and elevated CO_2; each climate stressor independently reversed prey selectivity between the two damselfish prey species, but the combined stressors override prey selectivity so that prey are consumed in equal proportions. In this case, specific sensory cues used by predator and prey were not tested, but further research may provide insight on which sensory modalities are driving these interactive effects. It is possible that warming can interact with elevated CO_2 by changing metabolic rates that affect cue production and reception, potentially amplifying any direct effects on the sensory transduction pathway (**Figure 1**).

There are very limited studies that examine how multiple stressors affect the same transduction pathway, and the underlying mechanisms. As previously mentioned, warming and elevated CO_2 effects have been tested in fish and crustaceans responding to visual and chemical cues (Goldenberg et al., 2018). Testing these stressors in isolation and together revealed that

only elevated CO_2 was driving behavioral responses when both stressors were present. A similar pattern was found in dogwhelks (Queirós et al., 2015) and sharks (Pistevos et al., 2015), where only elevated CO_2 disrupted chemosensory perception of prey while warming had no effect on these behaviors. Identifying multiple stressor effects on a single sensory modality can allow the correct identification of specific stressor effects on different senses [e.g., chemosensation (Dixson et al., 2010), vision (Ferrari et al., 2012), and audition (Simpson et al., 2011) in coral reef fish]. Again, we suggest that warming and elevated CO_2 may target different parts of the transduction cascade and may interact strongly in systems where cue production depends on activity.

Interaction of global stressors (i.e., warming and elevated CO_2) with local stressors also needs to be studied, as local stressors (e.g., sediment pollution: O'Connor et al., 2016) have been found to disrupt sensory cues and change behavioral responses. One study tested the global stressor of elevated CO_2 and local stressors of sediment runoff and pesticides separately, but did not cross the stressors (Lecchini et al., 2017); each stressor had negative effects on attraction to conspecific chemical cues for both fish and crustacean species, but the possibility of interactive effects (synergism or antagonism) between these stressors remains unexplored.

The continuation of these efforts, especially more realistic laboratory experiments and field experiments when possible, has the potential to identify and address the complex changes in future predator-prey interactions. This expansion will yield a better understanding of climate change impacts on sensory ecology, which can be applied to the success of conservation and restoration efforts for protecting and maintaining ecosystems.

AUTHOR CONTRIBUTIONS

AD conceived the manuscript, led the literature review, and produced the figures. MW contributed to the literature search and core concepts for this paper, and both authors contributed to writing and revising the final manuscript.

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Comparing Plasticity of Response to Perceived Risk in the Textbook Example of Convergent Evolution of Desert Rodents and Their Predators; a Manipulative Study Employing the Landscape of Fear

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Foragers process information they gain from their surroundings to assess the risk from predators and balance it with the resources in their environment. Measuring these perceived risks from the perspective of the forager can produce a heatmap or their "fear" in the environments, a so-called "landscape of fear" (LOF). In an intercontinental comparison of rodents from the Mojave and Negev Deserts, we set to compare families that are used regularly as examples of convergent evolution, heteromyid and gerbilline respectively. Using a LOF spatial-analysis on data collected from common garden experiments in a semi-natural arena we asked: (1) do all four species understand the risk similarly in the exact same physical environment; (2) does relative relation between species affect the way species draw their LOFs, or does the evolutionary niche of a species have a greater impact on its LOF?; and (3) does predator facilitation between vipers and barn owls cause similar changes to the shape of the measured LOFs. For stronger comparative power we mapped the LOF of the rodents under two levels of risk: low risk (snakes only) and high risk (snakes and barn owls). We found concordance in the way all four species assessed risk in the arena. However, the patterns observed in the LOFs of each rodent family were different, and the way the topographic shape of the LOF changed when owls were introduced varied by species. Specifically, gerbils were more sensitive to owl-related risk than snakes and at the opposite correct for heteromyids. Our findings suggest that the community and environment in which a species evolved has a strong impact on the strategies said animals employ. We also conclude, that given the homogenous landscape we provide in our arena and the non- homogenous patterns of LOF maps, risk assessment can be independent of the physical conditions under which the animals find themselves.

Keywords: community ecology, common garden experiments, convergent evolution, ecology of fear, evolutionary game theory, habitat selection, predator-prey interactions, spatial ecology

INTRODUCTION

Colloquially fear is defined as a psychological emotion that drives anti-predator responses of an individual animal to risk related information it collects from its environment (Laundré et al., 2010; Clinchy et al., 2013). However—ecologically fear should be defined as the strategic response of an individual animal to risk related information it collects from its environment. This definition stands in contrast with the vague term of psychological emotion. Mathematically this translates to the solution of a game theoretic model balancing the tradeoffs of resources, energetic and reproductive, and safety (Brown et al., 1999; Bleicher, 2017). Those strategic responses, when measured on the physical landscape can provide a visualization of the way a population of individuals perceives the risk in the landscape in form of a topographic (or heat) map, a landscape of fear (LOF; Laundré et al., 2001, 2010). In this article, we use LOFs to observe how similar species from two desert communities respond to the risk from the same set of predators, and most importantly in the exact same physical environment. The selective pressures imposed by historical predators over evolutionary time manifest themselves in contemporary populations as strategic decisions and behavioral patterns in response to a community of modern predators, both known and novel (invasive). Thus, employing the LOF framework in such a comparison permits an investigation of how the lethality of historical predators has impacted the current space-use and decision-making of species.

Predators affect plant communities both directly by consuming herbivores and indirectly through behavioral effects on their prey (Ale and Whelan, 2008; Orrock et al., 2008; Sih et al., 2010). Perhaps the best-known example comes from the reintroduction of wolves to Yellowstone National Park. The risk from wolves frightens the elk, especially females, causing them to forage in less-risky habitat away from rivers. This permits the survival and renewed growth of willows and aspens (Ripple and Beschta, 2006). In turn, these stabilize the stream banks (Ripple and Beschta, 2006, 2012; Beschta and Ripple, 2009; Eisenberg et al., 2014). Using this example, Laundré et al. (2001) proposed the LOF as a framework for understanding the consequences of behavioral trophic cascades in landscapes that are spatially heterogeneous in risk of predation. In a recent review, Bleicher (2017) suggests the LOF can be used to metaphorically understand the animal's *umwelt* (see Uexküll, 1909), the way it understands its environment. While physical aspects of a landscape may alter the cognitive responses of an individual animal to its surroundings, as in the example of waterways in Laundrè's seminal article (2001), the LOF should be studied as an attribute of a population which can but will not always, be independent of these features.

We define the LOF as a "map," a visualization, of how animals perceived the predation cost of foraging within the constraints of time and space. When an animal navigates the landscape in search of forage it balances the perceived risk through a number of energetically costly behaviors: vigilance, heightened senses, and even missed opportunities when the risk is deemed too high (Brown, 1999). The LOF, reflects the fundamental strategic-decisions an animal makes in its environment, habitat-use, and foraging behaviors. Thus, it is not surprising that the tool favored to measure the LOF has been the Giving-up Density (GUD) in over 60% of all manuscripts that chart the forager's LOF (Bleicher, 2017). The GUD, a model describing the quitting harvest rates of foragers as a function of foraging and predation costs (Brown, 1988) is now used as a tool. The optimal patch-use model, as it was known originally, states that given diminishing-returns of resources in a patch, the amount of food left behind at a location by a forager would equal the inflection point where the costs related to foraging outweigh the energetic benefits (Bedoya-Perez et al., 2013).

We present here a large biogeographical comparison of convergent species from different genera to test whether physical and morphological convergence leads to behavioral convergence in those species, specifically in their LOF and the way these change under variations in predation risk within a semi-natural arena (located in Sde-Boker, Israel). Similarly, we also use this experiment to test whether imperfect behavioral-convergence (see Bleicher et al., 2018a), will result in an imperfect convergence of the LOFs. Our experimental arena, vivarium, is a 17 × 17 m aviary that was constructed to facilitate common-garden experiments with predators and prey populations. This setup allows for the measurement of animal foraging in a non-invasive, near-natural environment while achieving full control of predation-risk variables and removing inter-specific competition (**Figure 1**).

Our example compares two well-studied families that exhibit many ecological, behavioral, and physical similarities—desert rodents from the family Heteromyidae mostly from North America and the subfamily gerbillinae from Africa and Asia. We address, compare, and contrast the behavior of these rodents using habitat selection, patch use, and in a broader sense, foraging dynamics. In general, a forager's LOF will be jointly influenced by its own species-specific aptitudes for detecting and evading predators, the structure of the physical environment insofar as particular features may favor the prey or the predator, and finally the perceived abundance and type of predators currently threatening the forager (Laundré et al., 2010; Bleicher, 2017).

While the intercontinental comparison is perhaps the most attention-drawing comparison this article provides, it is not our only objective. Within each family, we specifically chose one smaller species (20–35 g) and one larger (38–40 g) to determine whether body size differentially affects how rodents balance perceived risk and competition with other species. In both rodent families, the larger species is considered a stronger competitor (Thompson, 1982; Kotler et al., 1993b). There is strong evidence for anti-predator adaptations playing a key role in the mechanism of coexistence of these competing species (Kotler, 1984; Kotler and Brown, 1999). Additionally, series of manipulative experiments distilled the major environmental elements that allow for these competitors to coexist: most prominent among them being microhabitat partitioning between bush and open microhabitats (Rosenzweig, 1973; Kotler et al., 1993a); foraging substrate (Kotler et al., 2001); moonlight (Bouskila, 2001; Kotler et al., 2010), and temporal partitioning (Kotler et al., 1993b). To maximize our comparative ability, we

brought our study populations into the same seminatural-arena and into a controlled and relatively featureless environment in which we controlled foraging resources, population size, and risky habitat (offering foraging patches in open and bush microhabitats).

This set-up allows us to ask three questions using the comparison between these four rodent's LOFs:

(1) do all species of rodents exhibit similar LOFs across the various predation conditions?

If the rodents "understand" the risk in the same ways, areas ranked as high or low risk would be similar among the species. We expect the larger rodents to have similar patterns and the smaller competitors to have similar ones. This expectation leads to the second question.

(2) are similarities in the LOFs most striking for rodents of similar body sizes regardless of continent of origin or for rodents originating from the same desert even when they differ in body size?

We aim to separate the role of historical (evolutionary) risk of predation, the ghost of predator past, from the relative competitive ability of a species in shaping a population's LOF. If the two heteromyids respond in a similar way and the two gerbils respond in a different, but mutually similar way, it would undermine our assumption of evolutionary convergence between the rodent families, at least in their behavior.

(3) do owls cause changes only in the elevation of the LOF or in its shape as well?

This query is aimed to question the way these species assess risk; how they understand predation risk as a whole. A LOF that rises and falls based on the cumulative risk posed by predators, but maintains its shape (key topographic features), suggests that historical (evolutionary-scale) predation risk had a strong impact in the species' spatial distribution, i.e., the LOF reflects a fixed pattern that is tweaked (or turned on and off) based on the level of risk in the environment.

In contrast, a species could show plasticity in response to cumulative risk posed by predators. A species that redraws its LOF based on a focal feature of risk is most likely a product of evolution in a system where different predators applied different predatory pressures, and possibly at different rates of interaction. In such a situation, the cost-benefit analysis governing the foraging behavior of that species would focus on the greatest risk in the environment before factoring in lesser costs.

MATERIALS AND METHODS

Site

We conducted our experiments in a semi-natural, outdoor enclosure (vivarium 17 × 17 m) located on the Sede Boker Campus of Ben-Gurion University, Blaustein Institutes for Desert Research, Midreshet Ben-Gurion, Israel (N 30° 51' 25.978", E 34° 46' 51.284"). The vivarium was divided into quadrants where the southern half of the arena housed two vipers and the northern was a snake-free control (**Figure 1**). The vipers' movement was restricted by gates that permitted only the rodents to move freely between quadrants. The vivarium, covered with a wire net 7.5 m high, forms and aviary that allowed for barn owls to fly freely within the structure. The owls were housed and released, one at a time, from cages adjacent to the facility. To minimize actual depredation of rodents, the owls were fed prior to release and returned to their boxes at sunrise.

Species

We brought together one large and one small coexisting desert rodents from each continent, two common gerbils from the Negev Desert of Israel and a kangaroo rat and a pocket mouse from the deserts of the southwestern United States, to a common and controlled setting in the Negev Desert. The Negev Desert gerbils include the greater Egyptian gerbil *Gerbillus pyramidum* (GP), 40 g, and Allenby's gerbil *Gerbillus andersoni allenbyi* (GA), 30 g (Goodfriend et al., 1991). The North American Desert rodents include Merriam's kangaroo rat *Dipodomys merriami* (DM), 45 g (Lancaster, 2000), and the desert pocket mouse *Chaetodipus penicillatus* (CP), 22 g (Chebes, 2002). All are nocturnal desert granivores commonly found on sandy substrates such as sand dunes. All four rodents have adaptations to reduce the risk of predation, including saltatorial locomotion for enhanced escape abilities and auditory adaptations to increase hearing acuity. These adaptations are especially well developed in the kangaroo rats (Kotler, 1984; Randall, 1997).

We brought wild-caught vipers, trapped at locations where they would come in contact with wild populations of the above-mentioned rodents, to the same facility. We caught sidewinder rattlesnakes *(Crotalus cerastes)*, 35–60 cm mean length, from the Mojave Desert (Webber et al., 2016) and Saharan horned vipers (*Cerastes cerastes*), 30–60 cm mean length, from the Negev Desert (Anderson, 2011). Both snakes side-wind, burrow in the sand (usually under bushes) and feed on a variety of rodents and lizards (Ori, 2000; Anderson, 2011).

The animal collection was done respectively in the Mojave and Negev Deserts. The heteromyids were trapped between April-June 2012 predominantly in the Parker Dunes area (N 34°9'7.969", W 114°7'34.245") and supplemented by individuals from the San Bernardino (AZ) area (N 31°23'22.082", W 109°11' 22.851"). The sidewinders were collected in the Avra Valley alongside country roads (N 32°24'49.335", W 111°29'38.138") in two collection bouts in the spring of 2011, and 2012. The gerbils in Israel were collected in the Mashabim Dunes (N 31°0'14.531", E 34°44'47.31") and the horned vipers on the border between Israel and Egypt at Be'er Milka (N 30°57'4.609", E 34°23'10.821"). The gerbils were trapped in the spring of 2011 (GA) and 2012 (GP). The experiments were conducted within a year of trapping the animals to reduce the habituation to lab conditions. All the North American species were held in quarantine for 4 weeks prior to exportation to Israel. When in Israel, all the animals were held in climate-controlled animal-rooms at the Blaustein Institutes for Desert Research within 300 m from the experimental vivarium. The rodents we used in these experiments were all male except for GPs where the population had 60% males (due to a shortage

of males caught from the wild). We used only males in this experiment to comply with importation regulation for the Heteromyid rodents limiting the risk of releasing a possible invasive species (see Long, 2003).

Animal-Care

When not in experiments, the animals were housed in climate and light controlled animal husbandry rooms at the university. Rodents were caged in standard individual rodent cages lined with sterilized sand. They were fed nightly 3 g of millet, and the sand was replaced every 2-weeks according to animal care protocol. The animals were fed weekly with a handful of clover to sustain their water intake. Snakes were held in locked storage-bins 80 × 40 × 40 cm lined with sand and were given water (changed every few days) and a reptile heat lamp. Each snake was fed one live feeder mouse (*Mus musculus*) per week and their bins cleaned every 2 weeks.

Experimental Set-Up

Dates and Animals

The measurements were collected for each species in the absence of direct competition. This allowed us to make a comparative study of the effects of predation risk in the exact same setup in the absence of competition stressors. To allow for equal measurement of resource-use, we populated the vivarium with roughly the same biomass of rodents corrected for metabolic rate, leading to: 24 Allenby's gerbils from June-August of 2011, 16 Greater Egyptian gerbils from June-July of 2013, 16 Merriam's kangaroo rats from July-August of 2012, and 24 desert pocket mice from September-October of 2012. We ran each experiment for two lunar months. We measured the perception of risk each individual rodent perceived from the snakes in the experiment before entering the experiment and once it was taken out of the experiment (Bleicher et al., 2018a).

Based on RFID pit tags, implanted subcutaneously in each rodent, we tracked depredation in the vivarium. We found (in owl pellets, snake fecal matter and exit inventory) that a total of seven GA, five CP, three DM, and nine GP were depredated or died of natural causes during the experiment. During the experiment, for every pit tag found in an owl pellet, we released another individual in roughly the same location where the depredated individual was last observed (data from RFID antennae).

The Environment

Our vivarium mimics a dune habitat with a layer of 10 cm of sand covering a loess-clay subflooring with escape fencing buried to 1.5 m below ground. To replicate the sparse vegetation cover in semi-stabilized desert dunes we artificially created heterogeneity by adding 36 bramble-covered trellises in a grid of 6 × 6 each situated 2 m from the next trellis. Each trellis (80 × 50 × 15 cm) at each station was topped with a pile of cut brush to create a spherical "bush" approximately 1 m in diameter (**Figure 1B**). The environment beneath each trellis provides a sheltered environment mimicking a bush microhabitat, while the space between trellises replicates the open space naturally occurring between vegetation clumps in the deserts where our animals were trapped.

The vivarium was divided into two quadrants with a hardware-cloth fence (1 m above ground and 1.5 m

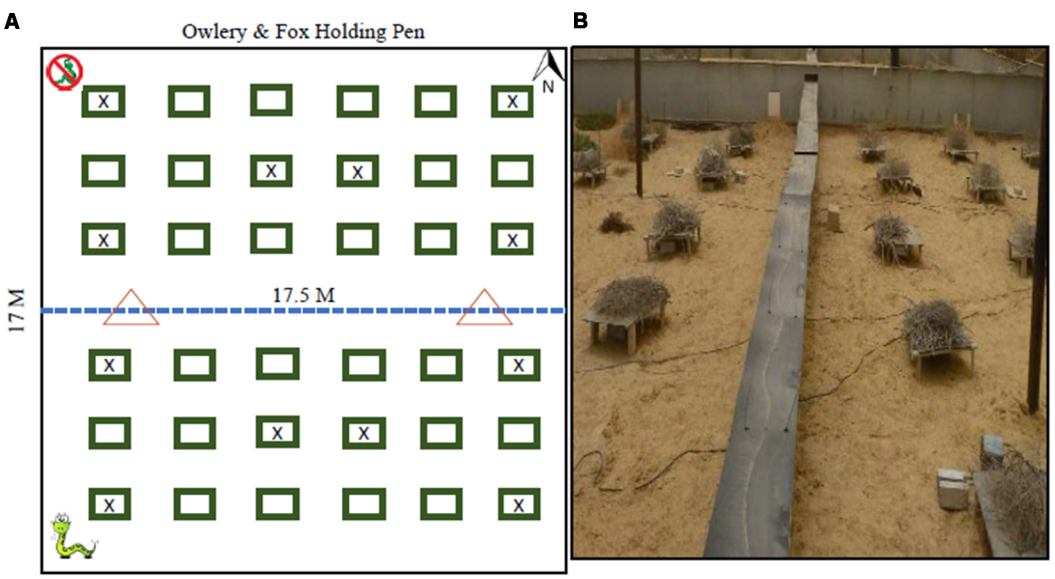

FIGURE 1 | Vivarium Layout **(A)** where the north (top) has no snakes and the bottom has two snakes, one Sharan Horned Viper and on Sidewinder Rattle snakes corresponding to the snakes with whom each of the rodent families evolved. The rectangles represent trellises replicating bush microhabitat. Trellises with an (x) did not have a foraging patch. The dotted line is a see-through hardware cloth fence reaching 1.5 m above ground and the same depth below ground to prevent tunneling. The triangles are the location of "S"-shaped gates that restrict the movement of snakes but allow the ideal free distribution of rodents across the entire arena. **(B)** A picture of the setup.

underground) that separated the northern and southern halves. On this divider, we installed two rodent gates, 8 m apart, that allowed rodents to pass freely from section to section. This measure allowed the rodents to distribute themselves according to an ideal free distribution. These gates were engineered to allow the movement of the rodents and restrict the movement of snakes. We avoided measuring the foraging of the rodents at the four corners and central two trellises of each quadrant. In addition, the distribution of these stations purposefully minimized and equalized the distance of patches from an edge of the enclosure keeping each station 3 m away from a fence or another station (**Figure 1A**).

Data Collection

We measured the LOF using a grid of 24 foraging patches sieved nightly to obtain a GUD measurement. Each patch ($38 \times 28 \times 8$ cm) held 3 liters of sand and was stocked each evening with 3 g of millet. At sunrise, each patch was sieved and weighed to 1/100 g and logged for analysis. Unforaged patches were collected and reset daily as well.

Every month, we ran two eight-night rounds. Each round comprised of four nights with owls and four nights without owls. Each month, one round was centered on the full moon and the other the new moon, for a total of 16 data collection nights per month and 32 nights per species.

We set the patches in rows of four with two under trellises recreating a bush microhabitat, and two placed adjacent (10 cm away) to two additional trellises representing the open microhabitat. Every 2 weeks we altered the patches' microhabitat at each of the 24 stations.

We have to state a major caveat for the experiment with the Allenby's gerbils (smaller Negev species). In that experiment, which was run first and acted as a pilot, we tested two additional layers of complexity not tested in the other species. We ran four six-night rounds per month centered around each of the four moonphases (new, waxing, full, waning), with only two nights per moonphase with the owls (Bleicher, 2014; Bleicher et al., 2016). Additionally, the experiment also added rotations with a muzzled red fox for two nights per moonphase. Based on these differences we ended up using a small subset of the data for this analysis and loosing statistical power from eight nights in other species to two per round in these smaller gerbils.

Data Analyses

General Effects

We ran a random-forest decision tree analysis in Statsoft Statistica. This analysis, best described as a categorical principal component analysis, uses a Bayesian approach to produce the likely major effects (splits) the data can produce organized by likelihood from most likely and important to the less robust effects at the final nodes. In this analysis, we input GUD as our independent variable, and species, microhabitat, snakes and owls as our dependent variables.

Station Effect

To account for station effects within quadrants, we used a general linearized model with GUD at a particular station as the dependent variable and rodent species, owls, quadrants, days (nested within owls), and station (nested within quadrants) as the independent variables. The objective of this analysis was to: (1) verify a station effect after accounting for quadrant or aviary-wide effects of the owl and snakes; and (2) determine the main effects of owls to see how they would influence the average "elevation" of the rodents' LOF. A significant station-effect suggests that the variation in perceived risk is spatially dependent as opposed to being explained wholly by the categorical division of risk treatments.

While the station effects may be significant as a whole for all rodent species, we also wanted to compare whether there was a fixed pattern to risk distribution each species associated with each station. Given an expected strong effect to presence and absence of snakes we ranked the level of fear in each of the 12 stations in each quadrant based on mean GUDs (combining owl treatments, microhabitat, moonphase, and the whole 2 months of data collection). The highest GUD, highest risk, was given a rank of 12 and the lowest GUD, safest station, given a rank of 1. We ran a Freidman's test of concordance for each quadrant with species as blocks ($n = 4$) and stations as treatments ($k = 12$). A significant concordance would suggest that the spatial distribution of risk in the arena was not random and that the rodents assessed the physical attributes outside of the experimental manipulations similarly (e.g., walls, fences, structural predator holding pens).

Landscape Shape

We wanted to examine the effect of changing risk on low-risk nights compared to the risk on high-risk nights. To do so, we regressed the GUD at each station on nights when owls were present in contrast with nights when owls were absent. A strong correlation would suggest that the LOF does not change qualitatively, but the features remain the same, and an increase in risk "elevation," i.e., a higher mean GUD, would reflect increased risk (see Laundré et al., 2010). Low correlation and flat slopes would suggest that the landscape features are redrawn based on the changed predator community.

LOF Maps

A visualization of the data was run by creating raster-maps using a smoothing function of distance, weighted least squares (DWLS) with the default tension of 0.5 (see Iribarren and Kotler, 2012). A map was created for each combination of owl treatments by rodent species for a total of eight maps.

RESULTS

The random forest analysis produced a model with training and test risk estimates of 0.47 ± 0.02 and 0.52 ± 0.3 respectively. The decision tree highlights the importance of the rodent species (importance rank 1.0), followed by the presence of owls [0.1], and both the microhabitat and snakes at the lower end [0.07]. The model offers a clear split between the importance of variables in the foraging decisions of heteromyids and gerbils (**Figure 2**, **Supplementary Material, Appendix I**). The heteromyids rank the predators, both snakes and owls as greater importance than habitat heterogeneity in the form of microhabitat. On the other hand, gerbils are sensitive to landscape variations and rank snakes as the lowest impact.

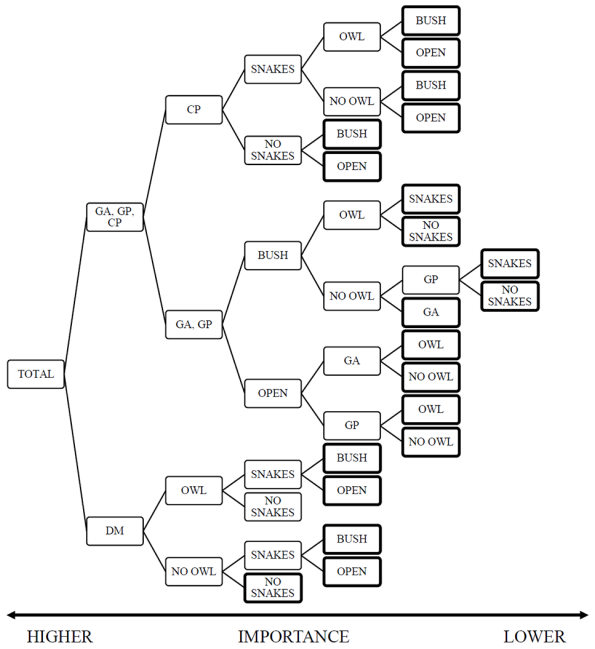

FIGURE 2 | Random-Forest Decision-Tree with Giving-up Density (GUD) as the dependent variable and species, snake-treatment owl and microhabitat as the independent variables. The figure is read from left to right with greater value to the initial nodes (left) than to final nodes marked with a bold outline. This analysis highlights the difference between the rodent families, where gerbils rank microhabitat as a greater importance than heteromyids. The heteromyids diverge in their focal predator, kangaroo rats ranking owls as a greater contributing factor to their foraging and pocket mice responding to snakes first. The data structure for this tree (**Supplementary Table S1**) provides the sample size, mean GUD and variance for each node).

The general linear model (N = 2,688, R^2 = 0.537) found that for each species, GUDs differed significantly among stations (**Table 1**). All species combined responded with preferences for the bush over the open microhabitat, preferred the control over the quadrant with snakes, and foraged more on nights without owl presence. The model also found a significant difference among species' foraging tenacity and for each species an interaction of owl presence with microhabitat and owl presence with snake treatment (quadrant). We do not offer in-depth examination here of these differences as they distract from the main purpose of this article, and are the basis for a number of articles published separately (Bleicher, 2014; Bleicher et al., 2016, 2018a; Kotler et al., 2016).

The average GUD, or mean "elevation" of the LOF, differed significantly for each species, as can be seen by the main effect of species on GUDs. The mean landscape elevation was similar for both gerbil species, GA and GP, with mean GUDs of 2.02 ± 0.05 g and 2.29 ± 0.04 g, respectively (**Figures 3E–H**). The mean elevation for the LOF of the heteromyid rodents, CP and DM, were at opposite extremes; low for DM with a mean of 0.71 ± 0.02 g and high for CP with a mean GUD of 2.50 ± 0.02 g (**Figures 3A–D**).

Overall, the rodents showed concordance in their perception of risk within each quadrant (**Table 2**). The overall pattern of distribution of risk increased towards the barrier between the quadrants likely as a result of permeability of the risk from snakes moving along the hardware cloth fencing (**Supplementary Material, Appendix II**).

To determine whether the LOF of the different species rises or falls with the risk of owl predation, we ran a regression analysis of mean GUD at a station when owls were present vs. when owls were absent. A positive slope with a tight correlation around the regression would show that the overall ranking of stations remains the same with and without owls; i.e., the LOF retains its shape with owls (**Table 3**, **Figure 4**). The heteromyids (kangaroo rat and pocket mouse) showed an increase in elevation of the LOF with owls and a tight relationship, suggesting little change in the topography of their respective LOFs. In contrast, the regressions for the two gerbils were non-significant. So while points on the landscape tended to rise with owls (a positive slope), these changes were not consistent (very low R^2) as stations on the landscape perceived as safer without owls were often perceived as the most dangerous with owls.

TABLE 1 | ANOVA comparing Giving-up Densities (GUDs) as testing for a station effect and effects of owl presence snake treatments and microhabitat on patch use combined for all four rodents (N = 2, 688, R^2 = 0.537).

Source	Type III SS	*df*	MS	*F*-Ratio	*p*-Value
LONGITUDE (X)	0.224	1	0.224	0.442	0.506
LATITUDE (Y)	1.886	1	1.886	3.732	0.053
Y × X	2.614	1	2.614	5.173	0.023
SPECIES × X × Y	22.717	3	7.572	14.983	0.000
OWL	68.992	1	68.992	136.506	0.000
MICROHABITAT	20.355	1	20.355	40.273	0.000
SPECIES	537.083	3	179.028	354.221	0.000
SNAKE	9.392	1	9.392	18.583	0.000
MICROHABITAT × OWL	0.567	1	0.567	1.122	0.290
SPECIES × OWL	5.195	3	1.732	3.426	0.016
SPECIES × MICROHABITAT × OWL	4.912	3	1.637	3.239	0.021
SNAKE × SPECIES × OWL	4.965	3	1.655	3.275	0.020
SNAKE × SPECIES × MICROHABITAT	2.453	3	0.818	1.618	0.183
Error	1,345.409	2,662	0.505		

Abbreviations: SS, sum of squares; df, degrees of freedom; MS, mean squares; p-Value, Probability of Type I error; owl, barn owl presence; species, rodent species; snakes, snake treatments (control or both snakes present in quadrant).

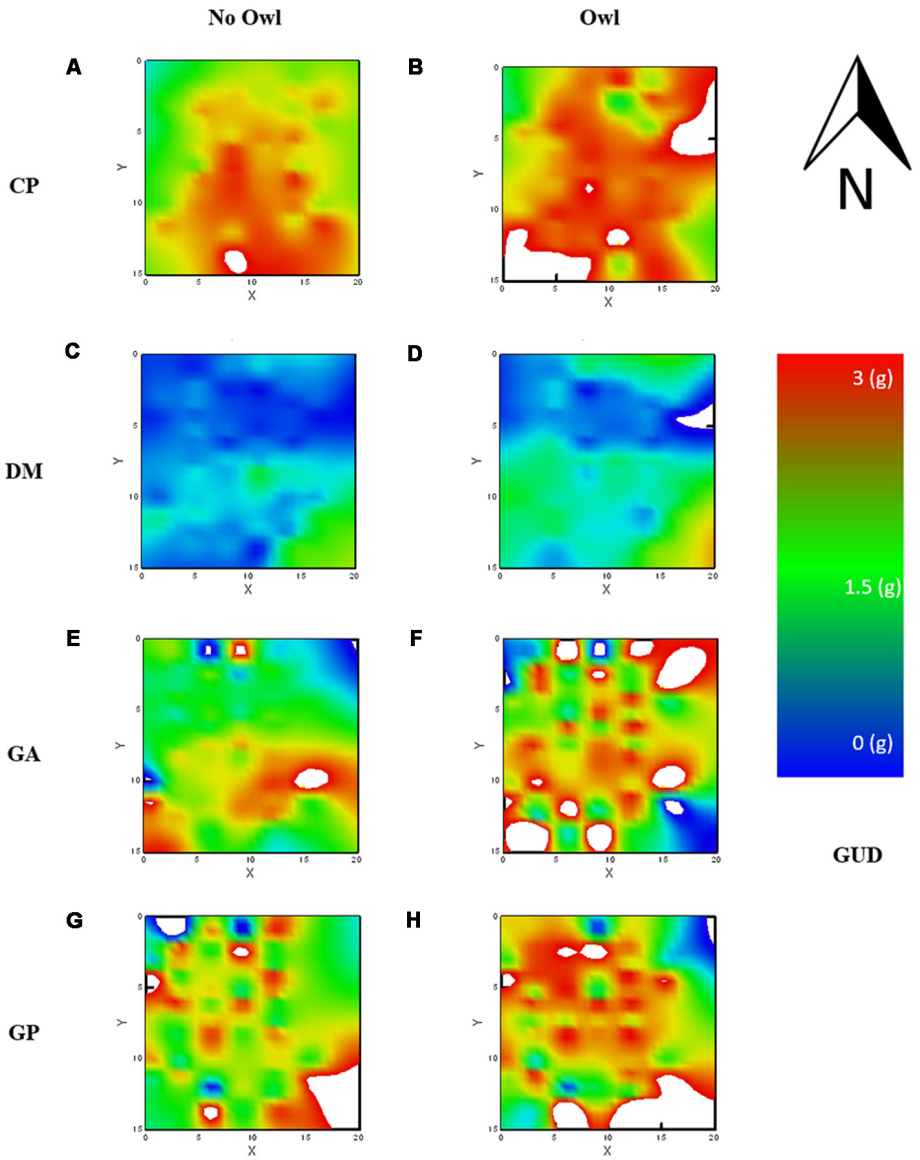

FIGURE 3 | Landscape of fear (LOF) charted using GUDs and using a distance, weighted least squares (DWLS) smoothing function to generate the raster. Warm colors [red (3 g)—yellow (2 g)] reflect perceived danger (high GUDs) and cold colors [blue (0 g)—green (1 g)] relative safety (low GUDs). Each chart is identified by its location along species rows and columns of without and with owl presence, for CP (**A,B**) respectively, for DM (**C,D**) respectively, for GA (**E,F**), respectively, and for GP (**G,H**) respectively. *Dipodomys merriami* (DM) showed relatively weak risk perception regardless of owl presence, *Chaetodipus penicillatus* (CP) showed the strongest risk perception regardless of owl presence, and both GA and GP showed stronger risk perception when owls were present.

DISCUSSION

This series of experiments applied the LOF, not as a theoretical model of spatial avoidance (Laundré et al., 2001), but as a measurable property of tradeoffs perceived by a population. The analyses we performed show that while the physical convergence is strong between the species, there appears to be a distinct pattern of divergence in the way heteromyids and gerbils comprehend the variations in risk based in the types of predators present and is generally distinct from the elements in the physical landscape.

We observed a degree of concordance between the four rodents which suggest that despite significant differences, all rodents perceived, or "understood," the distribution of risk in the vivarium in a similar manner. We specifically found an abhorrence towards the central divider between the quadrants. The most striking of the observed differences was a clear differentiation between heteromyid and gerbilline rodents in the way the presence of the barn owl affected the shape and elevation of their LOFs.

Below, we compare and contrast the LOFs of the four species; discuss results with regards to rodent body size and

TABLE 2 | Freidman's tests of concordance by quadrant (or snake treatment).

Quadrant	X_f^2	*p*-Value	*W*
North (Control)	160.77	<0.001	3.65
South (Snakes)	164.79	<0.001	3.75

TABLE 3 | Regression analyses for each species as a function of the GUD per station with and without an owl effect.

Species	Linear regression equation	R^2
C. penicillatus	y = 0.7046x + 0.9331	0.547
D. merriami	y = 1.1887x + 0.1518	0.807
G. andersoni allenbyi	y = 0.1825x + 1.9078	0.078
G. pyramidum	y = 0.412x + 1.574	0.117

taxonomic affiliation/continent of origin; and discuss the results with regards to the predator community.

(1) Did all species exhibit similar LOFs across the various conditions?

Landscape features may drive a common response in different species, as in the example of both elk and bison in Yellowstone avoiding waterways due to risk from wolf predation (Laundré et al., 2001). Features more likely to affect small mammal risk-perception may involve blocked sight lines (Embar et al., 2011) as in the case of gerbils in the same experimental vivarium and striped mice in South Africa (Abu Baker and Brown, 2010) avoiding habitat with thick vegetation cover. In these experiments, the rodents are clearly responding to the interaction of cues of predators as they interact with boundaries to movement, the partition between the quadrants. Not unlike the Yellowstone elk responding to the wolf risk by avoiding waterways, all rodents here avoid the fences when snake tracks and odors are present—suggestive of a combined effect of direct predatory cues and environmental information.

(2) Were similarities in the LOF most striking for species of similar body size, or ones originating from the same desert?

Our results did not show consistency between the patterns found in the LOFs of rodents within the same size classes nor within a family. Some patterns were replicated in different species, likely a result of similarities in behavioral traits.

(A) Size classes

In the small size class (GA and CP), the two species exhibited vastly different LOFs predominantly as a result of the height of the landscape (strongly evident in the random-forest). CP showed a high-elevation landscape (nearly unforaged), and the LOF for GA was of median magnitude (1/3 to half of the resources harvested). CP perceived the majority of the vivarium as risky, only willing to forage around the outer boundary of the vivarium. In contrast, the GA LOF was relatively "flat" and moderately safe in elevation (GUDs ∼2 g) and gradually rose towards risky "peaks" (see Laundré et al., 2010), or islands (see Abu Baker and Brown, 2010), in the landscape (**Figure 1B**).

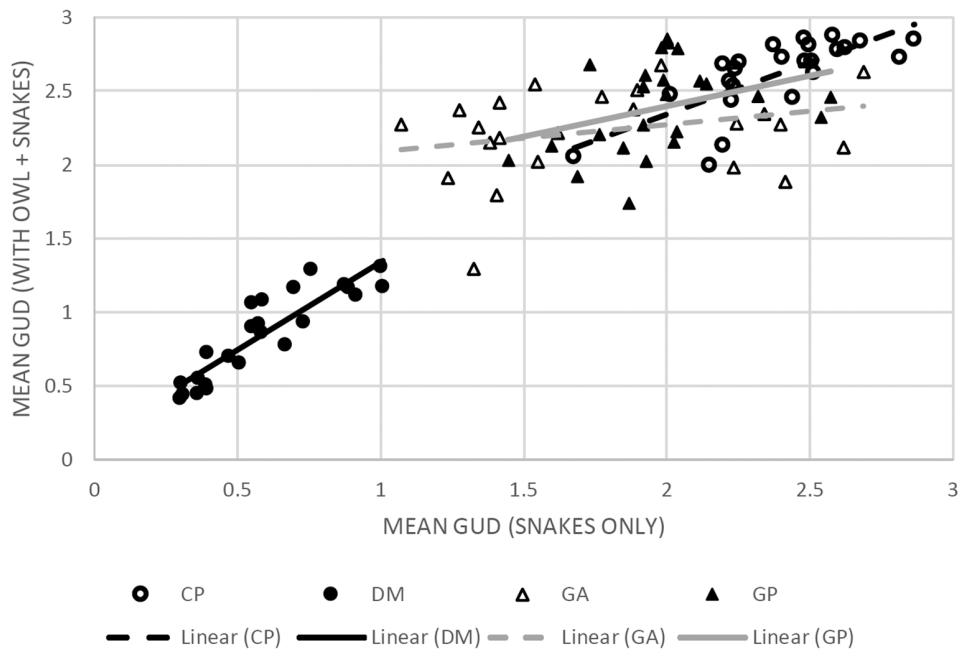

FIGURE 4 | Scatter plots with linear regression lines for each of the four species correlating the mean GUD at each of the 24 stations as collected on nights with owls (*y*-axis) and without (*x*-axis). The (●) markers and black trendlines represent heteromyid rodents and (▲) and gray trendlines mark gerbilline species. The solid markers and solid trend lines represent the larger species (DM and GP, respectively) and the empty markers and dashed trend-lines represent the smaller species (CP and GA, respectively). The corresponding statistics are available in **Table 2**. The figure shows that when owls are present the level of risk perceived increases in the heteromyid rodents (CP, DM) while maintaining a relatively similar spatial distribution of risk. Meanwhile, the wide dispersions of values and minimal slope in the gerbils suggest that the shape of the LOFs of each species varies in risk distribution under each of the predation conditions.

Presumably, this is a consequence of the trellises where snakes were ambushing and open microhabitat patches where they were most vulnerable to owls.

Why do the effects of the risky features in the CP LOF attenuate so gradually across the entire landscape, while they are more restricted in the GA LOF? We believe the answers are attached to the specific adaptation of the pocket mice to climb into the branches of Creosote thereby escaping their predators (Rosenzweig, 1973). Our experiment, mimicking a bush with a branch covered trellis means the bushes do not offer escape paths in the way a natural occurring bush would when escaping snakes. If we were to put ourselves in the eye of a pocket mouse, this would mean that the only safe escape available to us is unreachable. Pocket mice, as opposed to kangaroo rats, do not have the powerful hind legs that allow them to jump out of harm's way. With our trellis set-up designed to provide the maximum shelter from owls and foxes (see Embar et al., 2011) the similarity in GUDs under the bushes and in the open also suggests that the risk the pocket mice perceived from the snakes ambushing under the trellises and the risk from owls in the open was at least of equal value.

GAs, in comparison, take risks and forage under less favorable conditions. They forage on semi-stabilized dunes (Abramsky et al., 1990) and pick the "crumbs" left by stronger competitors (Kotler et al., 1993a). Both these behavioral patterns come at increased energetic and predation costs. To manage that risk they increase vigilance (Linder, 1988; Dall et al., 2001; Kotler et al., 2002). These behavioral adaptations result in an increase in GUDs across the landscape when risk is high, but also allow GAs to exploit more patches than CPs when risk is low. For CPs, their behavioral patterns observed in the wild suggest high selectivity towards safe habitats (Lemen and Rosenzweig, 1978; Brown et al., 1988) also reflected in a less diverse diet (Davidson et al., 1980). The CPs ability to enter torpor (Hayden and Lindberg, 1970) may also assist them in avoiding conflict and allows them to reduce movement in the environment when the conditions are not optimal. The relatively flat landscape suggests a limited dispersion of CPs even when the conditions were safer without the owls. These opposing strategies highlight the role of competition in these communities. The interplay of space, different anti-predator adaptations, and temporal use allow species in each community to co-exist with their close competitors. The LOF appears to support this pattern of behavior, a flat landscape when low risk from snakes is present, but turning to a spotty higher elevation (risky) map similar to GPs when the owls were flown in the vivarium.

The larger rodents exhibit substantially different LOFs both in their elevations and in "topographic" attributes. DM exhibit a flat landscape similar to its smaller family member, but as opposed to that of CP, that landscape is safe (low GUDs) and the safe areas are not centered on the edges. GPs showed a highly intricate weave of safe and risky patches, showing high sensitivity to variations in risk in the landscape (**Figure 3**).

With regard to its foraging patterns, GPs are regularly described as cream skimmers (Brown et al., 1997), meaning that they use their high harvest rates (small handling times) and low cost of changing patches (often due to fast locomotion) to move from patch to patch, discovering the richest patches sooner, and exploiting them when they are richest. This pattern was expressed beautifully in the LOF heat maps, which maintained an islands-of-fear pattern of localized risk even under low-risk conditions. This is when high harvest capacity within a short harvest time is most valuable. They move more, discover patches sooner, harvest them when they are richest, quit at high GUDs and move on in search of another rich patch especially at the time at which the environment is most dangerous (Dall et al., 2001; Kotler et al., 2002). The high sensitivity to risk, and where they are most likely to be ambushed, resulted in a LOF with a pattern similar to the one described by Abu Baker and Brown (2010) where striped mice avoid predation by genets in shrubs, creating a map of small islands of risk surrounded by safety zones.

In our system, when the level of risk increased in the environment (nights with an owl as well), GP altered the pattern of perceived risky zones, now predominantly the open microhabitat exposed to the owl and specific bushes fraught with high snake activity. This new landscape exhibits a focus on refuge identification as is best exemplified by the stark contrast between the LOF on nights when an owl was present and when the owl was not present (**Figure 3**).

The LOF of the kangaroo rats (DM) is flat and low, this suggests little fear. The management of predation risk in kangaroo rats (other *Dipodomys* species) is well documented and includes the following: high auditory acuity (Webster, 1961, 1962; Webster and Webster, 1971, 1979; Webster and Strother, 1972), foot drumming (Randall, 1997, 2001), kicking sand towards the predator (Bouskila, 1995), enlarged hind limbs (Biewener and Blickhan, 1988) and an ability to select safe habitats (Brown, 1988; Randall and Boltas King, 2001). Combined, these provide for a tremendous escape ability (Whitford et al., 2017). It is in this context (low risk) that the entire landscape slightly rises in response to the owl; overall, a less "dramatic" change than for the three other species.

(b) Intra-Continental Comparison

As indicated previously, the patterns of the four species were quite different from each other. Thus, even within the same rodent lineages (gerbilline and heteromyid) the way by which risk is assessed may not be inherited from their common ancestor, it appears to be more plastic and species-specific. Still, some general similarities exist within families. The plasticity in both gerbil species' LOFs suggests the effects of predators was not cumulative. Heteromyids, on the other hand, exhibited fixed-shape landscapes that rose and fell based on the intensity of risk of predation.

Using a macroevolutionary lens, these behavioral patterns offer a glimpse into the evolution of desert systems on both continents. The behavioral similarities in the response of the gerbils, i.e., the reorganization of the LOF, suggests the important role that owls took in shaping the granivorous rodent community through sensitivity to those predator cues (Bleicher, 2012; Bleicher et al., 2018b), foraging strategies (Kotler et al., 1993a; Embar et al., 2011) and temporal variability (Kotler et al., 1993b). When owls were present, they take the brunt of the attention.

In contrast, the heteromyid rodents decreased foraging slightly on the nights when predation risk by owls was added, even in the areas with snakes absent (**Figure 3**). We hypothesize that sharing an evolutionary history with snakes that have heat-sensing ability drives a baseline of risk on which any combination of predators has a cumulative impact and demands the basic anti-predator awareness. Thirteen species of rattlesnakes call the Great Basin deserts home, and all of these possess the infra-red sensory ability (Fowlie, 1965). In comparison, there are only five vipers in the Negev and the Sahara, and they all are limited in their hunting on moonless nights (Joger, 1984). The high diversity of lethal predators in North America suggests the pressure to manage the risk from snakes has been a lot more important in the evolution of heteromyids. From that importance stems their sensitivity and acuity to the presence and activity patterns of the snakes they encounter. This also suggests that the adaptations, both physical and behavioral, for managing the risk from snakes would be a lot more extreme, but benefit evasion from all predators (see Webster, 1962). With the species we studied, the kangaroo rats are able to take the risk due to the number of physical adaptations they have to manage the risk (as mentioned before) but none as effective as bipedal locomotion—allowing for reverse locomotion (Randall, 2001). Pocket mice, on the other hand, use a combination of habitat selection (dense vegetation) and when conditions are not favorable they have been observed to enter a torpor state to mitigate the energy loss resulting in limited foraging (Hayden and Lindberg, 1970).

(3) Did owls cause changes only in the elevation of the LOF or in its shape as well?

The answer to this question is not as straight forward as the analysis would suggest. As stated previously, the patterns of behavior are not consistent between species of the same size class, nor between species sharing evolutionary history within the same desert system. The spatial changes in risk in gerbilline species based on the predator community suggest completely new LOFs. The change in heteromyid LOFs predominantly exhibited an elevation rise; however, the rise only occurred at the risky stations, causing the landscape to fold and increase in rugosity. From the four examples this research provides, we can only draw a general conclusion—the species-specific adaptations characterize the manner in which the LOF changes in response to varying risk conditions. To conclude, spatial patterns of anti-predator behaviors are a useful tool to compare species response in manipulative experiments. They reveal behavioral patterns that address the assessment of risk and its interaction with environmental attributes in the landscape. These behavioral patterns provide insight into driving forces (ecological and macroevolutionary) that explain the interactions between (and within) trophic levels.

We have demonstrated that the accumulation of predation risk from multiple predators can change the LOF, the way species assess risk in their environment, in one of two major fashions: (1) A LOF will change in topographical features, specifically in species that assess the risk in the environment based on the highest risk factor as was exemplified by the gerbil species; and (2) a LOF can rise and fall, specifically in species where the response to predators is spatially fixed and risk is cumulative.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

ETHICS STATEMENT

The permits for this project were obtained from Ben Gurion University of the Negev Ethics in Animal Research Committee (Permit IL-73-11-2009) and animal shipping, handling and experimentation permits from the Israel Nature and National Parks Authority (INPA) permits (2011/38131 and 2012/12524). This is a publication 1015 of the Mitrani Department for Desert Ecology.

AUTHOR CONTRIBUTIONS

The data collection, analysis and main drafting of the manuscript was preformed by SB. JB and BK filled advisory rolls, were significantly involved in the experimental design, edited the manuscript and were responsible for the funding.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2019.00058/full#supplementary-material

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Squirrels Do the Math: Flight Trajectories in Eastern Gray Squirrels (*Sciurus carolinensis*)

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Animals are under strong selective pressures to make correct decisions when attempting to escape an approaching predator, and not surprisingly many studies have shown that animals adjust their flight initiation behavior in response to risk. However, we have a poor understanding of animals' capability to select an appropriate flight trajectory. We investigated whether eastern gray squirrels would adjust their flight trajectory based on the relative locations of the squirrel, the approaching threat, and potential refuges. We used a person running toward a focal squirrel ($N = 122$) as the threat and considered the three trees nearest the squirrel and taller than 8 m to be potential refuges. Squirrels were strongly affected by the angle (θ) formed by the locations of person, squirrel, and the three nearest trees. A squirrel was less likely to run to the nearest tree (Tree 1) when θ_1 was relatively acute, but also less likely to run to Tree 1 when θ_2 was obtuse, making Tree 2 a more attractive refuge. A squirrel was more likely to run to Tree 1 if it was close and if Tree 2 was relatively far. Subtle differences in the effects of θ_1 vs. θ_2 on squirrel refuge choice support the idea that squirrels prefer a nearby refuge. Squirrels were more likely to select Trees 2 and 3 rather than Tree 1 only when θ_2 was obtuse (105°). In contrast, most squirrels chose to run to Tree 1 when θ_1 was >65°; thus squirrels were more likely to choose Tree 1 even when doing so required running at least partly toward the approaching threat. The decisions made by focal squirrels provide evidence that this species' assessment of risk is highly nuanced. A great deal of variation has been reported in responses to predators within species. While part of the variation may be due to strategic unpredictability on the part of the prey, part of it may also be due to differences in flight trajectory and refuge preferences that have not been well-studied.

Keywords: flight initiation, flight trajectory, refuge, escape angle, squirrels

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INTRODUCTION

Fleeing is a common antipredator behavior across a wide range of animal taxa. When a potential predator is approaching, a prey animal must quickly determine both when to flee and which direction to go. A large body of literature on optimal escape theory has established that the decision of when to flee can reflect both an individual's ability to assess risk and the level of risk it is willing to accept. The decision of when to flee is typically measured as flight initiation distance (FID), i.e., the distance between the potential prey and an approaching predator at the moment the prey flees. Varied factors influence FID, including those related to the cost vs. benefits of flight (e.g., food availability or being engaged in interactions with conspecifics), characteristics and behavior

of the threat (predator size, gaze, and approach speed), characteristics of the prey (e.g., body size; Blumstein, 2006; Fernandez-Juricic et al., 2006) and habitat or location effects such as distance from refuge, position of the predator relative to a refuge, or amount of cover (Blumstein, 2003; Stankowich and Blumstein, 2005; Samia et al., 2016). In addition, urban populations of a species often have lower FIDs than rural ones (Møller, 2015; Møller et al., 2015).

Flight trajectories have been less studied in the field than FID, but animals similarly appear to make adaptive decisions about the direction in which they flee, as would be predicted given the high fitness cost of choosing incorrectly. In the lab, these decisions have been shown to be constrained by sensory and motor limitations [reviewed in (Domenici et al., 2011a,b)], and they may also be constrained by the advantages of alignment with a geomagnetic field in some species (Obleser et al., 2016). In both laboratory experiments and field studies, individuals from a wide range of taxa usually move relatively directly away from an approaching threat, thus maximizing or nearly maximizing their distance from approaching predators (Domenici et al., 2011a; Cooper, 2016a). However, the exact direction of flight relative to a threat varies within and across species, an inconsistency that may be a strategic unpredictability to decrease a predator's ability to predict prey response (Arnott et al., 1999; Domenici et al., 2011a). For example, in the Trinidadian stream frog *Mannophryne trinitatis* and two treefrog species (*Trachycephalus venulosus* and *Hypsiboas geographicus*), escape angles were generally predictable yet still highly variable; they jumped away from lateral or caudal stimuli but used a broad range of escape angles, and there was no directionality to their trajectory when approached frontally (Royan et al., 2010). Larval zebrafish (*Danio rerio*) used a mix of random and direct tactics when fleeing depending on the approach direction of the predator model; a zebrafish maximized distance from a threat when approached from the side but used random escape angles when approached by a threat in line with its heading, i.e., directly toward the head or tail (Nair et al., 2017). Further, some species vary their responses according to predator taxon. For example, Túngara frogs (*Engystomops pustulosus*) flee away from snake models but toward models of bats, as they seek to undercut the bat's flight path. Other exceptions to the general pattern occur in species that similarly try to undercut an approaching predator's path. Moths are most likely to escape pursuing bats when they decrease the escape angle, turning to move toward or perpendicularly to the bat when the bat is close and thus making it difficult for the bat to adjust its pursuit trajectory to reach the prey (Corcoran and Conner, 2016). Columbian black-tailed deer also preferentially selected acute escape angles when fleeing a nearby threat (Stankowich and Coss, 2007).

The presence of a refuge can also have a large effect on flight trajectories, and in some species, the location of the refuge is the primary determinant of flight trajectory (Hemmi and Pfeil, 2010). For example, broad-headed skinks (*Eumeces laticeps*) typically fled toward the nearest refuge regardless of their location relative to an approaching threat (Cooper, 1997), and side-blotched lizards (*Uta stansburiana*) fleeing from a model snake fled in a random direction with respect to the predator but toward a refuge (cliff) if it was nearby (Zani et al., 2009). Similarly, Mongolian gerbils (*Meriones unguiculatus*) in laboratory experiment tended to flee to the nearest refuge regardless of the position of a visual stimulus meant to induce escape behavior (Ellard, 1993). However, in some species the position of an approaching threat relative to a refuge can influence flight trajectory in at least some circumstances. Blue crabs (*Callinectes sapidus*) in the intertidal zone use the deeper water offshore as a refuge, and they will flee away from a human approaching from the shore in a direction that maximizes their distance offshore before the person will intercept them, although they flee generally toward the person if that person approaches from the sea (Woodbury, 1986). Thus, for these crabs the location of the approaching human can strongly alter escape trajectory, but only when the person is not blocking the path to the refuge. In staghorn sculpin (*Leptocottus armatus*), the location of a simulated aerial attack influenced flight trajectory. The sculpin fled toward a refuge at a 90° angle from the stimulus if they were already facing that direction. The sculpin also went toward a refuge placed so that they could move directly away from the stimulus to reach it, but their flight trajectories were random if they had to move toward the stimulus to reach a refuge or to turn around to reach a refuge at a 90° angle from the stimulus (Shi et al., 2017).

When a prey species typically has multiple refuges available, selecting one is likely to be a complex problem, and one that must be quickly solved when a predator is rapidly approaching. In this study, we investigated how eastern gray squirrels (*Sciurus carolinensis*) select among available refuges when approached rapidly by a human in parks or park-like settings. These squirrels often forage on the ground and run to a nearby tree as a refuge if a potential predator approaches. Although an older study that focused on flight initiation distance suggested that squirrels select the nearest tree as a refuge (Dill and Houtman, 1989), our casual observations suggested that this was not always the case. Previous studies of marmots (Kramer and Bonenfant, 1997) demonstrated that flight initiation distance increased when a prey has to move toward an approaching predator to reach a refuge, suggesting that prey perceive moving in the direction of a predator to be risky. This conclusion implies that refuge choice should be influenced not only by distance to refuge but also by the relative locations of prey, predator, and potential refuges, a prediction that was supported by recent theoretical models (Cooper, 2016b; Cooper et al., 2018). Cooper (2016a) consider a case in which one refuge was available and predicted that flight initiation distance would increase sigmoidally as distance to refuge increased, as predator approach speed increased, and as a potential prey was forced to run more directly toward an approaching threat to reach the refuge. (Cooper et al., 2018) modeled a situation in which threatened prey chose between two refuges. Based on the assumption that shorter flight initiation distances would be preferred, they predicted that prey would not always choose the nearer refuge but would be more likely to flee to the farther refuge if their path to the nearer refuge would take them more directly toward the approaching predator.

We tested whether eastern gray squirrels preferred the nearest tree as refuge vs. one of the two next-nearest trees and whether

refuge preference was affected by escape angle, i.e., the angle between the path of an approaching threat and the flight trajectories to the three possible refuges. Vulnerable animals like squirrels should minimize predation risk, so they should choose the nearest refuge to minimize costly flight distance, shorten flight initiation distance, and reduce exposure to predators during flight. However, the safety of the path to the nearest refuge may be compromised if it brings the squirrel closer to the predator. Based on this hypothesis, we predicted that squirrels would be more likely to select the nearest refuge but that this preference would be altered if other refuges were at similar distances and could be reached by moving away from the approaching threat. We also examined whether flight initiation distance was influenced by squirrel distance to refuge, escape angle, and the distance between person and squirrel at the start of the person's approach (starting distance). Both starting distance and distance from refuge affect flight initiation distance across a wide range of taxa, with FID typically increasing when starting distance and distance from refuge are longer (Stankowich and Blumstein, 2005).

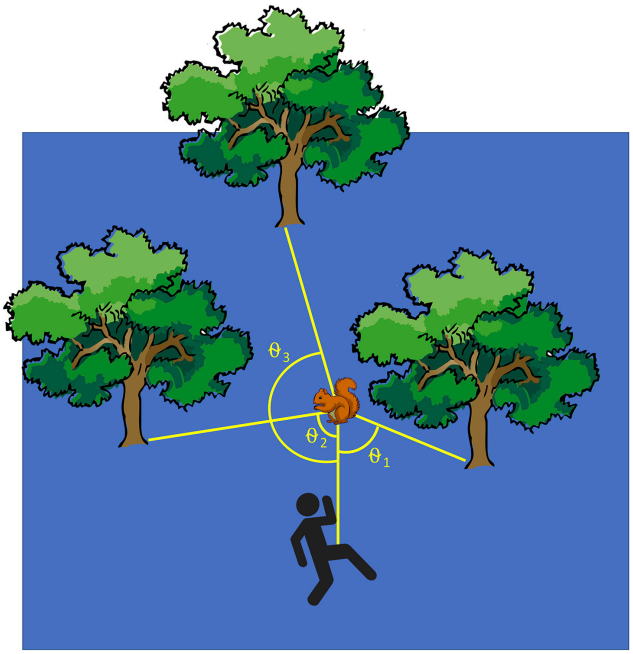

FIGURE 1 | The closest tree to the squirrel is denoted Tree 1 and the farthest is Tree 3. θ indicates the escape angle, i.e., the angle from person to squirrel to a tree.

METHODS

We conducted trials to investigate the flight behavior of eastern gray squirrels from September to November in 2014 and 2016 in Louisville, Kentucky. To maintain consistency in squirrels' activity levels, we conducted trials only from late afternoon until dusk and when the temperature was at least 11°C. Each trial met the following criteria: (1) the focal squirrel was foraging on the ground and not evidently responding to our presence, i.e., not alert, and not looking at us or sitting up on its hind legs; (2) the squirrel was at least 2 m from the nearest tree; (3) there were no squirrels within 15 m of the focal squirrel; and (4) there were a minimum of three trees within 25 m of the squirrel that were suitable refuges. The definition of suitable refuges was based on observations of squirrels' flight behavior before trials began; included trees were at least 8 m in height and had a trunk diameter >0.3 m. When these criteria were met, one person then sprinted at full speed toward the position of the focal squirrel from a distance of at least 10 m while the other two collaborators noted the locations of the focal squirrel when the runner started, when the squirrel became alert, and when it initiated flight. We marked the starting point of the runner and the squirrel locations with rocks immediately after the squirrel fled. The two collaborators stood approximately 3–4 m apart on opposite sides of the runner at the starting location to facilitate accurately marking the focal squirrel's locations. We also used small landmarks, e.g., fallen leaves, bare spots, chunks of bark, and sticks, to pinpoint each squirrel's locations. The runner simulated a potential threat of attack by a predator. Flat rocks were dropped by the runner to mark the locations of the runner when the squirrel alerted and when it fled. Focal squirrels were not within 3 m of any path, and the runner did not start from or follow any path toward the squirrel on any trial because varied species (Mainini et al., 1993; Miller et al., 2001; Eason et al., 2006), including *Sciurus carolinensis* (Bateman and Fleming, 2014), view humans approaching on a path as a lesser threat than humans approaching but not following a path. The mean running speed of the person was 3.1 ± 0.01 (SE) m/sec, which was calculated from 5 sprints of 20 m that were 10 min apart and not part of the trials.

We recorded the distance in meters from the squirrel to the runner at the start of the trial, the alert distance, and flight initiation distance, using a tape measure to measure all distances to the nearest 10 centimeters. We also recorded the distances between the squirrel and the three nearest trees and between the runner and each of those same trees at the moment the squirrel became alert. The nearest tree to the squirrel was defined as Tree 1, the second closest as Tree 2, and the farthest as Tree 3. A sighting compass was used to record the compass direction to each of the three trees from the positions of both the squirrel and the runner, as well as the direction the squirrel was facing. The tree directions were then standardized so that the starting position of runner was always placed at 0°. The standardized directions were used to calculate escape angle, i.e., the angle (θ) between the person, squirrel, and tree for each tree (θ_1, θ_2, and θ_3; **Figure 1**) at the moment the squirrel became alert. Angles were calculated on a 180° scale, with a θ of 180° representing a squirrel that ran directly away from the runner, and a θ of 0° representing a squirrel that ran directly toward the runner.

The squirrels in our trials were not individually identifiable, and accordingly we took measures to reduce the probability of re-sampling individuals. In 2014, we conducted trials on grassy lawns at five sites: the University of Louisville campus ($N = 32$; 21 ha) and four urban parks, including Central Park ($N = 10$; 7

ha), George Rogers Clark Park ($N = 11$; 19 ha), Shawnee Park ($N = 5$; 128 ha), and Tom Sawyer Park ($N = 7$; 23 ha). In 2016 we performed trials only at University of Louisville ($N = 30$) and Central Park ($N = 27$), the sites that had the highest densities of squirrels. Based on a mark-recapture study from 2010–2013, the number of squirrels on the University of Louisville campus was estimated as 434 ± 46 (±SE; William Persons, pers. com.). At all sites we moved systematically from one side of the site to the other to reduce the probability of re-sampling. At both University of Louisville campus (2014 and 2016) and Central Park (2016), we performed trials over two consecutive days, sampling approximately half the site each day. Approximately 40% of the squirrels we observed in 2014 had ear tags, but in 2016 we saw only two individuals with tags and did not include any tagged individuals in trials. We conducted a total of 122 trials, and all trials were combined for analyses. In two additional trials, which are not included in the above counts or in analyses, one focal squirrel chose the fourth nearest tree and the other chose the fifth nearest tree.

Our alternative hypothesis predicted that squirrels would flee to the closest tree, and accordingly we analyzed squirrel refuge choice as either "Chose Tree 1" or "Did not choose Tree 1." For statistical regression analyses we used generalized linear models (glm) in R Core Team (2016). We performed a logistic regression to assess squirrel refuge choice. We were most interested in the effects of squirrel and person position relative to the potential refuges. Accordingly, the following explanatory variables and all their interactions were initially included in the logistic regression on squirrel refuge choice: all squirrel distances to trees, person distance to Tree 1, and θ for each of the three trees. In addition, we included the main effects of the following explanatory variables with no interaction terms: flight initiation distance, starting distance, and alert distance. We could not include the interaction terms for this second set of variables because the model became overfitted. We could not include the person distances to Trees 2 and 3 even as main effects because they were co-linear with person distance to Tree 1. Of these three variables, we chose to include person distance to Tree 1 because our study design focused on whether the squirrel selected Tree 1 as a refuge or not. Alert distance and FID were linearly correlated ($r = 0.82$) so we used a model comparison approach to determine which of these two variables to include. The initial model with alert distance had a lower AIC (150.05) than did the initial model with FID (157.65), so we performed backward elimination using the model containing alert distance.

We performed a linear regression to determine the factors that influenced flight initiation distance, with the explanatory variables squirrel distance to refuge and θ_{refuge} (the escape angle for the tree chosen by a fleeing squirrel) as linear terms and the explanatory variable starting distance as the quadratic term. In a separate analysis, to determine whether refuge choice was independent of the direction a squirrel was facing when they fled, we performed a Chi square goodness-of-fit test on the number of times squirrels chose the tree they were most nearly oriented toward, the tree they were second closest to facing, and the tree they were oriented farthest away from at the time of the threat. All means are given ± SE.

RESULTS

Out of 122 trials, 60 squirrels chose Tree 1 as a refuge and 62 did not, with 45 choosing Tree 2 and 17 choosing Tree 3. Squirrel mean distances to the three potential refuges were 5.3 ± 0.18 m (Tree 1), 8.8 ± 0.23 m (Tree 2), and 12.4 ± 0.29 m (Tree 3). Refuge choice was independent of the direction squirrels were facing (Chi square goodness-of-fit test: $X^2 = 3.26$, $N = 122$, $p = 0.20$). Person distance to Tree 1, starting distance, alert distance, and θ_3 did not have significant main or interaction effects on squirrel refuge choice and were dropped from the logistic regression model. The final model had an AIC value of 132.79 (see **Supplementary Table 1**).

Both θ_1 and θ_2 had significant main effects, but no significant interactions. As θ_1 increased, the probability of the squirrel choosing Tree 1 also increased ($p < 0.0001$; **Figure 2A**). For θ_1, the escape angle at which squirrels were equally likely to choose Tree 1 or a more distant tree was 65°; in other words, at $\theta_1 = 65°$ the probability that a squirrel would choose Tree 1 as a refuge was 0.5. When $\theta_1 \leq 65°$, only 25% of squirrels chose Tree 1 as a refuge ($N = 60$; **Figure 2B**), but when $\theta_1 > 65°$, 73% of squirrels ($N = 62$) chose Tree 1, the nearest refuge. As θ_2 increased, i.e., as the trajectory toward Tree 2 allowed the squirrel to run away from rather than toward the approaching threat to reach that refuge, the probability of the squirrel choosing Tree 1 decreased ($p = 0.002$; **Figure 3A**). For θ_2, the angle at which squirrels were equally likely to choose either Tree 1 or a more distant tree was 105° (**Figure 3B**). When $\theta_2 \leq 105°$, 67% of squirrels ($N = 58$) chose Tree 1 as a refuge, but only 33% of squirrels ($N = 64$) chose Tree 1 when $\theta_2 > 105°$. Squirrel distance to Tree 2 interacted with squirrel distance to Tree 1 ($p = 0.01$; **Figure 4**) and marginally interacted with squirrel distance to Tree 3 ($p = 0.066$; **Figure 5**). Squirrels were more likely to choose Tree 1 when close to it and far from Tree 2, and when very far from both Tree 2 and Tree 3.

Flight initiation distance ranged from 1.6–18.8 m (mean FID = 9.2 ± 0.33 m). In the linear regression to determine which factors affected FID, squirrel distance to refuge and θ_{refuge} did not have significant effects on FID and were dropped from the model. Flight initiation distance had a significant quadratic relationship with starting distance ($p = 0.002$; **Figure 6**), increasing as starting distance increased up to approximately 25 m and then declining (see **Supplementary Table 1**).

DISCUSSION

Squirrels used complex criteria to choose a refuge when fleeing from an approaching threat. A squirrel incorporated the spatial relationships among itself, the approaching threat, and the potential refuges in its flight trajectory decision. Approximately half (49%) of focal squirrels chose the nearest refuge, with 37% of the squirrels fleeing to the next-closest tree and 14% choosing the farthest of the three nearest trees. The escape angles for the two nearest refuges (θ_1 and θ_2) both had significant main effects on squirrel refuge choice (**Figures 2**, **3**), with squirrels generally preferring larger escape angles so that they would be moving less directly toward the approaching threat. Relative proximity to refuges was also a factor in their decisions: a squirrel was

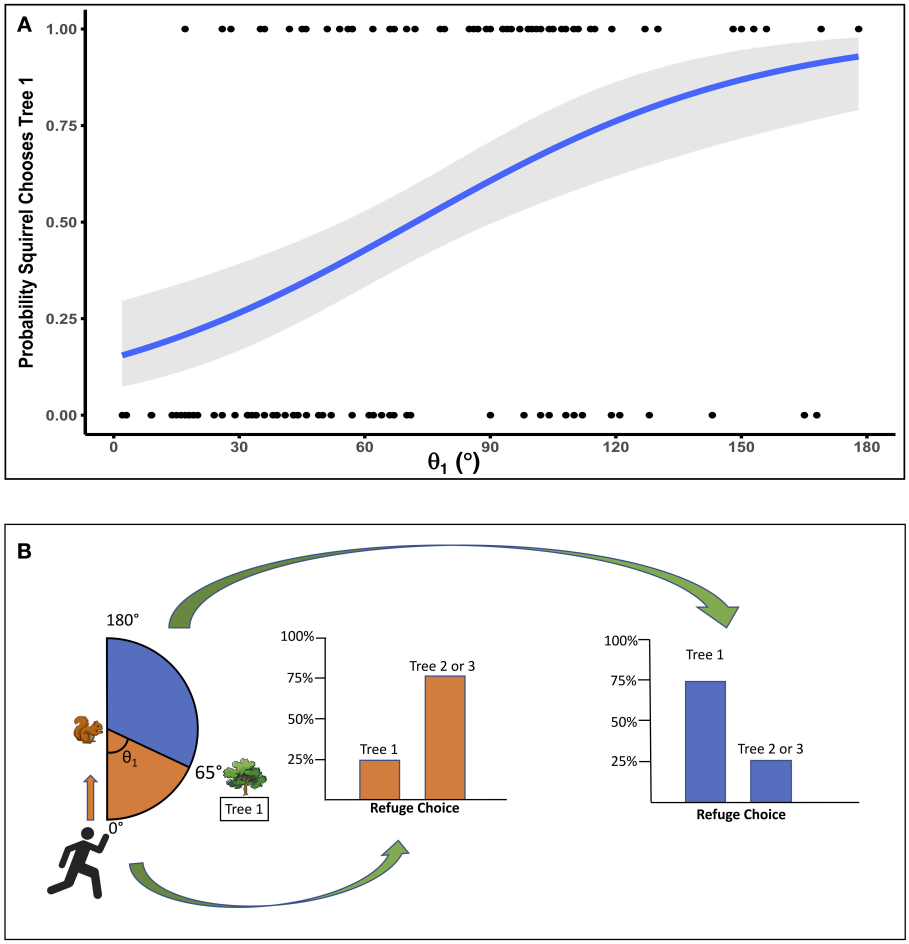

FIGURE 2 | (A) The probability that a squirrel will choose Tree 1 increases as the escape angle to Tree 1 (θ_1) increases, so that the squirrel runs more directly away from the approaching threat to reach that refuge. The probability of a squirrel choosing Tree 1 is 0.5 when θ_1 is 65°. Gray area indicates SE, and dots show squirrel refuge choices, with a value of 1 for a squirrel that chose Tree 1 and a value of 0 for a squirrel that chose Tree 2 or Tree 3. **(B)** Squirrels were less likely to select the nearest tree (Tree 1) as a refuge when θ_1 was relatively acute, meaning they had to run in the general direction of the approaching person to reach that tree. When Tree 1 angle (θ_1) was $\leq$ 65°, 25% of squirrels chose Tree 1 and 75% chose Tree 2 or 3. When θ_1 was $>$ 65°, 73% of squirrels chose Tree 1 and 27% chose Tree 2 or 3.

more likely to choose the nearest refuge (Tree 1) if both of the other two refuges were very far away (**Figure 5**), or if the squirrel was close to Tree 1 and far from Tree 2 (**Figure 4**). Subtle differences in the effects of escape angles for Tree 1 vs. Tree 2 on squirrel refuge choice also support the idea that squirrels prefer a nearby refuge. Squirrels were more likely to select one of the two more distant refuges rather than the nearest refuge only when the escape angle for Tree 2 (θ_2) was obtuse (105°). In contrast, most squirrels chose to run to Tree 1 when its escape angle was >65°; thus squirrels were more likely to choose Tree 1 even when doing so required running at least partly in the direction of the approaching threat. Taken together, these results suggest that proximity of a refuge can modulate the negative effects of a relatively poor escape angle and that a more favorable escape angle can compensate for greater distance to a refuge. This finding provides general support for a prediction in the theoretical model of (Cooper et al., 2018), which addressed the problem of choosing between two refuges and predicted that a fleeing prey would select the more distant refuge if the trajectory to that refuge was more away from the predator and the trajectory to the nearer refuge.

In some studies, flight trajectory has appeared to be random with respect to the approaching threat, behavior that has been explained as creating unpredictability (Arnott et al., 1999; Domenici et al., 2011a), or possibly due to microhabitat differences, where differences in trajectory in different habitats confound overall conclusions (Martín and Lopez, 2000). Most previous studies have suggested that animals are reluctant to run toward an approaching threat (Domenici et al., 2011a; Cooper, 2016a), as was the case in this study for refuges that were farther away. The common preference for obtuse escape angles could be due to a general aversion to moving toward a predator, but may also reflect the greater probability of being cut off from a refuge by the predator if a prey were moving toward both predator and refuge. In contrast, individuals of some species will take an acute escape angle when a predator is very near, undercutting

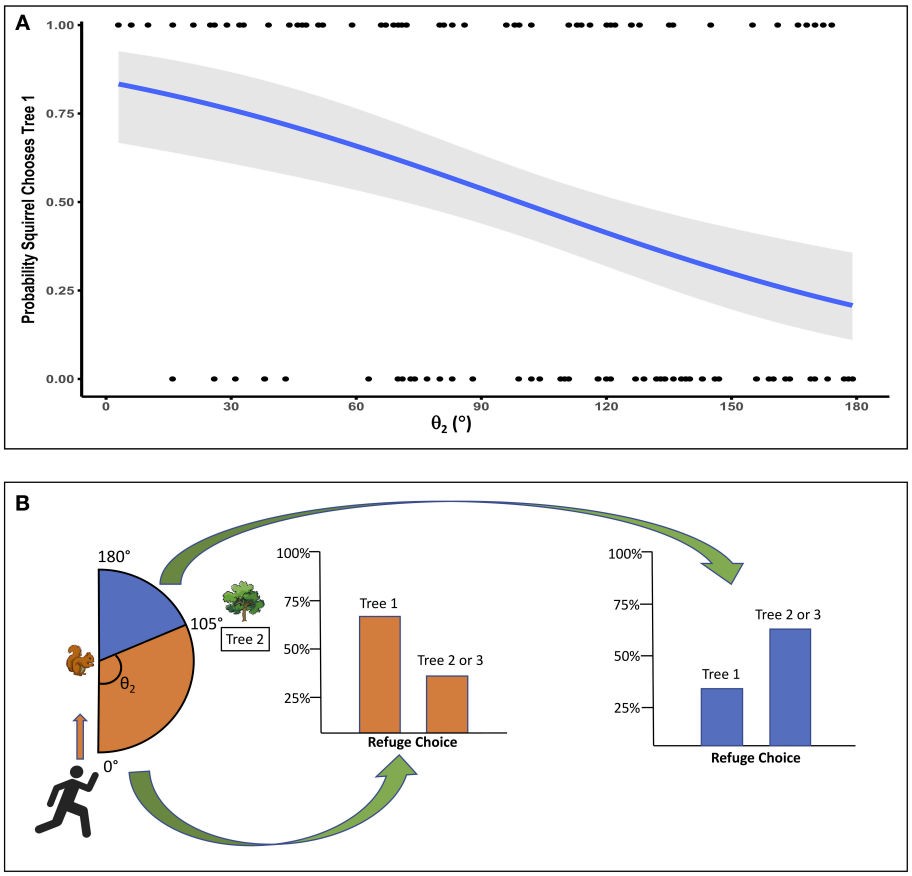

FIGURE 3 | (A) As θ_2 increases, Tree 2 becomes a more attractive (less risky) refuge, and the probability that a squirrel will choose Tree 1 decreases. However, a relatively large angle for Tree 2 ($\theta_2 \geq 105°$) is required to make the probability of choosing Tree 1 ≤ 0.5. Gray area indicates SE, and dots show squirrel refuge choices, with a value of 1 for a squirrel that chose Tree 1 and a value of 0 for a squirrel that chose Tree 2 or Tree 3. **(B)** Squirrels were less likely to select Tree 1 as a refuge when θ_2 was relatively large, making the flight trajectory to Tree 2 less risky. When Tree 2 angle (θ_2) was $\leq 105°$, 67% of squirrels chose Tree 1 and 33% chose Tree 2 or 3. When θ_2 was $> 105°$, 33% of squirrels chose Tree 1 and 67% chose Tree 2 or 3.

an approaching predator when that predator is unlikely to be able to change direction swiftly enough to capture the prey (Corcoran and Conner, 2016) or causing the predator to make energetically costly changes in direction to be able to follow the prey (Stankowich and Coss, 2007) However, to reach the nearest refuge, squirrels in this study sometimes preferred a trajectory that took them closer to the threat. The density of refuges is also likely to affect escape behavior. Based on our results, we expect that escape angle and distance to refuge would both influence flight trajectory when multiple refuges are close enough to the prey to be suitable refuges and the distances between the prey and the refuges differ enough to be readily distinguishable. However, if refuges are at high densities and thus very near one another, a threatened prey may be unable to determine which refuge is closest or may not need to flee to the closest refuge because travel time is short and differs only slightly among nearby refuges. In this scenario, prey may prioritize flight trajectory over flight distance, more frequently choosing the refuge most directly away from the threat. If refuges are far apart, prey may tend to remain relatively near a refuge and more frequently choose that closest refuge even when the escape angle to reach it is relatively poor, forcing the prey to move at least indirectly toward an approaching threat.

For many species FID is positively correlated with distance from refuge (Cooper and Wilson, 2007), and in some species, variation in indirect risk (distance from cover) is more important to risk-assessment than variation in direct risk [distance from humans; white-browed sparrow-weavers (*Plocepasser mahali*), (Fong et al., 2009)]. Thus, while they engage in other activities, animals clearly monitor their distance from potential refuges where the likelihood of discovery or capture by a predator would be lessened. This vigilance suggests that animals are also likely to be aware of the direction they should take to reach that refuge. We do not currently have a good understanding of how many potential refuges members of different species typically track or recognize. In the eastern gray squirrel, that number at our study site and under our experimental conditions appeared to be three, with only two individuals of 124 moving to the fourth or fifth nearest tree. The number of potential refuges an animal considers will obviously depend in part on prey taxon, with sensory and

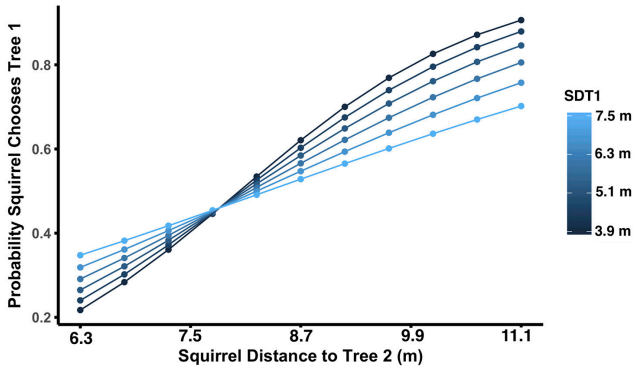

FIGURE 4 | As squirrel distance to Tree 2 increases, the probability of choosing Tree 1 (the nearest tree) as a refuge increases. There is a significant interaction between squirrel distance to Tree 1 (SDT1) and squirrel distance to Tree 2. Lines depict the relationship between squirrel distance to Tree 2 and the probability of choosing Tree 1, with line color indicating the level of squirrel distance to Tree 1. When squirrel distance to Tree 1 is small, squirrel distance to Tree 2 has a stronger effect on the likelihood that the squirrel will choose Tree 1.

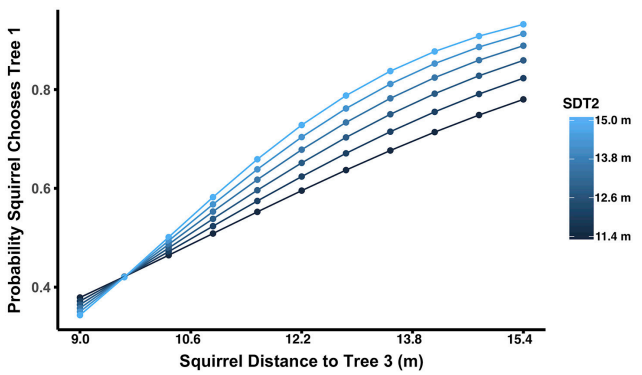

FIGURE 5 | As squirrel distance to Tree 3 increases, the probability of choosing Tree 1 increases. Lines depict the relationship between squirrel distance to Tree 3 and the probability of choosing Tree 1, with line color indicating the level of squirrel distance to Tree 2 (SDT2). When SDT2 is large, squirrel distance to Tree 3 tends to have a stronger effect on the likelihood the squirrel will choose Tree 1, although this was only marginally significant ($p = 0.066$).

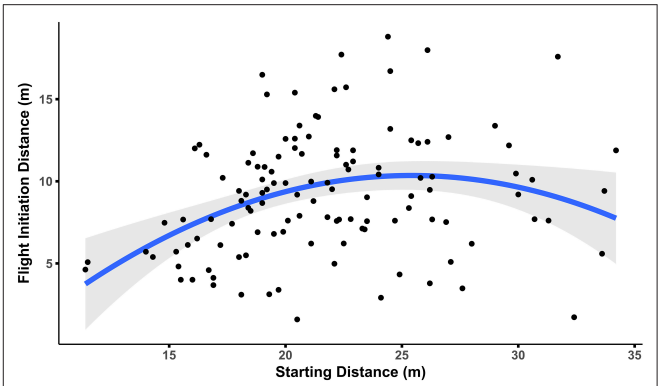

FIGURE 6 | Starting distance has a quadratic relationship with FID. Squirrel FID increases as starting distance increases, but only up to ∼25 m after which FID declines. Gray area indicates SE.

cognitive abilities providing limitations. That number will also depend on refuge availability and the kind of refuges a species or individual uses. Some species will have only one refuge in the vicinity, and thus only need to keep track of the direction of one feature to be able to flee efficiently, and in some cases that refuge may be a large landscape feature, which would facilitate orientation if rapid flight is undertaken. For example, lizards that preferentially flee to a cliff (Zani et al., 2009) or blue crabs that flee to deeper waters (Woodbury, 1986) may need to keep track of only one direction in which to flee in the event a threat arises while they are engaged in foraging or other activities, although of course if that direction is unavailable they need to quickly find alternatives or be aware of them beforehand. Slower approaches by a predator are likely to allow consideration of a greater number of refuges, where they are available, although that very slowness may reduce the need to travel to a refuge other than the nearest.

Starting distance had a significant effect on FID, with squirrels fleeing at greater distances when the person started his approach from a greater distance. Starting distance is commonly positively correlated with FID in vertebrate taxa (Blumstein, 2003; Cooper, 2005; Stankowich and Blumstein, 2005; Stankowich and Coss, 2006; Samia and Blumstein, 2015; Holmern et al., 2016; Evans et al., 2017), although these two metrics are sometimes not correlated in urban habitats (birds: Tätte et al., 2018). This effect of starting distance supports the flush early and avoid the rush (FEAR) hypothesis, which posits that a positive correlation between starting distance and FID exists because waiting can be costly for prey, either because of increased risk or decreased benefits due to the need to monitor the approaching predator (Blumstein, 2003; Cooper and Blumstein, 2014). In this study, however, FID decreased slightly with starting distances greater than about 25 m. This suggests that in this urban population, squirrels do not experience increased risk of capture beyond that starting distance for terrestrial predators they would normally contend with. In addition, these squirrels may not monitor their surroundings for threats beyond that distance (Blumstein, 2003) and may not immediately detect predators when they enter the zone within which the squirrels do monitor threats (Stankowich and Coss, 2006). This delay in detection occurs because the prey animal is not constantly scanning for threats (Stankowich and Coss, 2006). In Columbian black-tailed deer (*Odocoileus hemionus columbianus*), FID decreased at very long starting distances in deer that were not alert to being approached by humans, but in deer that were aware of the approaching threat, FID had a logarithmic relationship with starting distance (Stankowich and Coss, 2006). This result suggests that more wary individuals respond to predators approaching from far away differently than do less reactive individuals and may view such predators as more dangerous (Stankowich and Coss, 2006). As is the case with other species, squirrels in urban environments have moderated responses to humans (Bateman and Fleming, 2014;

Uchida et al., 2016). Less habituated squirrels in rural habitats may have longer monitoring distances where visibility permits, and rural squirrels might also have a logarithmic relationship between FID and starting distance rather than the quadratic relationship found in this study.

Although starting distance influenced FID, squirrel distance to refuge did not have a significant effect. Previous studies have typically found that FID is positively correlated with distance to refuge (e.g., Dill, 1990; Cooper and Wilson, 2007; Guay et al., 2013), as optimal escape theory would predict (Ydenberg and Dill, 1986; Cooper and Frederick, 2007). One meta-analysis concluded that the effect of distance to refuge on FID was ubiquitous across taxa and significant (Stankowich and Blumstein, 2005), and a second found broad support for this relationship across 28 lizard species (Samia et al., 2016). There are some exceptions to this pattern. For example, in the lacertid lizard *Psammodromus algirus*, FID was not correlated with distance to refuge, possibly due to differences in microhabitat and refuge quality (Martín and Lopez, 2000). In our study species, *Sciurus carolinensis*, a positive correlation between FID and distance to refuge was previously found in a study that used a motorized stuffed cat (*Felis domesticus*) as a predator, albeit over a small range of distances to refuge (1–5 m; Dill and Houtman, 1989). However, Engelhardt and Weladji (2011), who examined *S. carolinensis* responses to an approaching human over a wider range of distances to refuge (0.3–26 m), found as we did that distance to refuge did not influence FID in *S. carolinensis*, and the results in Dill and Houtman (1989) suggested that the relationship between distance to refuge and FID may have been logarithmic, with distance to refuge having little effect above 3 m (Bonenfant and Kramer, 1996).

In sum, the decisions by focal squirrels we observed provide evidence that in this species assessment of risk is highly nuanced. It is also potentially resilient to atypical predator behavior; the squirrels in this study made reasonable choices despite the fact that the human threat sprinted at them in a way that does not reflect the majority of their experience with humans, who are a frequent presence in their environment and are usually only moving at walking speed. A great deal of variation has been reported in responses to predators within species. While part of the variation in FID, for example, may be due to strategic unpredictability on the part of the prey, part of it may also be due to differences in flight trajectory and refuge preferences that have not yet been well-studied. Species that use particular sites or objects as refuges provide a particularly rich opportunity for assessing the effects of flight trajectory on other metrics commonly used to assess flight behavior, including FID, and distance fled.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

No animals were captured or touched during this experiment. The Institutional Animal Care and Use Committee of the University of Louisville approved this project.

AUTHOR CONTRIBUTIONS

PE initiated and designed the study, observed and measured squirrel responses, wrote the initial draft of the manuscript, and revised later versions. LN observed and measured squirrel responses, performed statistical analyses, and edited the draft of the manuscript. JA participated in the study design, observed and measured squirrel responses, and edited the draft of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2019.00066/full#supplementary-material

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frontiers in Ecology and Evolution

ORIGINAL RESEARCH
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Multi-Trophic Species Interactions Shape Seascape-Scale Coral Reef Vegetation Patterns

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How species interactions shape habitat structure is a longstanding question in ecology. A curious phenomenon reflecting ecological self-organization around reef habitat structures exists on coral reefs: large-scale (hundreds to hundreds of thousands of m^2) halo-like patterns surrounding patch reefs, i.e., individual coral reefs that are often separated by seagrass or macroalgal meadows. These "halos," long known to occur in various locations worldwide, reflect a distinct band of unvegetated sediments surrounding coral patch reefs. However, the full suite of mechanisms controlling them have never been rigorously explored, perhaps due to the common assumption dating back nearly 50 years that they arise solely from reef-based herbivory patterns shaped by anti-predator behavior. Here we provide empirical evidence from a set of halos within Australia's Great Barrier Reef that risk-averse foraging and a previously unrecognized functional group contribute to halo formation, demonstrating that these halos cannot be explained by any one mechanism in isolation. Our results show that halos are a more complex ecological phenomenon than previously assumed by the majority of studies of halos. Specifically, risk-averse grazing by herbivores is likely a key mechanism behind the formation of halos, as generally assumed, but bioturbators also play a central role. This knowledge furthers our understanding of how small-scale species interactions can structure habitat at landscape scales. These large-scale habitat features are important because they affect at least one important ecosystem function, carbon storage, and potentially others (e.g., biological nutrient transfer). These results also raise the question of whether other self-organized ecological patterns may be more nuanced than is currently assumed. This study capitalizes on recent advances in high resolution satellite imagery accessibility that allow ecologists to measure landscape-scale habitat features nearly everywhere on land and in shallow seas. Our results suggest that halos may hold potential as the basis for a tool for remotely observing ecological interactions and measuring large-scale ecosystem change on coral reefs.

Keywords: coral reef, reef fish, halo, behavior, self-organization, species interactions

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INTRODUCTION

Ecologists have long been fascinated by the diverse ways in which species interactions can shape the habitats in which they live. From early studies in California grasslands (Bartholomew, 1970) to more recent studies of self-organized landscape patterns arising from different ecological processes across terrestrial (Getzin et al., 2016; Tarnita et al., 2017) and marine (Ruiz-Reynés et al., 2017) ecosystems, studies of how interactions among species shape their physical habitat abound. In many of these cases, vegetative patterns in particular result from the interplay of organisms' abundance and/or behavior with existing physical habitat structure. Self-organized patterns deriving from small-scale species interactions have recently been shown to increase ecosystem resilience to perturbations in marine systems (de Paoli et al., 2017). Understanding how species interactions shape their physical environment thus has increasing relevance for ecological systems in the Anthropocene.

A striking example of repeated habitat features over large spatial scales of hundreds to hundreds of thousands of m^2 can be seen from high spatial resolution (in this case, $\sim$2 m pixel^{-1}) aerial and satellite imagery of tropical coasts. These halo-like patterns occur where an absence of seagrass or benthic algae leaves a distinct band of un-vegetated sediments surrounding coral patch reefs. Patch reefs are individual coral reefs that are often separated by seagrass or algal meadows and most often cover spatial extents of tens to hundreds of m^2. These halo-like patterns, long known to occur in a handful of locations (e.g., Randall, 1965; Ogden, 1976) and often referred to as grazing halos (hereafter halos), occur in coral reefs worldwide (E. Madin, pers. obs; Precoda, pers. obs; C. Roelfsema, pers. obs.). However, the exact mechanisms controlling this common tropical and sub-tropical vegetation pattern have never been conclusively established, despite a common assumption dating back nearly 50 years that they arise from risk-averse herbivory (Randall, 1965; Ogden et al., 1973; Armitage and Fourqurean, 2006; Madin et al., 2011).

Halos offer a unique window into understanding how self-organized species interactions around existing habitat features can re-structure habitat at landscape scales (Rietkerk and van de Koppel, 2008). Ogden et al. (1973) pioneering work in the US Virgin Islands, in which halos disappeared after herbivorous sea urchins were experimentally removed, indicated that herbivory was sufficient to form halos and was necessary for their maintenance. Most studies of halos have implicitly or explicitly assumed that herbivory is the direct mechanism causing halos (Ogden et al., 1973; Sweatman and Robertson, 1994; Valentine and Heck, 2005; Armitage and Fourqurean, 2006; Valentine et al., 2007; Madin et al., 2011; Downie et al., 2013; Turgeon et al., 2014) and that predation risk is the indirect mechanism behind halos, i.e., predators create landscapes of risk that maintain spatially-constrained foraging patterns by herbivores (Randall, 1965; Ogden et al., 1973; Macintyre et al., 1987; Valentine et al., 2007; Madin et al., 2011; Downie et al., 2013; Atwood et al., 2018). However, Alevizon (2002) described halos around patch reefs in the Bahamas that had no apparent herbivore assemblage, calling the former assumption into question. These contrasting findings suggest that different systems may have different mechanisms behind halo formation, or that these mechanisms are more complex than has generally been assumed.

Understanding the key mechanisms behind halo formation, and the degree to which halos share common characteristics and mechanisms across systems, could help scientists and managers understand large-scale ecosystem changes on coral reefs. However, in order to take advantage of what halos can tell us about how an ecosystem is operating or potentially changing, we first need clarity on the particular mechanisms at play. Specifically, understanding which functional groups and/or processes lead to these landscape-scale habitat patterns in a given system may allow us to detect or infer ecosystem change if and when halos change in their occurrence and/or size.

To address this need, we used underwater and satellite remote imaging technologies to (1) quantify spatial characteristics of halos around a suite of Indo-Pacific patch reefs and (2) document species presence and interactions within these halos. Our unique approach of applying high-resolution satellite imagery to quantify halo characteristics allows us to do so at scales not accessible with traditional *in-situ* monitoring methods. This approach further provides a proof of concept for using this technology to quantify changes in "footprints" of species interactions on shallow seabed habitat over large spatial scales.

METHODS

Characterizing Halos From Remote Sensing Imagery

Halos were visually identified and manually digitized from high spatial resolution remote sensing images of the sheltered lagoonal habitat ($\sim$27 km^2) adjacent to Heron Island, Australia with pixel size varying between 1 and 4.0 m. These images were acquired as part of ongoing benthic habitat mapping research on Heron Reef (Roelfsema et al., 2018) and included a series of multi-year, sequential remote sensing images of Heron lagoon from satellites Worldview 2/3, Quickbird 2, and Ikonos and an airplane for CASI imagery. A subset of satellite imagery representing lagoonal area was extracted using a boundary previously identified as part of a geomorphic zonation map (Phinn et al., 2012). When halos were present, 24 reefs (including the same 22 patch reefs used for *in-situ* surveys) and their halos were outlined in 11 lagoon satellite images spanning 2002 to 2014. Halos were present in fewer than half of the images for any given reef (**Table S1**). Given the thin, wispy, and often sparse nature of the benthic algae assemblage within Heron lagoon, it is unclear if halos were not present in the years they did not appear in satellite imagery, or rather if the level of background algae was too sparse to enable detection from the imagery. Halo width was calculated by subtracting the radius of a circle with the same area as the reef from the radius of a circle with the area enclosed by the outer edge of the halo as discerned by a single observer for all halos. Further details of halo measurement are in **Supplementary Information**. Reef and halo polygons were measured using QGIS 2.14.8-Essen (QGIS Development Team, 2016).

In-situ Observation of Species Interactions and Algal Patterns

Underwater remote video (i.e., cameras recording in the absence of divers) was used to monitor fish and invertebrate behavior and abundance at 22 patch reefs within the Heron reef lagoon system (**Figure S1**) in May of 2013. This habitat is typically shallower than 5 m and features patch reefs isolated by expanses of bare or algae-covered sand. Benthic algae in Heron lagoon is a mixed-species assemblage, comprised in our study area largely of *Enteromorpha* sp. (synonymized now under *Ulva*; Chlorophyta), *Hincksia* sp. (Phaeophyta), *Cladophora* sp. (Chlorophyta), and benthic micro algae (BMA) mats. Video surveys were conducted during both daytime and dusk/nighttime with GoPro underwater video cameras (www.gopro.com) deployed in a line outward from patch reefs, with an average of 201 min per video (**Figure S1**). To estimate the percent cover of benthic algae at different distances from the reef, benthic quadrats were laid near each camera station and points on the substrate were manually classified from images as sand or algae. **Table S2** provides a summary of the data collected.

After assigning species-level identifications from remote videos (see **Supplementary Information**), fishes were grouped into the following functional categories: (i) herbivores that primarily feed on plant material and detritus, which included all surgeonfishes, rabbitfishes, parrotfishes, chubs, and unicornfishes; (ii) piscivorous top predators that feed mainly on other fishes and have few or no predators themselves, which included all sharks; (iii) piscivorous mesopredators, which included barracuda, jacks/trevally and groupers, that feed mainly on other fishes and are potentially preyed upon by the top predators; and (iv) invertivores that feed largely on benthic invertebrates, disturb sediment and dislodge associated benthic algae through bioturbation, which included grunts (which includes sweetlips), snappers, and emperors. Only macro-invertebrates (sea cucumbers, sea urchins, and cephalopods) and sea turtles that could be reliably identified were censused from our videos and included in our dataset. Sea urchins, turtles, and cephalopods were observed in only a handful of instances, precluding analysis of their occurrence data.

Daytime and dusk/night fish presence (percent of total video seconds) and bite rate (both aggregate and per capita) were calculated for trophic groups and selected genera. Sea cucumbers were counted and their percent of total video seconds recorded.

RESULTS

Characterizing Halos From Remote Sensing Imagery

Halo widths in Heron lagoon were determined in two ways: first, by averaging the halo widths over remotely sensed images at the patch reefs where the surveys were conducted (**Figure 1A**), and second, by averaging benthic algal cover in 2013 from *in-situ* quadrats at the same reefs (**Figure 1B**; details in next section). Our remotely sensed outlines provide a binary metric of halo presence (i.e., an area is either inside or outside of the halo boundary) and indicated that halos at these reefs were generally no larger than ∼15 m wide (**Figure 1A**).

Additionally, a comparison of halo widths and patch reef sizes within the Heron lagoon showed a positive relationship between halo width and patch reef area (**Figure S2**). However, visual inspection of analogous data from other locations globally shows no clear relationship, suggesting that this Heron lagoon reef pattern is not ubiquitous (K. Precoda, unpublished data).

In-situ Observation of Species Interactions and Algal Patterns

Our *in-situ* benthic quadrats across the halos, which provide a metric of spatial changes in benthic algal cover at standardized points up to 30 m from the reef edge (i.e., both within the halo and beyond the halo boundary in the algal meadows; **Figures 1B, S3**), indicated that algal cover increased most dramatically between 15 and 25 m from reef edges. Halo boundaries were observed up to 14 m from reef edges, with a mean of ∼9 m (**Figure 1A**). Together with our remotely sensed data, these data suggest that (a) halo boundaries in this system are visible in satellite imagery where algal percent cover roughly exceeds 10–12%, and (b) algal coverage increases up to (at least) 25 m from reef edges.

Our video surveys showed that most activity by herbivores and large bioturbating fishes occurred at distances shorter than or comparable to typical halo widths in Heron lagoon. Herbivores' mean aggregate bite rate dropped precipitously by about 10 m from the reef [**Figure 1C**; families Acanthuridae, Siganidae, Labridae (Scarinae only; formerly family Scaridae), and Kyphosidae]. Sea cucumbers (class Holothuroidea), which bioturbate the substrate while scavenging for detritus, had higher densities farther from patch reefs and were most commonly found ∼20–25 m from reefs (**Figure 1D**). The large bioturbating sweetlips (family Haemulidae) foraged roughly evenly from the reef edge up to ∼20 m from reefs (**Figure 1E**). Bioturbating emperors (family Lethrinidae) had the highest aggregate bite rate at ∼10 m from reef edges, but foraged at lower rates out to ∼25 m from reefs (**Figure 1F**). Stingrays (family Dasyatidae), shovelnose rays (family Rhinobatidae), and eagle rays (family Myliobatidae) were all occasionally observed. Two of the shovelnose rays in the field of view (FOV) at 12.2–17.8 m were feeding and thus bioturbating the substrate; others were passing and were not observed to bioturbate the substrate (**Figure S4**). Rays were seen passing by at all camera distances from the reef; the three rays that were feeding or resting on the substrate were observed between 7.2 and 17.8 m.

The outer boundary of where herbivores were observed was ∼10 m from patch reef edges, with the vast majority of feeding activity, and highest per-capita bite rate no farther than 5 m (**Figure 1C**).

Large predatory fishes (sharks, family Carcharhinidae; jacks, family Carangidae; and barracudas, family Sphyraenidae) were found at all distances surveyed from patch reefs (i.e., up to 25 m from reef edges) during daytime observations, but their activity was concentrated primarily within ∼5–10 m (**Figures 1G,H**) from the reef's edge. Sharks exhibited a smaller, secondary peak in activity at ∼20–25 m from the reef's edge (**Figure 1G**).

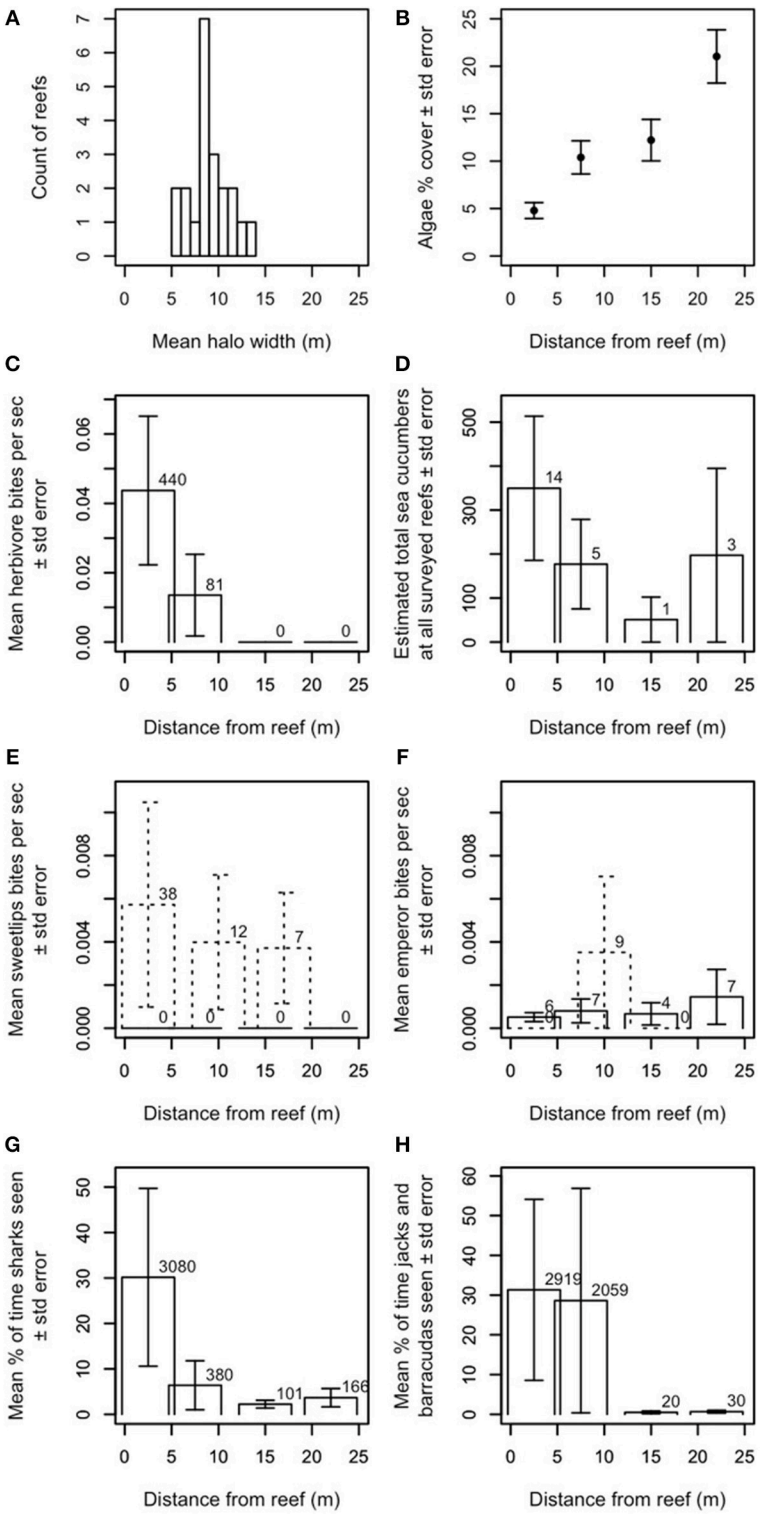

FIGURE 1 | Ecological characteristics of halos and contributors to halo formation. **(A)** Approximate widths of halos at Heron Island, measured from satellite images, and averaged over time. Twenty-one reefs are included because three of the 24 reefs used in the study never had measureable halos in the satellite images. **(B)** Average benthic algal cover in 2013 surrounding the 22 study reefs where *in-situ* data could be collected relative to distance from the reef. **(C)** Mean aggregate bite rate of herbivorous fishes by distance from the reef (number above bar indicates N bites observed). Herbivorous fishes were seen in our dusk/night dataset, but were never observed feeding. **(D)** Estimated total number of sea cucumbers at all surveyed reefs by distance from the reef (number above bar indicates N individuals

(Continued)

FIGURE 1 | observed). Daytime observations are shown. Dusk/night observations yielded too few observations to report statistically (i.e., one sea cucumber in the band from −0.3 to 5.3 m from the edge of the reef and three sea cucumbers in the band from 7.2 to 12.8 m from the edge of the reef). **(E,F)** Mean aggregate bite rate for **(E)** sweetlips and **(F)** emperors, by distance from the reef (number above bar indicates N bites observed). Solid lines indicate daytime observations; dashed lines indicate dusk/night observations. Panels **(C,E–H)** show data averaged over all available (daytime $N = 22$, dusk/night $N = 6$–9) reefs at Heron. All bite rates are calculated relative to the total length of the video recordings at each distance from the reef. **(G,H)** Mean percent of time spent for reef-associated piscivores **(G)** sharks (genera *Carcharinus, Galeocerdo, Negaprion, Triaenodon*) and **(H)** jacks and barracuda (genera *Caranx, Carangoides, Sphyraena*), by distance from the reef. Percents are calculated relative to the total length of the video recordings at each distance from the reef and number above bar indicates N seconds individuals of each group were observed. Only one shark was observed off the reef at dusk/night; no jacks or barracudas were observed off the reef at dusk/night. None of the system's large reef-based piscivores (genera *Plectropomus, Cephalopholis, Gymnothorax*) were seen off the reef during daytime observations or anywhere during dusk/night observations. *Y*-values in panels **(C–H)** are extrapolated to cover entire area at that distance (see **Supplementary Information** for details).

Spatial patterns of foraging away from a central location such as patch reefs could be related to competition among reef-based foragers (e.g., mobile herbivores). Specifically, higher herbivore densities on the reef could necessitate more distant foraging to meet nutritional requirements if sufficient food resources are not present on or within the reef matrix. We therefore examined whether densities of the herbivorous species observed foraging in the halo zone were related to either the maximum excursion distance away from patch reefs (**Figure 2A**) or to halo width (**Figure 2B**), and whether herbivore density increased with increasing reef area (**Figure S5**). In each case, no pattern was apparent. A rank-order, tie-corrected Spearman correlation produced rho = 0.35 (**Figure 2A**) and rho = 0.25 (**Figure 2B**). We used a permutation test (100,000 permutations) to calculate *p*-values and obtained $p = 0.06$ (**Figure 2A**) and $p = 0.15$ (**Figure 2B**), suggesting that while we detected no statistically significant effects of density-dependence (**Figure 2A**), a non-significant trend toward density-dependent foraging herbivore distances may exist. Multiple models confirmed that regardless of which, if any, outliers were excluded from analysis, the slope of the relationship between reef area and herbivore abundance was statistically indistinguishable from zero.

Alternately, foragers' spatially constrained movement could be the consequence of consumption by predators, where foragers that venture farther from the reef's shelter are more likely to be consumed and thus fewer individuals are found in more distant areas. We therefore quantified attacks by predators on prey. We observed no successful attacks by predators on any forager (e.g., herbivore or invertivorous bioturbator) in 395 h of daytime footage (with 311 of these in the halo zone and algal meadow) and 52 h of dusk/night footage.

A third possibility is that predator presence, even in the absence of frequent consumption of prey, is sufficient to constrain foragers' excursions to areas adjacent to and near reef shelter. We therefore examined the relationship between mobile (i.e., non-cryptic) predator presence and aggregate herbivore bite rate (**Figure 3**) and per-capita bite rates by herbivores, sweetlips, and emperors (**Figures 4A–C**) as a function of distance from reef shelter. **Figure 3** suggests that both shark and mesopredator presence may be inversely correlated with aggregate herbivore bite rate and that overall foraging by herbivores may be suppressed when predation risk is higher. Per-capita bite rates by herbivores, sweetlips, and emperors each peaked away from the reef's edge, with herbivores peaking between 4.7 and 10.3 m (**Figure 4A**), sweetlips peaking between 7.2 and 12.8 m (**Figure 4B**), and emperors peaking between 12.2 and 17.8 m from reef's edge (**Figure 4C**).

DISCUSSION

The longstanding question in ecology of how species interactions self-organize to shape habitat structure continues to spark interest across a vast array of ecosystem types (Rietkerk and van de Koppel, 2008). Understanding the interplay between established physical structure (in this case, coral patch reefs), species behaviors and interactions, and vegetative patterns is of particular relevance in the current era of rapid biological and environmental change (Hobbs et al., 2009). This study sheds light on the mechanisms underlying one well-known example of ecological self-organization around reef habitat structures: coral reef halos. We do so by documenting the species abundance, activity, and behavioral relationships at a set of Indo-Pacific patch reefs surrounded by concentric, vegetation-free halo patterns and using remote sensing imagery to quantify halo sizes averaged over time. Collectively, these results provide evidence that individual-level species interactions likely scale up to generate landscape-level patterns in habitat structure on scales visible from high-spatial-resolution (∼2 m pixel size) satellites. By empirically demonstrating that risk-averse herbivory and a novel functional group—bioturbators—can contribute to the formation of halos, these findings shed new light on the prevailing assumption within marine ecology (e.g., Randall, 1965; Ogden et al., 1973) regarding the mechanisms underlying self-organized halos (**Figure 5**). In so doing, this study also highlights the utility of applying high-resolution, emergent imaging technologies toward uncovering answers to classic questions in ecology.

Our finding that herbivores rarely (if ever) forage as far as the halo boundary in our study location, but that bioturbators behave in a manner consistent with halo formation by reaching this boundary, adds a new dimension to a long-assumed suite of species interactions behind these large-scale habitat features. Specifically, bioturbation by sweetlips and emperors may explain the discrepancy in Heron lagoon between the outer boundary of where herbivores were observed (∼10 m from patch reef edges, with the vast majority of feeding activity and highest per-capita bite rate no farther than 5 m; **Figure 1C**) and where some halo boundaries were observed [up to 14 m from reef edges, with a mean of ∼9 m (**Figure 1A**), with increasing algal coverage up to 25 m (**Figure 1B**)]. Since halos were first documented in the Caribbean over 50 years ago (Randall, 1965), most studies have assumed that herbivory was the sole direct species interaction

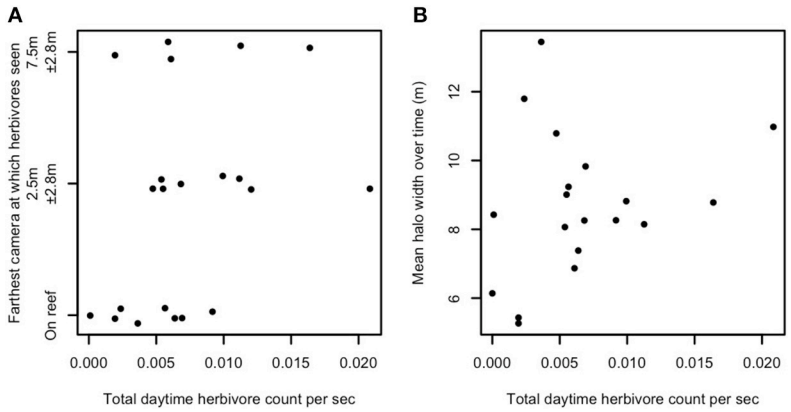

FIGURE 2 | Relationship between herbivore abundance, behavior, and halo width. Herbivore abundance per unit time against **(A)** field of view (FOV) of farthest camera visited by herbivores (vertically jittered for better visibility) and **(B)** time-averaged halo width, in Heron lagoon. Twenty-one patch reefs are shown in **(A)**, as no herbivores were seen on one patch reef which had video cameras. Nineteen patch reefs are shown in **(B)**, as no halos were measured around three patch reefs which had video cameras. Y-axis values in **(A)** represent the range of horizontal distance along the benthos from the edge of each patch reef that was within each camera's FOV. Herbivores in this plot include all species observed feeding in any of the halos (i.e., *Acanthurus* spp., *Siganus* spp., *Zebrasoma veliferum*, and *Naso unicornis*). Abundances are extrapolated to estimate total abundance at each reef.

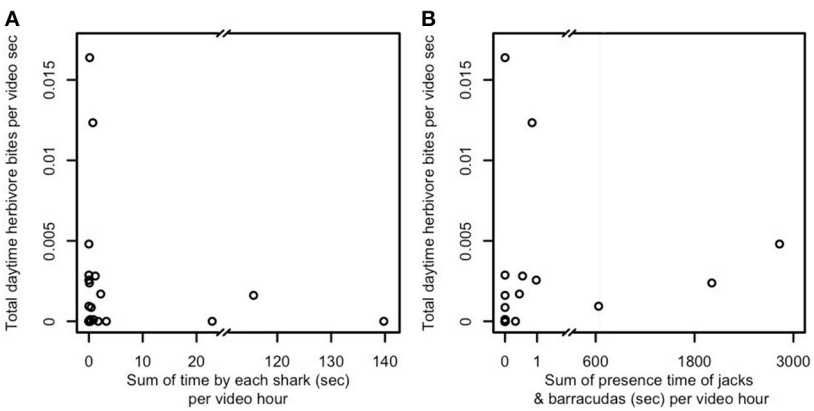

FIGURE 3 | Relationship between predator presence and herbivore bite rate for **(A)** sharks and **(B)** jacks and barracudas. Predator presence is measured as total time predators were observed at the study's 22 focal patch reefs in Heron lagoon (no herbivores were seen on one patch reef; sharks were observed around seven patch reefs and jacks and barracudas around eight). When multiple predators were present, the time each individual was observed was summed to obtain total presence time. Presence time is normalized per hour of video time. Herbivores in this plot include all species observed. Because of the variation of one to three orders of magnitude in predator presence time, because predators were observed at only a minority of patch reefs, and for lack of an a priori model that is sufficiently detailed to explain bite rate observations over such a wide range of predator presence times, we present here the raw data for examination.

underlying them (Ogden et al., 1973; Sweatman and Robertson, 1994; Valentine and Heck, 2005; Armitage and Fourqurean, 2006; Valentine et al., 2007; Madin et al., 2011; Downie et al., 2013; Turgeon et al., 2014), with predation risk assumed to be an indirect species interaction governing herbivore foraging patterns (Randall, 1965; Ogden et al., 1973; Macintyre et al., 1987; Valentine et al., 2007; Madin et al., 2011; Downie et al., 2013; Atwood et al., 2018). Indeed, algal transplant assays conducted elsewhere (Valentine et al., 2008) and within a subset of the patch reefs studied here (Madin et al., 2011) demonstrate that grazers will forage into the halo in a distance-sensitive manner to graze on algae when it is artificially made available, supporting this assumption. Despite this common assumption, however, previous studies have also pointed to a range of other mechanisms as possible drivers of halos, such as local water movement due to current eddies (Steiner and Willette, 2014), sediment deposition (Garrett et al., 1971), incidental bioturbation via refuge-seeking animals (Glynn, 1985), nutrient toxicity from enhanced nutrient levels surrounding patch reefs (Alevizon, 2002), and spatially constrained bioturbation (Alevizon, 2002; Steiner and Willette, 2014). Although these mechanisms are not mutually exclusive, and one or more could theoretically be at work, all but foraging by herbivorous and bioturbating fishes were found to be insufficient in isolation to explain halos found in our and/or other study systems (**Supplementary Information**). To our knowledge, no previous studies have shown bioturbator

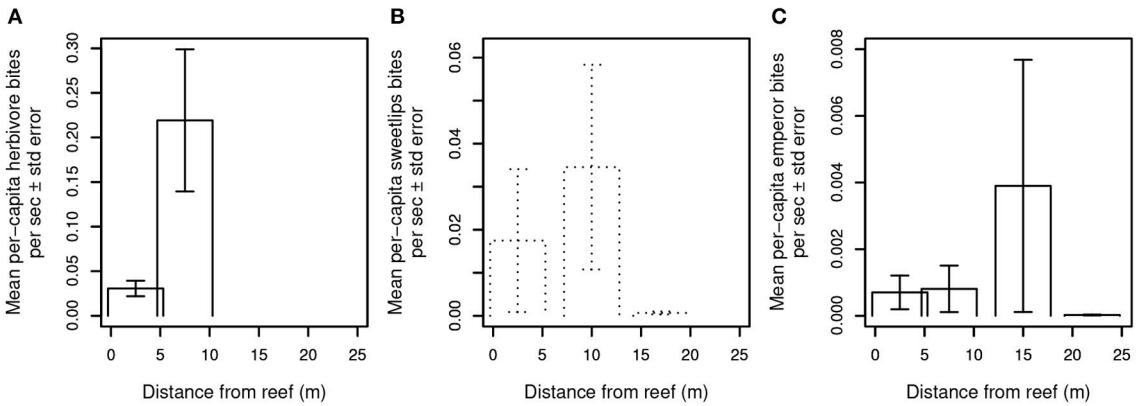

FIGURE 4 | Per capita bite rates of herbivores and bioturbating fishes. Bite rates are calculated per second of video footage for **(A)** herbivores, **(B)** sweetlips, and **(C)** emperors. Daytime observations are indicated with solid lines and dusk/night with dotted lines. No herbivores were observed in the dusk/night videos, and no feeding by sweetlips occurred in the daytime videos. In the dusk/night videos, emperors were seen taking a total of nine bites at the intermediate-distance camera on a single patch reef, and a total of three non-feeding emperors were recorded at the near and far cameras on other reefs.

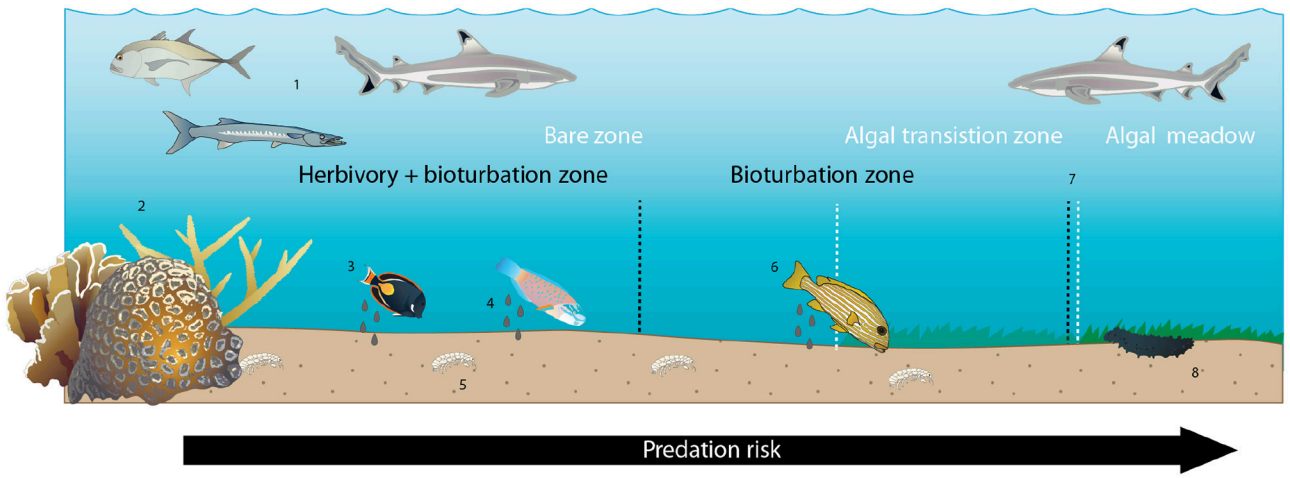

FIGURE 5 | Conceptual diagram of proposed and hypothesized drivers of halos at Heron Island. Zones indicated by white text and dashed lines are benthic algal zones; zones indicated by black text and dashed lines are ecological process zones. Numbers below refer to numbered icons that reflect patterns and processes known, observed in this study, or assumed to occur. (1) Predators generate predation risk through the lagoon seascape. (2) Coral patch reefs provide shelter from predators for smaller-bodied potential prey, creating a gradient of predation risk that increases with distance from patch reef shelter. (3) Herbivorous prey species graze benthic algae in a distance-sensitive manner, preferentially foraging in the immediate vicinity of the patch reef. (4) Foraging fishes defecate where they forage, creating elevated detrital input in the vicinity of the patch reef. (5) Some small benthic and infaunal invertebrates preferentially inhabit areas of higher fish activity, presumably due to elevated detrital resources, though others do not. (6) Larger-bodied bioturbating fishes preferentially forage in the vicinity of the reef, presumably due to elevated infaunal prey surrounding the reef and/or increased predation risk farther from the reef. (7) Declining net herbivory + bioturbation at increasing distances from the reef leads to increasing coverage of benthic algae over this gradient. (8) Larger-bodied scavenging invertebrates (sea cucumbers) preferentially forage near the reef and in the algal meadow beyond the halo boundary, presumably due to elevated faecal- and algal-derived detrital food availability, respectively.

foraging patterns that are consistent with halo formation. Our results suggest that herbivory is a key mechanism in forming halos within Heron lagoon to a maximum of ∼10 m from patch reefs, and that bioturbators are likely key to extending halo boundaries beyond this range (**Figure 5**; see **Supplementary Information**).

Our observations of bioturbating fishes (sweetlips and emperors) foraging in a distance-sensitive manner relative to patch reefs (**Figures 1E,F**) are important for two reasons. First, one of these groups (emperors) is heavily targeted by fisheries within the Great Barrier Reef (Castro-Sanguino et al., 2017), and both are common targets in other reef systems globally, particularly where other fish stocks have been previously reduced by fishing. Removing these groups from an ecosystem may therefore have consequences for habitat structure, specifically formation of halos, and subsequently sedimentary carbon storage (Atwood et al., 2018). Second, using halos as indicators of species interactions, an idea proposed as an alternative to the high cost

and limited feasibility in remote locations of traditional *in-situ* monitoring practices (Madin et al., 2011), will only be possible if we fully understand the suite of interactions and other conditions leading to their occurrence.

In line with the conclusions of recent studies quantifying coral reef predator effects on patterns of prey foraging (Rizzari et al., 2014; Gil et al., 2017; Rasher et al., 2017; Atwood et al., 2018), the most parsimonious explanation for the patterns we document of spatially constrained foraging is that predation risk limits foraging excursions of herbivorous and potentially also invertivorous (bioturbating) fishes, though other mechanisms may also be involved. **Figure 3** suggests that herbivores may collectively forage at lower rates in the presence of large, mobile predators. Spatially-constrained foraging in the presence of predators is consistent with risk allocation theory (Lima and Dill, 1990; Brown, 1999) and is expected under central place foraging (Orians and Pearson, 1979). More specifically, predation risk is known to constrain the upper bound, but not necessarily the lower bound, of risky prey fish behaviors such as foraging (Madin et al., 2010b). This is because prey under low risk are able to engage in risky behaviors, but will not necessarily do so at all times, whereas prey under high risk must balance risk-taking with predator avoidance, leading to a consistently constrained upper bound of foraging distances. The wedge-shaped distribution of points in **Figures 3A,B** is consistent with this expectation. Additionally, herbivores' per-capita bite rate peaked away from the reef's edge between 4.7 and 10.3 m (**Figure 4A**). This finding is consistent with those of both Catano et al. (2016) and Gil et al. (2017) from other coral reef locations that found reduced aggregate herbivory, yet higher individual bite rates, in riskier habitats. Analogous patterns of spatially constrained foraging by herbivores, detritivores, and planktivores in another reef system have been found through a combination of theoretical and empirical work to be the result of predation risk (Madin et al., 2010a,b). None of the possible explanations other than predation risk would be expected to lead to the spatially constrained foraging pattern we observed in herbivores (**Table S3**). Despite the fact that herbivores' potential predators spend greater amounts of time near patch reefs than farther away (**Figures 1G,H**), coinciding with areas frequented by herbivores, this area contains a means to avoid or escape interactions with those predators, i.e., the reef matrix. The lack of physical refuge within the bare halo and the algal meadows beyond, combined with the composition of Heron lagoon's predator assemblage (i.e., including many large, mobile predators) means that although predator encounters in those areas are less frequent, such encounters increase in risk for herbivores the farther they are from the reef refuge (**Figure 5**; Gil et al., 2017). When feeding in sand areas adjacent to patch reefs—where predators are most abundant—herbivores reduce encounter probability with predators through tidally-driven temporal behavioral adjustments (Atwood et al., 2018).

Bioturbating fishes such as emperors and sweetlips are also at greater risk of predation with increasing distance from reef shelter, but to a lesser extent than smaller herbivores (∼25–70 cm adult TL max; smaller for juveniles) given bioturbators' larger (∼85–100 cm TL max) body size. Similar to herbivores, per-capita bite rates for both of these families peaked away from the reef's edge, with sweetlips peaking between 7.2 and 12.8 m (**Figure 4B**) and emperors peaking between 12.2 and 17.8 m from reef's edge (**Figure 4C**). Again, this pattern is consistent with that which would be expected under a gradient of predation risk on coral reefs (Catano et al., 2016; Gil et al., 2017). Specifically, in risky, exposed areas, prey must divide their time between vigilance and taking bites from the substrate (which precludes vigilance), resulting in a higher per capita bite rate due to the need to acquire sufficient energetic resources in a shorter amount of time. However, these patterns may also be explained by changes in the quantity or quality of invertebrates, or by differences in small-scale abiotic factors (such as water motion). Bioturbating sweetlips were observed foraging exclusively at night and emperors foraged during both day and night, whereas large potential predators of these fishes (i.e., sharks) were observed exclusively during daytime. This temporal separation precludes analysis of the relationship between shark presence and these bioturbators' activity, yet it suggests that adaptive temporal niche partitioning may be occurring—a well-known risk-mitigating strategy in marine animals (Heithaus et al., 2012; Rasher et al., 2017; Atwood et al., 2018). It is also possible that bioturbating fishes' spatially constrained foraging may be a response to food distribution or parasite avoidance (**Table S3**). Ollivier et al. (2018) found that overall community composition of benthic (infaunal) invertebrates varied significantly with distance from Heron lagoon patch reef edges, but that taxon-specific abundance patterns were variable and did not indicate clear trends in any particular taxa relative to distance from reefs.

Bioturbating sea cucumbers cannot necessarily detect nor move rapidly away from predators, nor are they likely prey for the dominant fish predators we observed. While sea cucumber diets are known to include both plant/algae and animal-based detrital food sources, stable isotope analyses from another location and species have shown that benthic macro- and micro-algae constitute the bulk of their diets (i.e., 30.9–67.4%; Sun et al., 2013). Algal-derived detrital food availability for these scavengers presumably increases with distance from patch reefs due to the higher standing stock and lower consumption by herbivores of algae farther from patch reefs. Food availability is therefore a likely explanation for sea cucumbers' distribution. Because sea cucumbers move extremely slowly relative to fishes and have relatively low densities per reef in our system, their net effect on algae removal via bioturbation may be more limited than that of more mobile fishes. This may help explain why algal meadows persist in the areas of highest sea cucumber density (**Figure 1**).

In summary, we propose that predation risk is likely a key mechanism leading to the spatially constrained foraging patterns of herbivores and potentially bioturbating fishes that create halos, and that a secondary effect of these patterns is spatially structured sea cucumber foraging (**Figure 5**).

The use of observational data necessarily results in limitations to what can be concluded. For example, attribution of causality based on correlational data alone is difficult, particularly with low samples sizes such as in the case with our dusk/night surveys. However, when considered in conjunction with previous results from the literature and the expectations generated from

competing hypotheses (**Table S3**), our observational data allow us to rule out a number of possible mechanisms (Sagarin and Pauchard, 2010) for the spatial foraging patterns we observed. As with many observational approaches applied to ecological questions on large spatial and temporal scales, these results provide insights that could not be generated from more definitive, yet smaller-scale, manipulative studies (Sagarin and Pauchard, 2010).

Additional research into the suite of species interactions that collectively govern the formation and regulation of halos is necessary to understand this well-known tropical phenomenon. For example, disentangling the relative roles and importance of the various ecological players involved in the species interactions behind halos could potentially be achieved through the collection of both behavioral and species abundance data within "natural experiments," where herbivore and/or predator populations have been altered by human actions such as fishing or marine reserves. Alternatively, large-scale, *in-situ* species manipulations and exclusions would lend valuable insight, though logistical constraints may render such studies impossible at the spatial and temporal scales required in some systems and settings. Where they are possible, however, doing so at multiple locations globally will lend the greatest insight into the mechanisms behind halos and will help us understand their potential as the basis of scientific and conservation tools. Importantly, smaller-scale experiments that generate time-series data on halo creation, and in particular how each functional group contributes to halo formation, would yield valuable insight.

Our finding that multiple types of small-scale species interactions may collectively structure habitat at landscape scales in a self-organized way (Rietkerk and van de Koppel, 2008) suggests that halos could serve as indicators of large-scale change within coral reefs. For example, human activities such as fishing that reduce particular functional groups (e.g., herbivorous or bioturbating fishes) might be expected to indirectly impact halo occurrence and/or width by virtue of reducing their role in halo formation. Such human activities have been shown in other systems to dramatically affect species interactions, in turn altering physical habitat over very large spatial and temporal scales (e.g., the classic killer whale/sea otter/urchin/kelp forest example; Estes et al., 1998). Changes to halo presence or size may also have further ecosystem-level effects. For example, halos may be hypothesized to affect ecosystem processes, such as coral recruitment, in systems where hard-bottom (e.g., "pavement") substrate underlies bare halo zones (e.g., Downie et al., 2013) because herbivory is known to both promote (via reducing algae) and inhibit (via scraping of coral recruits) coral recruitment (Mumby, 2009). Similarly, halos have been shown to affect at least one ecosystem function, sedimentary carbon storage, through the same pathway of predator-prey interactions (Atwood et al., 2018). Further, benthic primary producers play a critical role in a wide range of other ecosystem processes and functions, such as provision of food and habitat for mobile species, stabilization of sediments, and nutrient cycling, among many others. Halos may therefore provide large-scale indicators of how some species densities, species interactions, ecosystem processes, and/or ecosystem functions are changing over space or time. Because of their relatively large spatial scale, remote methods of observation (e.g., satellites, drones, and other aerial tools) will provide a means of doing so that may not be otherwise feasible. The accessibility of these type of remote sensing data sets is rapidly increasing with "cube satellites" revisiting every place on earth daily with 3 m pixel resolution, providing an ideal tool to explore high temporal resolution patterns as well (Thompson et al., 2017). By coupling these new and emerging technologies with behavioral ecology, halos hold potential as a tool for remotely observing ecological interactions and assessing both natural and human-induced ecosystem change now and into the future.

AUTHOR CONTRIBUTIONS

EM conceived the study. EM and AH designed and led field studies. EM, AH, TA, and OL collected field data. OL processed and analyzed video imagery. CR provided satellite imagery. KP analyzed satellite imagery and performed data analyses. KP and EM wrote the first draft of the manuscript. All authors provided intellectual input and contributed to manuscript revisions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2019.00102/full#supplementary-material

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frontiers in Behavioral Neuroscience

MINI REVIEW
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Recent Advancements Surrounding the Role of the Periaqueductal Gray in Predators and Prey

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Recent advances in neural circuitry techniques, like optogenetics and chemogenetics, have allowed for a greater understanding of the periaqueductal gray (PAG) and its importance in predator and prey behaviors. These studies in rodents have highlighted the role of the rostrolateral PAG in hunting behaviors, and have demonstrated functional differences across the dorsal-ventral/rostral-caudal axes of the PAG associated with defensive behaviors. Human imaging studies have further demonstrated that the PAG is active during situations involving imminent threat suggesting that the function of the PAG is likely largely conserved across species. This mini-review article highlights some of the recent advancements towards our understanding of the functional neuroanatomy of the PAG and its importance in the predator and prey behaviors that are critical for survival.

OPEN ACCESS

Keywords: periaqueductal gray, hunting, defensive behavior, freezing, escape, fear

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The periaqueductal gray (PAG) is essential for the expression of both the hunting behaviors performed by predators and the defensive behaviors performed by prey. Anatomically, it is largely bordered dorsally by the superior colliculus, and ventrally by the dorsal raphe (DR) and midbrain reticular nucleus. It can be further sub-divided into four columns arranged around the cerebral aqueduct (dorsomedial, dorsolateral, lateral, ventrolateral; **Figure 1**). These columns can be identified in part by their cytoarchitecture, by the presence of nicotinamide adenine dinucleotide phosphate (NADPH)-positive neurons in the dorsolateral PAG, and by distinct afferent and efferent connections (Vianna and Brandão, 2003). The PAG is sometimes defined in functional terms within the context of its dorsal (including dorsolateral and dorsomedial columns) and ventral (including lateral and ventrolateral columns) regions.

The PAG recognizes and generates appropriate behaviors in response to a variety of aversive stimuli including pain and hypoxia (Lau and Vaughan, 2014; Schenberg et al., 2014) but the focus of this mini-review is its role in predation and prey behaviors. While it is clear that the PAG is critical in producing behaviors in the hunting and the hunted, the involvement of different subareas differs depending on the behavior expressed. For example, in the rat, exposure to a cat results in the highest expression of Fos, an immediate early gene used as a marker of cell activation, in the rostral dorsomedial and dorsolateral PAG, and in the caudal lateral and ventrolateral PAG (Canteras and Goto, 1999; Comoli et al., 2003). This differs from the neural activation resulting from insect predation, where Fos expression is highest in the rostrolateral PAG (Comoli et al., 2003). This mini-review integrates recent circuit-based studies to discuss the role of different sub-areas of the PAG in the predator and prey behaviors which are essential to survival.

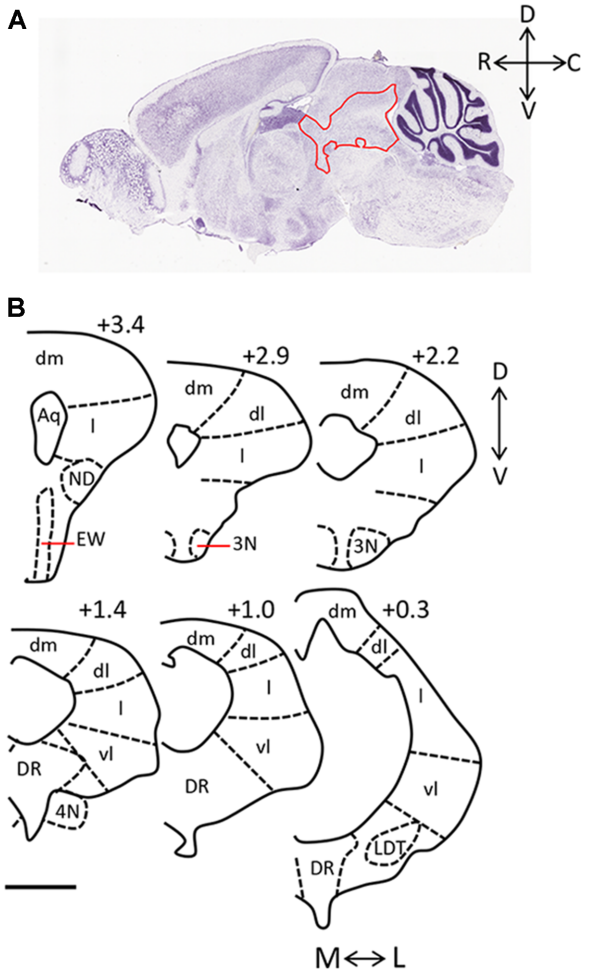

FIGURE 1 | Anatomy of the periaqueductal gray (PAG) in the rodent. **(A)** Sagittal section of mouse brain (Image credit: Allen Mouse Brain Nissl Sagittal Atlas, Image 21, 2004). The PAG is outlined in red and rostral (R), caudal (C), dorsal (D), and ventral (V) axes are indicated. **(B)** Camera lucida drawings of coronal sections taken through the rat PAG, with the dorsomedial (dm), dorsolateral (dl), lateral (l) and ventrolateral (vl) columns labeled (adapted from Comoli et al., 2003, with permission from Elsevier). Location of each section relative to the interaural line is included (+3.4, +2.9, +2.2, +1.4, +1.0, 0.3), and the medial (M)—lateral (L), and D-V axes are indicated. Scale bar, 1 mm. Abbreviations: 3N, oculomotor nucleus; 4N, trochlear nucleus; Aq, aqueduct; dl, dorsolateral; dm, dorsomedial; DR, dorsal raphe; EW, Edinger-Westphal nucleus; l, lateral; LDT, laterodorsal tegmental nucleus; vl, ventrolateral.

THE ROLE OF THE PAG IN PREDATORY HUNTING

The rostrolateral PAG, most often defined at the level of the oculomotor nucleus, has emerged as an area that is critical for predatory behaviors in cats, rats and mice (Shaikh et al., 1987; Sukikara et al., 2006; Mota-Ortiz et al., 2012; Tulogdi et al., 2015). Lesions of the rostrolateral PAG in male rats greatly reduce the incidents of attacking or chasing insect prey (Mota-Ortiz et al., 2012). Furthermore, the rostrolateral PAG plays a critical role in the switch from maternal behaviors to predatory hunting which occurs in morphine-sensitized dams that have been further exposed to low doses of morphine (Sukikara et al., 2006). Morphine-treated dams will ignore their pups, and instead will increase their predatory behavior towards insects, but morphine-treated dams with lesions of the rostrolateral PAG perform reduced insect hunting and more maternal care. This suggests that the rostrolateral PAG is an important part of an opioid-dependent, potentially adaptive response, which can be recruited in times when food sources are limited. The rostrolateral PAG has also been implicated in predatory aggression, unrelated to a food source, in experiments comparing neural activation in muricidal rats with non-muricidal rats (Tulogdi et al., 2015). Rats who have committed muricide display a shift in brain activation towards ventral areas compared to dorsal areas of the PAG, and this occurs, particularly in the rostral and not caudal areas. Overall, these findings are part of a growing number of studies describing the PAG as a brain area able to integrate information concerning different motivational states to express appropriate adaptive behaviors.

Studies suggest that the role of the PAG in predatory behavior may be associated with a more general role for the PAG in reward processing. A recent study using a $GABA_A$ agonist to inactivate the rostral lateral and dorsolateral columns of the PAG demonstrated that inhibition of this region reduces food consumption and that this may be due to reward-responsive neurons in the lateral or dorsolateral PAG (Tryon and Mizumori, 2018). Further, evidence for the role of the PAG in reward processing comes from a study focused on the efferent projections from the rostrolateral PAG to orexinergic neurons in the lateral hypothalamus which are thought to be critical in determining motivation to chase prey (Mota-Ortiz et al., 2012); orexinergic neurons have been reported to be important for reward processing in general and are associated with the rewarding aspects of both food and drugs (Harris and Aston-Jones, 2006). Whether the PAG plays a significant role in reward processing that is contributing to its regulation of predatory drive has yet to be determined.

Projections to the PAG, that could be mediating the predator behaviors described, include substantial projections from areas in the prefrontal cortex, amygdala, and hypothalamus (Floyd et al., 2000; Han et al., 2017; Li et al., 2018; Park et al., 2018). Although, these projections do not target the rostrolateral regions of the PAG specifically, optogenetic and chemogenetic experiments identifying pathways required for predatory behaviors have identified projections to and from the ventral PAG as being critical. The central nucleus of the amygdala mainly has inhibitory projections to the ventral PAG, and activation of this pathway leads to enhanced prey pursuit (Han et al., 2017). Similar to projections from the amygdala, the projection from the lateral hypothalamus to the ventral PAG is also made up of mainly GABAergic neurons and it is these neurons that drive predatory attack (Li et al., 2018). Interestingly, stimulation of excitatory, rather than inhibitory, afferent connections leading from another hypothalamic area, the medial preoptic area, to the ventral PAG increases hunting behaviors towards both an inanimate object and natural prey in mice (Park et al., 2018). This suggests that the glutamatergic projections from the medial

preoptic area, and the GABAergic projections from the central amygdala and lateral hypothalamus, may target different cell types within the microcircuitry of the PAG such that both excitatory and inhibitory input ultimately leads to a coordinated behavioral output.

THE ROLE OF THE PAG IN PREY

Prey rely on a range of behavioral responses to increase the likelihood of survival in the face of dangerous and life-threatening situations. These include active fight/flight responses and passive freezing behaviors, which can be either innate or learned (conditioned). Environmental factors, like the presence of escape routes, and the proximity to the threat, contribute to the type of defensive behavior elicited. In the case of distal threats, rodents will engage in freezing behaviors, and will switch their defensive response to escape-related behaviors like flight and jumps as the likelihood of attack increases (Bolles and Fanselow, 1980). Interestingly, this diverse range of complex behavioral responses are all mediated, in part, by the PAG. Perhaps not unexpectedly, there is widespread activation in the PAG, as measured by c-fos, after exposure to a predator odor (Dielenberg et al., 2001). Evidence for region-specific functional roles related to the varied and sometimes opposite behavioral responses are discussed below.

Evidence for a Dorsal-Ventral Functional Division

Common understanding of PAG function in defensive behaviors is that the dorsal and ventral PAG have two apparently opposing actions on behavior; traditionally, dorsal PAG activity is largely associated with escape and flight, while ventral PAG activity is responsible for freezing behaviors (Adamec et al., 2012; Assareh et al., 2016; Silva et al., 2016; Tovote et al., 2016; Watson et al., 2016; Vieira-Rasteli et al., 2018). However, recent studies using single-unit recordings and optogenetic/chemogenetic techniques have painted a more complex picture.

Overall, neurons in the dorsal PAG increase their firing rate more in response to exposure to a predator odor than neurons in the ventral PAG (Watson et al., 2016). In support of the theory that the ventral PAG generates freezing behaviors, optogenetic manipulations have demonstrated that an inhibitory pathway leading from the central nucleus of the amygdala to GABAergic neurons in the ventrolateral PAG acts to disinhibit glutamatergic outputs to the magnocellular nucleus of the medulla; this pathway is critical for producing learned freezing behaviors in response to a footshock (Tovote et al., 2016). This projection appears to be independent of the projection from the central nucleus of the amygdala to the PAG associated with hunting behavior. Glutamatergic neurons in the ventrolateral PAG are also required for the animal to express normal freezing behaviors when exposed to a moving object that visually mimics a predator, highlighting these output neurons as important mediators of freezing behaviors (Tovote et al., 2016).

Interestingly, optogenetic activation of GABAergic neurons in the ventrolateral PAG, or the glutamatergic neurons in the dorsal PAG which project to these neurons, result in flight; this neural microcircuitry likely facilitates the rapid switch between active and passive defensive responses observed in rodents who perceive imminent danger (Tovote et al., 2016). Findings that optogenetic activation of glutamatergic projections from the lateral hypothalamus to the ventral PAG result in escape behaviors (fleeing and jumping) from a moving object (Li et al., 2018) suggest that neurons in the hypothalamus may be acting *via* their actions on GABAergic neurons in the ventral PAG or glutamatergic neurons in the lateral PAG, both of which receive similar amounts of projections from the lateral hypothalamus (Tovote et al., 2016).

In support of the theory that the dorsal PAG is responsible for escape behaviors, a recent study using optogenetics and calcium imaging has demonstrated that glutamatergic neurons in the dorsal PAG encode the decision to escape an aversive stimulus and the speed at which this escape occurs (Evans et al., 2018). However, in addition to flight, the dorsal PAG has the ability to also control risk assessment and freezing (Vianna and Brandão, 2003; Bittencourt et al., 2004; Aguiar and Guimarães, 2009; Assareh et al., 2016; Deng et al., 2016). Whether stimulation of the dorsal PAG results in freezing or escape behaviors appears to depend on the strength of the stimulus; higher levels of electrical current applied to the dorsal PAG (Vianna and Brandão, 2003; Bittencourt et al., 2004) and in the lateral PAG (Assareh et al., 2016) resulting in escape behaviors. Interestingly, cell-type specific activation of excitatory neurons in the dorsal PAG can mediate both these diverse responses; optogenetic stimulation of CamKIIα-positive neurons in the dorsal PAG results in both increased flight and freezing (Deng et al., 2016). Single-unit *in vivo* electrophysiology performed during exposure of a mouse to a predator (rat) identified distinct subsets of dorsal PAG neurons that are responsible for risk assessment, flight, and freezing with a very small percentage of cells firing in association with more than one of these behaviors (Deng et al., 2016).

Acute predator exposure in rodents is used as a model of post-traumatic stress disorder because of its long-term and persistent effects. The long-term neural plasticity induced by a single exposure to a predator observed in the PAG differs according to dorsal and ventral subdivisions. Phosphorylated cAMP response element binding protein (pCREB) is a protein that regulates expression of many synaptic plasticity-related genes, and thus its expression can be used as a marker of neural plasticity. pCREB expression is increased transiently 20 min following predator exposure in the lateral PAG (Adamec et al., 2003, 2009) but this is associated with a potentiation in transmission from the central nucleus of the amygdala to the lateral PAG (Adamec et al., 2003). Artificially increasing pCREB expression in non-predator exposed rats in the lateral PAG can also increase potentiation from the central nucleus of the amygdala to the lateral PAG and is anxiogenic mimicking what is observed in predator exposed rats (Adamec et al., 2009). pCREB expression is also persistently increased in the dorsal PAG, and decreased in the ventral PAG, 1 and 7 days after predator exposure (Adamec et al., 2011). While this data supports a role for the PAG in fear memory formation resulting from

predator exposure, this is controversial. Experiments involving chemogenetic silencing of the dorsal PAG in mice suggested that the dorsal PAG is needed for the expression of acute fear behaviors on exposure to a predator, but that this is not required for the formation of the fear memory; mice are able to show learned fear to the context in which the predator exposure took place despite not showing an acute fear response at the time of predator exposure (Silva et al., 2016).

Evidence for a Rostral-Caudal Functional Division

While many studies investigating the role of the PAG in rodent defensive behaviors consider columns of the PAG, less focus has been placed on differences that may occur from rostral to caudal ends, and few studies have directly compared the function of rostral and caudal regions. However, the studies that have investigated rostral-caudal position as a factor have suggested key differences in function across the rostral-caudal axis. Cat-induced activation of the rat PAG, as measured by Fos expression, suggests that a distinct rostral-caudal gradient occurs in combination with a dorsal-ventral gradient (Canteras and Goto, 1999; Comoli et al., 2003). This differing activation across a rostral-caudal axis has also been observed at a causal level in experiments using local infusions of an NMDA antagonist, AP5, to block activity in the rostral or caudal dorsolateral PAG (Souza and Carobrez, 2016). Excitatory transmission *via* NMDA receptor activation in the rostral dorsolateral PAG is associated with the expression of innate defensive responses to predator odor and the subsequent expression of fear to the context previously paired with the odor. Souza and Carobrez additionally described that, while excitatory transmission in the rostral dorsolateral PAG is not required for consolidation of predator odor-induced contextual fear, NMDA-mediated activity in the caudal dorsolateral PAG is required to maintain contextual fear conditioning (Souza and Carobrez, 2016). These findings differ from Silva et al. (2016), described above, which suggested that the dorsal PAG is associated predominantly with the motor output of the fear response rather than formation of the contextual fear memory itself. The difference between these two findings may highlight the potential importance of the fear stimulus itself; Souza and Carobrez (2016) used a predator odor while Silva et al. (2016) used a live rat raising the possibility that engaging multiple sensory modalities, rather than solely olfaction, recruits several pathways that can result in formation of a fear memory independent of the dorsal PAG. This again underlines the complexity, as well as the controversy, surrounding the role of the PAG in memory. In addition, to this point, a rostral-caudal functional differentiation has focused on the dorsal PAG; further work is required to better understand whether rostral and caudal, lateral and ventrolateral areas are also responsible for differing aspects of innate and learned fear responses associated with predator exposure.

Human PAG Activation Associated With Fear of Predators

Human imaging studies have clearly demonstrated that the PAG is activated when humans are exposed to stimuli that suggest danger. Blood-oxygen-level dependent (BOLD) activity in the PAG increases as innately threatening stimuli (i.e., a virtual predator or a tarantula) becomes more imminent (Mobbs et al., 2007, 2009, 2010; Coker-Appiah et al., 2013). BOLD responses in the PAG are also increased on exposure to negatively valenced, aversive pictures and this is correlated with fear bradycardia (heart rate deceleration), the result of activation of the parasympathetic nervous system, akin to freezing behavior in rodents (Hermans et al., 2013). Activity in the PAG resulting from exposure to aversive pictures is also correlated with increased activity in the amygdala suggesting that the amygdala-PAG pathway is involved in the processing of aversive stimuli (Hermans et al., 2013). This pathway has similarly been shown to be important in the processing of threat (Mobbs et al., 2009) further paralleling studies on the amygdala-PAG pathway described in rodents above.

Perhaps due to the limited spatial resolution of functional magnetic resonance imaging (fMRI) technology, much of this research has not sub-divided the human PAG into its dorsal and ventral sub-areas (Linnman et al., 2012). However, one study investigating resting-state functional connectivity in the dorsolateral and ventrolateral PAG of post-traumatic stress disorder patients does suggest that a functional subdivision associated with active vs. passive coping is present in humans, similar to that observed in animals (Harricharan et al., 2016). Overall, these studies in humans suggest that the function of PAG recruitment on exposure to a threat may be largely conserved between rodents and humans thus strengthening the utility of rodent studies aimed at understanding the complex role that the PAG plays in prey behaviors.

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TF is the sole author of this review article.

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frontiers in Behavioral Neuroscience

REVIEW
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Stress Across Generations: DNA Methylation as a Potential Mechanism Underlying Intergenerational Effects of Stress in Both Post-traumatic Stress Disorder and Pre-clinical Predator Stress Rodent Models

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Although most humans will experience some type of traumatic event in their lifetime only a small set of individuals will go on to develop post-traumatic stress disorder (PTSD). Differences in sex, age, trauma type, and comorbidity, along with many other elements, contribute to the heterogenous manifestation of this disorder. Nonetheless, aberrant hypothalamus-pituitary-adrenal (HPA) axis activity, especially in terms of cortisol and glucocorticoid receptor (GR) alterations, has been postulated as a tenable factor in the etiology and pathophysiology of PTSD. Moreover, emerging data suggests that the harmful effects of traumatic stress to the HPA axis in PTSD can also propagate into future generations, making offspring more prone to psychopathologies. Predator stress models provide an ethical and ethologically relevant way to investigate tentative mechanisms that are thought to underlie this phenomenon. In this review article, we discuss findings from human and laboratory predator stress studies that suggest changes to DNA methylation germane to GRs may underlie the generational effects of trauma transmission. Understanding mechanisms that promote stress-induced psychopathology will represent a major advance in the field and may lead to novel treatments for such devastating, and often treatment-resistant trauma and stress-disorders.

Keywords: PTSD, predator stress, intergenerational, DNA methylation, glucocorticoid receptors, FKBP5

Abbreviations: PTSD, post-traumatic stress disorder; OCD, obsessive compulsive disorder; GAD, generalized anxiety disorder; GR, glucocorticoid receptor; hGR, human glucocorticoid receptor; Fkbp5, FK506 binding protein 5; Bdnf, brain-derived neurotrophic factor; Dlgap2, Dlg associated protein 2; Avp, vasopressin; PND, postnatal day; Barx1, BARX homeobox; AIMS, Amplification of inter-methylated sites; ADCYAP1R1, ADCYAP receptor Type I; CDV, Community and domestic violence; CFTR, Cystic fibrosis transmembrane conductance regulator; COMT, Catechol-O-methyltransferase gene; CORIN, Corin, serine peptidase genes; IGF2, Insulin-like growth factor 2; IL8, Interleukin 8; IL16, Interleukin 16; IL18, Interleukin 18; Nr3c1, Nuclear receptor subfamily 3 group C member 1; Nr3c2, Nuclear receptor subfamily 3 group C member 2; SMYD3, SET And MYND domain containing 3; SLC6A4, Solute carrier family 6 member 4; qPCR, Quantitative PCR.

POST-TRAUMATIC STRESS DISORDER (PTSD)

Individuals exposed to highly traumatic experiences (e.g., physical assault, rape, natural disaster, kidnapping, combat, etc.) can develop post-traumatic stress disorder (PTSD). The DSM-V classifies PTSD as a stress and trauma-related disorder, defined by a set of symptom clusters that appear for at least 30 days following severe trauma. Symptoms include *re-experiencing* of the trauma (unwanted intrusion of the memory in the form of thoughts, nightmares, and flashbacks, intense upset evoked by conditioned cues or environmental stimuli), *avoidance* of cues related to the traumatic event (situations, places, activities), and *hyperarousal* (increased startle response, irritability, sleep problems). Negative disturbances in mood and cognition (dissociative amnesia, negative affect, and anhedonia) are also recognized as core symptoms (American Psychiatric Association, 2013). Women are twice as likely to develop PTSD as men, and the disorder is often comorbid with other anxiety disorders, as well as depression and substance abuse (Kessler et al., 1995). Not only can exposure to trauma affect the psychological status of an individual, but it can also have deleterious consequences on the individual's social, professional, and family life. As a result, the fallout from trauma carries a large burden for individuals and society (Kessler et al., 1995).

Epidemiological studies have found that between 37% and 92% of people report past exposure to at least one traumatic event (Van Ameringen et al., 2008; Kilpatrick et al., 2013; Atwoli et al., 2015; Benjet et al., 2016; Kessler et al., 2017). Of those, between 25% and 35% of trauma survivors go on to develop PTSD (Yehuda, 2001; Kessler et al., 2005). These figures contribute to the lifetime prevalence of the disorder which is currently estimated at 6.1% in the United States and 9.2% in Canada (Van Ameringen et al., 2008; Goldstein et al., 2016). PTSD is, therefore, one of the most common psychiatric disorders as most anxiety and affective related disorders [e.g., generalized anxiety disorder (GAD), agoraphobia, panic attacks, obsessive compulsive disorder (OCD), major depressive disorder, bipolar disorder] for comparison have relatively lower lifetime prevalence, ranging from 0.7% to 5% (Kessler et al., 2012; Bandelow and Michaelis, 2015; Statistics Canada, 2015). Among "highly exposed" groups (e.g., low-income, low social support, urban populations), lifetime rates of PTSD can be as high as 40% (Breslau et al., 1998). In the wake of large-scale disastrous events, incidence and prevalence of PTSD to populations endemic to the area involved can often by shifted sharply upward, like those affected in the New York City area or the Mississippi Delta following the 9/11/2001 terrorist attacks or Hurricane Katrina in 2005, respectively (Galea et al., 2002, 2008). Nonetheless, trauma event type confers differences in risk for PTSD. Direct interpersonal victimization related to rape, physical abuse, and kidnapping has the highest conditional risk, while other trauma event categories such as automobile accident or natural disaster are at low risk for PTSD prevalence (Kilpatrick et al., 2013; Benjet et al., 2016; Kessler et al., 2017). In addition, a dose-response relationship exists between symptom severity and frequency of trauma experience: the more traumatic events a person experiences, the greater the intensity of their PTSD symptoms (Binder et al., 2008).

Although many individuals who present with the symptoms of PTSD actively seek out psychotherapy, psychopharmacology, or both (Van Ameringen et al., 2008) only about 60% are responsive to interventions (Önder et al., 2006), with only about a third of patients achieving full remission (Berger et al., 2009). The disparity in those achieving full remission, coupled with epidemiological studies estimating the lifetime prevalence of PTSD at 5%–10% for the general population (Van Ameringen et al., 2008), suggests a desperate need for understanding the mechanisms that contribute to the vulnerability, development, and maintenance of PTSD ultimately leading to the identification of candidate treatments (Reul and Nutt, 2008; Hauger et al., 2012; Steckler and Risbrough, 2012).

As noted above, traumatic stress can have deleterious effects on an individual's mental health. However, recent data suggests that the harmful effects of traumatic stress during one's lifetime can propagate into future generations (Blaze and Roth, 2015), perhaps leading to an increased vulnerability to the development of psychopathology. This review will examine recent work on the intergenerational effects of stress in both human (with PTSD) and rodent (predator stress) studies, with a focus on DNA methylation as a potential underlying mechanism. As predator stress is ecologically relevant and considered one of the most comprehensive animal models of PTSD (see Deslauriers et al., 2017), our discussion will focus on research using this model. For the purposes of this review, we define intergenerational effects of stress similar to that described by Klengel et al. (2015). Briefly, intergenerational transmission involves direct stress exposure to the parental (F0) generation and subsequent offspring generation (F1) by means of either the developing germ cell or fetus. If the stress exposure occurred while the fetus (F1) was developing *in utero*, intergenerational transmission occurs in the F2 generation. This is in contrast to transgenerational whereby the germ cells have *not* been exposed to stress.

HUMAN STUDIES: STRESS EFFECTS ACROSS GENERATIONS

There is a variety of research showing intergenerational effects of stress. In a landmark study by Solomon et al. (1988), Israeli veterans of the 1982 Lebanon War who were offspring of Holocaust survivors were more likely to develop PTSD than other Israeli soldiers following their military experiences who did not have parents interned in Nazi concentration camps (Solomon et al., 1988). Likewise, PTSD in former Israeli soldiers captured and held as prisoners of war during the 1973 Yom Kippur War positively correlated with offspring PTSD (Zerach et al., 2017). Furthermore, in a study of Cambodian refugees living in the United States, parental PTSD predicted higher rates of child PTSD for older children. A gradient effect was found such that when neither parent had PTSD, 12.9% of the children had PTSD; when one parent had PTSD, 23.3% of the children had PTSD; and when both parents had PTSD, the percentage of children with PTSD jumped to 41.2% (Sack et al., 1995). In a

more recent report, offspring of parents suffering from PTSD that survived the 1994 Tutsi genocide in Rwanda showed greater secondary traumatization symptoms and were less resilient (Shrira et al., 2019). In addition to intergenerational effects, data suggests transgenerational effects of stress. For example, offspring of Israeli Holocaust survivors had a higher incidence of PTSD, mood disorders, anxiety disorders, and substance abuse disorders three generations downstream (Barocas and Barocas, 1979; Dasberg, 1987; Scharf, 2007; Yehuda et al., 2008, 2015). Overall, these data suggest that parental traumatic stress make offspring more vulnerable to mental illness.

Stress Hormones

Stress hormones appear to play a key role in the development of, and vulnerability to, PTSD. Cortisol (principal human stress hormone; corticosterone is the main glucocorticoid in most other species) coordinates and prepares the body to respond to environmental demands and stressors to achieve systems homeostasis. Selye (1956) was the first to demonstrate a common pathway of physiological activity in response to stress. This pathway was later dubbed the hypothalamic-pituitary-adrenal (HPA) axis. During a stressful event, cells of the paraventricular nucleus of the hypothalamus respond by secreting corticotropin-releasing hormone (CRH) into capillaries in the median eminence of the hypothalamus. CRH released into this portal capillary system stimulates neurosecretory cells in the anterior pituitary which in turn release adrenocorticotropin hormone (ACTH). From there, ACTH travels through the blood stream and acts on the cortex of the adrenal gland where it stimulates secretory cells to release glucocorticoids, particularly cortisol, into the general circulatory system. Cortisol prepares the body to adapt to current stressors by suppressing immune system activity, counteracting insulin, supporting increased glucose availability (e.g., gluconeogenesis), and regulating water retention and electrolytic balance in the kidneys (Khani and Tayek, 2001; Dunlop and Wong, 2019). Cortisol also acts to decrease the activity of paraventricular nucleus and anterior pituitary, negatively influencing its own release. This regulator mechanism limits the stress response helping return the body to homeostasis and is often referred to as the negative feedback loop (Sapolsky et al., 1985). As the term stress is ambiguous and not well defined, it is important to note that various daily events that many might interpret as innocuous or pleasurable, such as exercise or sex, evoke this canonical HPA axis stress response (Stranahan et al., 2008; Jokinen et al., 2017; Finke et al., 2018). Nevertheless, unlike sex and exercise, the stressors that satisfy Criteria A in the DSM-5, while quite varied, are traumatic, sudden, unexpected, involuntary, and uncontrollable (American Psychiatric Association, 2013; Kilpatrick et al., 2013; Kessler et al., 2017). Dysregulated or aberrant HPA axis activity, especially in terms of cortisol, is often postulated as part of the etiology and pathophysiology of PTSD in response to traumatic events. The direction of this altered neuroendocrine activity that might confer a susceptibility to developing and maintaining PTSD, however, has yielded inconsistent findings (Zoladz and Diamond, 2013).

Mason et al. (1986) were the first to report lower mean 24-h basal urinary cortisol levels from inpatient PTSD combat veterans. Although this study only compared different psychiatric groups, subsequent studies have shown similar low cortisol levels relative to healthy controls (Yehuda et al., 1993; Kanter et al., 2001; Rohleder et al., 2004). Conversely, other studies have reported increased basal cortisol levels (Lemieux and Coe, 1995; Carrion et al., 2002; Young and Breslau, 2004) or no difference in individuals with PTSD compared to healthy controls (Duval et al., 2004; Yehuda et al., 2004; Otte et al., 2007). Discrepancies in reported cortisol levels concern issues around, but not limited to, sex, length of combat exposure in veterans, differences in trauma type, childhood trauma, current PTSD status, differences in naturally fluctuating levels of cortisol throughout the day (at awakening or peak, nadir, fastening state), comorbidity with other psychiatric disorders such as major depressive disorder and substance use disorders, plasma vs. urinary vs. cerebral spinal fluid cortisol levels, and lack of statistical power (Zoladz and Diamond, 2013; Dunlop and Wong, 2019). Given such inconsistences, these findings suggest that basal cortisol abnormalities might only represent a subset of the manifestation of PTSD. As such, basal cortisol is a rather dubious biomarker for PTSD. Zoladz and Diamond (2013), suggest investigating a more comprehensive role for cortisol in multiple physiological processes. In particular, it is suggested that impaired HPA axis functioning diminishes negative feedback and increases sympathetic nervous system and immune system activity, both linked to PTSD etiology and pathophysiology (Zoladz and Diamond, 2013).

Similar to the heterogeneous findings of baseline cortisol activity, mixed evidence linking altered cortisol levels in the immediate aftermath of an acute traumatic experience to PTSD have been reported. Specifically, one study found a negative correlation between diminished cortisol levels peri-trauma and to the development of PTSD (Ehring et al., 2008), whereas other studies have found no correlation (Resnick et al., 1995; Heinrichs et al., 2005), or a positive correlation, especially in relation to childhood trauma and the development of PTSD months later (Lipschitz et al., 2003; Delahanty et al., 2005; Pfeffer et al., 2007). Here, it is probable that the ontogeny of HPA axis during childhood contributes differently to the development of PTSD compared to maladaptive adulthood responses. In adults, administration of hydrocortisone to patients undergoing very invasive surgeries show fewer PTSD symptoms in follow-up sessions (Schelling et al., 2004, 2006), providing credence for perturbed cortisol functioning in adults' response to acute traumatic stress that goes on to develop PTSD. Moreover, studies investigating cortisol levels in the dexamethasone suppression test and trier social stress test of individuals with PTSD have shown rather consistently robust inhibition of cortisol in response to synthetic glucocorticoids or psychosocial stress (Yehuda, 2002; Zoladz and Diamond, 2013; Wichmann et al., 2017).

Interestingly, alterations in stress hormone release have also been observed in the offspring of individuals with PTSD. Yehuda et al. (2000) found lower serum cortisol levels and greater cortisol suppression in the offspring

of Holocaust survivor's suffering from PTSD compared to healthy subjects who were not offspring of Holocaust survivors (Yehuda et al., 2000). Further, pregnant women who developed PTSD in response to the September 11, 2001, World Trade Center collapse had infants with lower cortisol levels compared with infants of mothers who did not develop PTSD (Yehuda et al., 2005). Similarly, Yahyavi et al. (2015) found that offspring of Iranian combat veterans from the Iran-Iraq war with a current or past history of PTSD had significantly lower serum cortisol levels than offspring of combat veterans without a history of PTSD. Moreover, beyond diurnal changes in stress hormones, youth offspring of mothers with PTSD also show a blunted, maladaptive, cortisol saliva response to an acute laboratory stressor (Danielsona et al., 2015), a response seen likewise in female PTSD patients (Pierrehumbert et al., 2009; Zaba et al., 2015; Wichmann et al., 2017).

EPIGENETIC MODIFICATIONS: DNA METHYLATION AND PTSD

Epigenetic modifications have been espoused to help explain the transmission of experience to future generations. Epigenetic modifications are changes in the transcriptional potential of a cell by the environment, which occur independent of alterations in the gene sequence. Epigenetic modifications provide a mechanism that links genes and environment while playing an important role in the modulation of behavioral responses to stress (Day and Sweatt, 2011). Although the mechanism by which epigenetic modifications lead to the transmission of environmental influences to subsequent generations is not known, one possibility involves the transmission of DNA methylation (Meaney, 2001; Day and Sweatt, 2011; Dias and Ressler, 2014).

DNA methylation is one of the most broadly studied and well-characterized epigenetic modifications. DNA methylation plays a significant role in the maintenance of cellular identity and heritable changes in gene expression throughout the cell cycle, typically by prohibiting DNA transcription by the addition of a methyl group to a gene promoter region. Indeed, DNA methylation may be important in fundamental mechanisms for the induction and stabilization of PTSD (Zovkic et al., 2013).

As can be seen in **Table 1**, several studies have identified alterations in methylation states of specific genes in people with PTSD. Of the gene methylation studies in people with PTSD, the most commonly studied target is the glucocorticoid receptor (GR), a major regulator of the HPA-axis. GR, also known as Nr3c1 (nuclear receptor subfamily 3, group C, member 1), is the receptor to which cortisol and other glucocorticoids bind. The GR is expressed in almost every cell in the body and regulates genes controlling the development, metabolism, and immune response. In the absence of cortisol or corticosterone hormone, GR resides in the cytosol complexed with a variety of proteins including heat shock protein 70 and 90 (hsp70 and 90), and the protein chaperone FK506-binding protein (Fkbp5). Labonté et al. (2014) found that individuals with lifetime PTSD had lower morning cortisol release, higher mRNA expression of the human GR (hGRtotal, 1B, and 1C) and lower overall methylation levels in hGR 1B and 1C promoter regions. Similarly, lower *Nr3c1* 1F promoter methylation was observed in combat veterans with PTSD compared with combat veterans without PTSD (Yehuda et al., 2014). Interestingly, DNA methylation of the Nr3c1 promotor appears to be sex-specific as methylation at the *Nr3c1* 1F promoter was linked to traumatic memories and PTSD risk in male, but not in female genocide survivors in Rwanda (Vukojevic et al., 2014). However, much more research is necessary to fully assess the role of sex on DNA methylation of specific targets in people with PTSD.

As described by Klengel et al. (2015), it was once thought that DNA methylation was completely erased during development of the primordial germ cells and during fertilization. However, this erasure, or reprogramming, appears not to be complete. Evidence for certain loci that escape reprogramming is growing with examples including imprinted genes and repetitive elements (Kobayashi et al., 2012; Radford et al., 2014; Tang et al., 2015). Hence, altered GR promoter methylation may be one mechanism by which parental stress is translated into changes in gene expression and physiology, ultimately resulting in psychologically vulnerable offspring phenotypes (see **Table 1**). Perroud et al. (2011, 2014) examined the impact of the Tutsi genocide on the children of women who were pregnant while genocide was ongoing in Rwanda. In 2011, more than 20% of the Rwandan population met the criteria for PTSD. Peripheral blood leukocytes were obtained and methylation levels of the promoter regions of the GR *NR3C1* was examined in trauma-exposed woman and their children. As expected, both mothers exposed to genocide and their children had significantly higher levels of PTSD than the control group. They also showed higher methylation levels at exon 1F promoter of *Nr3c1*, at CpG3-CpG9. Furthermore, there was a negative correlation between *Nr3c1* methylation and glucocorticoid levels in plasma (Perroud et al., 2011, 2014). Changes in methylation of the GR receptor appears to be dependent on parental status of PTSD. Offspring with just paternal PTSD showed higher GR-1F promoter methylation, whereas offspring with both maternal and paternal PTSD showed lower methylation (Yehuda et al., 2014). Interestingly, in comparison to demographical controls, Holocaust survivors showed increased methylation of the promoter region for FK506 binding protein 5 (FKBP5), a protein that lowers the affinity of cortisol when it is bound to GR; thereby potentially hindering the negative HPA axis feedback loop (Binder, 2009), while methylation at this site was lower in Holocaust survivor offspring (Yehuda et al., 2016). Although gender-specific effects cannot be disentangled in this study, Yehuda et al. (2016) suggest that *Fkbp5* hypermethylation, leading to decreased *Fkbp5* expression and increased GR sensitivity in the F0 mothers, may result in lowered circulating glucocorticoid levels during pregnancy, promoting demethylation in the fetus to optimize or increase glucocorticoid levels. Alternatively, preconception or postnatal social influences may also influence offspring cortisol levels and hence regulate *Fkbp5* methylation levels. Currently, whether changes in glucocorticoids in offspring reflect intergenerational consequences of parental exposure or offspring recalibration of glucocorticoid regulation is not known. Future

TABLE 1 | Selective human studies supporting the role of DNA methylation in post-traumatic stress disorder (PTSD).

Reference	Candidate gene	Epigenetic changes	Sample size	Generation study carried out through
Koenen et al. (2011)	*Slc6a4*	Lower *Slc6a4* methylation levels in blood tissue and higher number of traumatic events increased risk for PTSD	100	F0
Ressler et al. (2011)	*Adcyap1r1*	*Adcyap1r1* methylation levels were observed in the peripheral blood of PTSD subjects compared with control	94	F0
Rusiecki et al. (2013)	*Il8, Il16, Il18*	Increased serum *Il18* methylation in combat veterans who developed PTSD, but decreased *Il18* methylation levels in veterans without PTSD	150	F0
Norrholm et al. (2013)	*Comt*	Higher blood level methylation of *Comt* are associated with impaired fear inhibition	270	F0
Yehuda et al. (2013)	*Nr3c1, Fkfbp5*	Pre psychotherapy *Nr3c1* 1F promoter methylation in blood tissue positively correlated with improvements in symptoms, while decreased *Fkbp5* methylation occurred concomitantly with recovery from PTSD	16	F0
Labonté et al. (2014)	*Nr3c1*	Increased *Nr3c1*mRNA expression and decreased overall *Nr3c1* 1B and 1C promoter methylation levels in individuals blood with lifetime PTSD	46	F0
Vukojevic et al. (2014)	*Nr3c1*	In the peripheral blood, methylation of *Nr3c1* 1F promoter is linked to traumatic memories and PTSD risk in male survivors of the Rwandan genocide	152	F0
Yehuda et al. (2014)	*Nr3c1*	*Nr3c1* 1F promoter methylation inversely correlated with symptoms severity in the blood tissue of combat veterans with PTSD	122	F0
Yehuda et al. (2014)	*Nr3c1*	Offspring with paternal PTSD showed higher *Nr3c1* 1F promoter methylation in the blood tissue if maternal PTSD was not present. Offspring with maternal and paternal PTSD showed lower methylation	95	F0, F1(Intergeneration)
Perroud et al. (2014)	*Nr3c1, Nr3c2*	Higher plasma levels methylation at promoter 1F for *Nr3c1* in female survivors of genocide and their offspring compared to controls, but no differences observed in *Nr3c2* methylation	25	F0, F1(Intergeneration)
Yehuda et al. (2016)	*Fkbp5*	Holocaust survivors showed increased methylation of the promotor region for *Fkbp5*, while their offspring showed the opposite in their blood tissue	71	F0, F1(Intergeneration)
Kertes et al. (2017)	*Bdnf*	Maternal experiences of war trauma were associated with higher *Bdnf* methylation in umbilical cord blood, placental tissue, and lower methylation in maternal venous blood.	24	F0, F1(Intergeneration)
Serpeloni et al. (2017)	*Barx1, Cftr, Corin, Smyd3*	Grandmaternal exposure to CDV during pregnancy was significantly associated with decreased methylation in *Corin*, *Smyd3*, and *Barx1*, and increased methylation of *Cftr* in grandchildren's saliva samples	121	F0, F1, F2, F3 (Intergeneration)
Kim et al. (2017)	*Bdnf*	Subjects with PTSD showed a higher methylation *Bdnf* promoter I region in their blood tissue compared with those without PTSD	248	F0
Voisey et al. (2019)	*Bdnf*	Decreased methylation at three *Bdnf* sites were observed in combat exposed PTSD veterans blood compared with control.	96	F0

Abbreviations: adenylate cyclase-activating polypeptide Type I receptor gene (Adcyap1r1), amplification of inter-methylated sites (Aims), BARX homeobox 1 (Barx1), brain-derived neurotrophic factor (Bdnf), catechol-O-methyltransferase gene (Comt), community and domestic violence (CDV), corin, serine peptidase genes (Corin), cystic fibrosis transmembrane conductance regulator (Cftr), FK506 binding protein 5 (Fkbp), nuclear receptor subfamily 3 group C member 1 gene (Nr3c1, glucocorticoid receptor gene), insulin like growth factor 2 (Igf2), interleukin like chemokines (Il8, Il16, Il18), nuclear receptor subfamily 3 group C member 2 gene (Nr3c2, mineralcorticoid receptor gene), SET and MYND domain containing 3 (Smyd3), solute carrier family 6 member 4 (Slc6a4).

studies, perhaps using animal models (discussed below), are necessary to fully understand the role of GR methylation in development and vulnerability to mental illness.

While epigenetic studies investigating the intergenerational effects of stress have primarily looked at changes in the methylation state of GR, recent reports suggest that other targets may play a role in this transmission (see **Table 1**). While methylation of the *Bdnf* gene promoter region has been associated with the development of PTSD (Kim et al., 2017; Voisey et al., 2019), a recent report suggests that maternal trauma exposure may be linked to high *Bdnf* methylation levels in offspring (Kertes et al., 2017). Among 24 mothers and newborns in the eastern Democratic Republic of Congo, a region with extreme conflict and violence to women, maternal experiences of war trauma and chronic stress were associated with higher *Bdnf* methylation in umbilical cord blood, placental tissue, and lower methylation in maternal venous blood. While the studies described above examined parental and first-generation methylation status of specific genes, Serpeloni et al. (2017) examined the grandchildren of grandmothers exposed to psychosocial stress during pregnancy. Grand maternal exposure to community and domestic violence (CDV) during pregnancy was significantly associated with decreased methylation of the *CORIN* (corin, serine peptidase), *SMYD3* (SET and MYND domain-containing protein 3, is a histone methyltransferase), and *BARX1* (BARX Homeobox 1 is a protein-coding gene) genes, as well as increased methylation of the *CFTR* (cystic fibrosis transmembrane conductance regulator) gene in the grandchildren. *CFTR* and *CORIN* genes are involved in circulatory system processes and congenital abnormalities and dysregulation of these genes impact the release of vitamin D, blood pressure regulation, heart failure and hypertension. *SMYD3* and *BARX1* genes are involved in embryonic development, craniofacial development, odontogenesis, and stomach organogenesis. The exact role methylation of these genes plays in the intergenerational effects of stress, however, has not been elucidated.

In all, there appears to be strong support for epigenetic regulation, specifically DNA methylation, in human PTSD (see **Table 1**). Furthermore, evidence suggests that these epigenetic changes may be passed on to future generations and lead to a vulnerability to psychiatric illnesses in the offspring. Although these findings are exciting, several limitations should be noted. For instances, low sample size, age variability, a lack of replication and sufficient controls, and a paucity of information on sex effects/differences and ancestry information (Rady et al., 2010; Zannas et al., 2016; Lacal and Ventura, 2018) plague these studies. Furthermore, maternal and paternal lineages may have differing effects on the epigenetic transmission of stress. Moreover, the exact nature of the transmission is dependent on which parent underwent the stressor (and when) as the maternal lineage can be observed in the F0, F1 and F2 generations, and the transgenerational phenotype in F3, whereas the paternal lineage can be seen in the F0 and F1 generations, and the transgenerational phenotype in F2 (for review, see Bale, 2011; Gabory et al., 2009). Hence, more research is necessary to fully understand the role DNA methylation plays in the development of, or vulnerability for, PTSD. In addition to these issues, there are several confounds that must be considered in the human research that question the role of epigenetics in the transmission of stress effects across generations. For instance, it may be that the children of survivors of trauma adopt ineffective coping techniques like their parents. Alternatively, the children may have shared trauma with their parents, and/or been exposed to parental PTSD symptoms, or experienced parental emotional abuse or neglect. These, and others not listed, may contribute to the increased likelihood of a child developing a stress-induced psychopathology (Yehuda et al., 2005; Sturge-Apple et al., 2012; Lehrner et al., 2014). Both ethical limitations and the logistical constraints associated with human research limit full understanding of the mechanisms underlying these transgenerational effects. For this reason, recent efforts have been made to assess transgenerational effects of stress in rodents. Rodent models are useful because they: (A) simulate a human condition in a controlled setting; (B) allow the disease to be studied as it develops; and (C) facilitate the preliminary evaluation of pharmacological and other treatments for humans.

PREDATOR STRESS AS AN ANIMAL MODEL OF PTSD

While there is no one ideal animal model of PTSD that recapitulates all symptoms of the disorder, Predator Stress paradigms are arguably one of the more comprehensive approaches used by researchers (see Deslauriers et al., 2017). Predator stress typically involves acute exposure of a prey species (mouse or rat) to a predator or predator cue (typically a cat, rat, or ferret). A considerable volume of literature exists demonstrating that cat exposure generates high levels of anxiety-like behavior, avoidance of trauma-related cues, hyperarousal, and impaired spatial memories (Adamec and Shallow, 1993; Adamec, 1998; Adamec et al., 1999, 2004, 2005, 2008; Blundell et al., 2005; Diamond et al., 2006; Cohen et al., 2009; Lebow et al., 2012; Fifield et al., 2013, 2015; Goswami et al., 2013; Zoladz et al., 2015; Lau et al., 2016; Schöner et al., 2017; Cohen et al., 2018). Other similarities to PTSD include the fact that female mice appear more susceptible to predator stress (Adamec et al., 2006) and common pharmacological treatments of PTSD (e.g., sertraline) are efficacious in reducing anxiety-like behaviors and hyperarousal following predator stress (Adamec et al., 2004; Matar et al., 2006). In addition, predator stressed rodents show the modifications in glucocorticoid levels, gene expression, and the release of CRH in the amygdala similar to that seen in PTSD (Adamec and Shallow, 1993; Adamec et al., 1998; Blanchard et al., 2001; Dielenberg et al., 2001; Cook, 2002; Hebb et al., 2003; Rosen, 2004; Schulkin et al., 2005; Takahashi et al., 2005; Roseboom et al., 2007; Armario et al., 2008; Campeau et al., 2008; Yao et al., 2008; Zoladz et al., 2008; Clinchy et al., 2011; Whitaker and Gilpin, 2015; Lau et al., 2016). Furthermore, predator stress induces long lasting potentiation of rodent amygdala afferent and efferent transmission in right hemisphere, the degree of which is highly predictive of changes in rodent anxiety (Adamec et al., 2005). Finally, amygdala dendritic length is increased following predator stress (Adamec et al., 2012; Cohen et al., 2014).

RODENT STUDIES: PREDATOR STRESS EFFECTS ACROSS GENERATIONS

While predator exposure alters behavior indicative of stress-induced psychopathology in humans (see section above), there is growing laboratory evidence that predator stress during pregnancy has deleterious outcomes to rodent offspring. In mice, exposure to predator rat urine during the 1st week of pregnancy leads to a decrease in the litter size and survival of offspring (de Catanzaro, 1988). Predator odor exposure also induces heightened levels of circulating corticosterone in pregnant female rodents and alters offspring development (Weinstock et al., 1988). In rats, exposure to a live predator prenatally leads to a predisposition to seizures associated with alterations in hippocampal plasticity in the offspring (Ahmadzadeh et al., 2011; Saboory et al., 2011; Korgan et al., 2014). More recently, adult offspring (postnatal day 90) of mice exposed to a predator odor during the last half of pregnancy display increased predator-avoidance behavior, alterations in social behavior, novelty-induced anxiety-like behaviors, and increased corticosterone levels (St-Cyr and McGowan, 2015; St-Cyr et al., 2017, 2018). This is consistent with studies showing dexamethasone (synthetic glucocorticoid) administration during pregnancy leads to reduced HPA axis sensitivity in adult offspring by attenuating the GR and mineralocorticoid receptors in the hippocampus, which results in enhanced anxiety-like behaviors and stress responsivity (Levitt et al., 1996; Welberg and Seckl, 2001). While these data suggest that predator stress in the parental generation impacts offspring, much more research is required to fully elucidate these effects.

Epigenetic Modifications: DNA Methylation and Predator Stress

As can be seen in **Table 2**, several studies have identified alterations in methylation states of specific genes following predator stress. For instance, Dlgap2, a gene that encodes a postsynaptic density protein, is more likely to be unmethylated in animals that display an anxious phenotype following the predator stress paradigm (Chertkow-Deutsher et al., 2010). Similarly, predator stress induces phenotypic variability in stress coping responses that can be linked to the degree of methylation of the hormone vasopressin (Avp) in the amygdala (Bowen et al., 2014). However, it is currently unknown whether changes in Dlgap2 and Avp DNA methylation persist into future generations.

Similar to human literature described above, methylation of the GR and Fkbp5 appear to play a role in the transmission of predator stress effects to future generations (see **Table 2**).

Female offspring from prenatal predator odor-exposed dams showed increased transcript abundance of both the GR gene (Nr3c1; on the day of birth) and Fkbp5 (in adulthood) in the amygdala (St-Cyr et al., 2017). Moreover, increased Fkbp5 expression was inversely correlated with decreased DNA methylation for this product's gene (St-Cyr et al., 2017), a finding consistent with the human literature (Yehuda et al., 2016). In a related study, female offspring of mice exposed to predator odor during pregnancy had decreased *Bdnf* transcript abundance which was positively correlated with a concomitant decrease in DNA methylation of *Bdnf* exon IV in the hippocampus (St-Cyr and McGowan, 2015). Epigenetic alterations of the *Bdnf* gene have been linked to impaired brain functioning, memory, stress, and neuropsychiatric disorders (Fuchikami et al., 2010; Ikegame et al., 2013; Andero et al., 2014). These results are consistent with other work in which predator scent stress induced a significant down-regulation of *Bdnf* mRNA in the CA1 region of the hippocampus (Kozlovsky et al., 2007). Hypermethylation of hippocampal *Bdnf* DNA may be a cellular mechanism underlying the persistent hippocampus-specific cognitive deficits which are prominent features of the pathophysiology of PTSD. Indeed, selective hypermethylation of the *Bdnf* gene in the dorsal hippocampus appears to be an important component of local synaptic structure, plasticity, and maintenance of intrusive memories of the trauma in an active state following exposure to a life-threatening event (Zoladz et al., 2012). Despite these findings, more research is necessary to fully

TABLE 2 | Selective rodent studies supporting the role of DNA methylation in predator stress model.

Reference	Candidate gene	Epigenetic changes	Sample size	Generation study carried out through
Chertkow-Deutsher et al. (2010)	*Dlgap2*	Higher *Dlgap2* methylation and reduced mRNA expression in predator odor exposed rat's hippocampus.	Not mentioned	F0
Bowen et al. (2014)	*Avp*	Predatory stress was associated with decreased *Avp* promoter methylation in the medial amygdala	48	F0
St-Cyr and McGowan (2015)	*Bdnf*	Female offspring of mice exposed to predator odor during pregnancy had decreased *Bdnf* transcript abundance positively correlated with a concomitant decrease in methylation of *Bdnf* exon IV in the hippocampus	42	F0, F1(Intergeneration)
St-Cyr et al. (2017)	*Nr3c1, Fkfbp5*	Female offspring from prenatal predator odor-exposed dams showed increased transcript abundance of both the glucocorticoid receptor gene (*Nr3c1*; on the day of birth) and *Fkfbp5* (in adulthood) in the amygdala	24	F0, F1(Intergeneration)

Abbreviations: amplification of inter-methylated sites (Aims), arginine vasopressin (Avp), brain-derived neurotrophic factor (Bdnf), disks large-associated protein 2 (Dlgap2), FK506 binding protein 5 (Fkbp5), nuclear receptor subfamily 3 group C member 1 gene (Nr3c1, glucocorticoid receptor gene).

assess the role of DNA methylation in the transmission of stress effects across generations (Blouin et al., 2016). Overall, laboratory predator stress controlled experiments such as cross-fostering, *in vitro* fertilization, or experiments across multiple generations will provide invaluable information on the biological epigenetic transmission and behavioral transmission which is virtually impossible to achieve for ethical and practical reason in humans.

CONCLUSION

Both human and rodent research suggests that stress effects can be passed on to future generations. However, there is a paucity of research on the mechanism of such a transmission. One possibility is epigenetic modifications. While the mechanism by which epigenetic modifications lead to the transmission of stress to subsequent generations is unknown, one possibility involves the transmission of DNA methylation. In humans, altered methylation of the GR, and/or its co-chaperone, FKBP5 genes, have been identified in the offspring of people with PTSD (Yehuda et al., 2004, 2014, 2016, 2015; Erhardt and Spoormaker, 2013). This is consistent with laboratory studies in rodents in which predator exposure during pregnancy alters methylation of the GR and FKBP5 DNA in offspring (St-Cyr et al., 2017). Future research is needed in both humans and animal models, however, to fully understand the role that DNA methylation (of specific targets such as GR) plays in the transmission of stress. Understanding mechanisms that promote PTSD will represent a major advance in the field and may lead to novel treatments for such a devastating, and often treatment-resistant disorder.

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