DETERMINANTS AND CONSEQUENCES OF PERCEIVED PREDATION RISK: FROM INDIVIDUAL BEHAVIOR TO TRANSGENERATIONAL EFFECTS

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EDITORIAL
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Editorial: Determinants and Consequences of Perceived Predation Risk: From Individual Behavior to Transgenerational Effects

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Editorial on the Research Topic

Determinants and Consequences of Perceived Predation Risk: From Individual Behavior to Transgenerational Effects

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Predation is a major evolutionary and ecological process that shapes population dynamics and community structure directly, *via* lethal effects, but also indirectly, through non-lethal "fear" effects. The papers in this Research Topic provide new information on how these effects take place, from community to individual level and *via* transgenerational effects.

The fear of being killed may result in animal movements being shaped according to the distribution of predators. Prey move to reduce the risk of encountering predators while predators will move toward areas rich in prey. For prey, predation risk creates a mosaic of risky/safe areas, the so-called "landscape of fear." Scrosati reviews the evidence for non-lethal effects in a fascinating coastal study system, where the dog whelk prey upon barnacles and mussels. These sessile prey organisms are able to use water-borne cues from dogwhelk to avoid settling in areas of predator presence, a behavior that may have evolved to reduce predation risk and thus increase survival. Burgas et al. show that such an effect of a predator on the distribution of a prey can be long lasting for a whole community. The large prey species preferred by the northern goshawk are less abundant close to goshawk nests, an effect that also lasts for years after the hawk no longer nests there. For smaller prey species the trend seems to be opposite, as their abundance can be even higher close to the top predator nest—potentially due to the protection provided by the goshawk against smaller predator species.

Although prey may avoid settling close to their predator, they rarely can avoid it altogether and are thus exposed through their life to various degrees of predation risk. Predator presence alone creates non-consumptive effects on individual prey fitness by affecting prey foraging behavior and habitat-use. Kelleher et al. use auditory calls of predators to assess the interactive effects of habitat, refuge availability and predator type, fox (cursorial) vs. owl (ambush predator), on the foraging behavior of white-footed mice. Foraging behavior of prey was most reduced and altered (increased use of refuges) when exposed to foxes at the open forest edge (the habitat where mice are the most vulnerable to predation), suggesting that environmental context can modulate prey responses to risk.

Predator presence can also alter prey development and physiological stress responses. Abondano Almeida et al. show that increased predation risk (through a simulated bird attack) influences warning signal efficacy and several life-history traits like growth and molting events in wood tiger moths. Larvae exposed to predation increased melanin deposition, reached a smaller body size, and molted more often. Phenotypic plasticity is thus a potential mechanism to respond to novel predation risk in aposematic species. These species also deter predators by using a combination of defenses, e.g., visual signaling or aversive tastes. Using an experimental approach, Winters et al. investigates the multimodal aposematic defense in the wood tiger moth to blue tit predators. They show that color, taste and smell are used as a warning signal impacting predator attack at different stages. In addition, moth color and smell impacts their survival, as moths of the white morph with methoxypyrazine smell were captured and killed less often by birds.

In addition to the direct effects to individuals, predation risk perceived during breeding can flow on between generations, as "stressed" parents modify their offspring's phenotype. Mohring et al. investigates hormonal profile and reproductive investment of female eider ducks breeding under high predation risk. Females breeding in high risk areas had higher levels of prolactin, thus showing surprisingly high parental investment, which resulted also in higher offspring condition. This could be linked to selective disappearance of breeders in low body condition in high risk sites. In addition to affecting parental behavior and condition, predation risk perceived by the parents can also directly affect offspring. Sievert et al. indeed show that pups of predator-exposed bank vole females grow faster in smaller litters whereas pups of mothers exposed to conspecific alarm cues show the opposite pattern.

The effects of predation risk can not only affect the first generation of offspring, but may persist in the population through multiple generations. In these cases, the time necessary for the insurgence of inducible antipredator defenses can be key in the effectiveness of the antipredator response in later generations. Graeve et al. tests if inducible response indeed get faster in *Daphnia spp.* if predation risk persists in the population over several generations. In the third generation, individuals had a much faster response and also mounted a stronger response. Moreover, embryos directly exposed to predator cues, in addition to mother's exposure, will have an even stronger response at birth. Thus, combined direct and indirect experience of predation risk may strengthen prey response across multiple generations.

The strongest predator effect on animal communities globally is undoubtedly that of humans. In their hypothesis-paper, Smith et al. discuss novel insights on how animals in the anthropogenic world may exhibit mismatches between the actual risk they are facing and their response to them. Animals may perceive a risk by human activity that is not real, and respond to it even if there would be no need to (assessment mismatch hypothesis). It is easy to think of many species in the modern world that are afraid of people who in the current times would no longer harm them. Animals may also show an under-response to cues of human activity (selection mismatch hypothesis) or the response to the cue could simply be wrong (response mismatch hypothesis), such as freezing behavior in response to a fast approaching vehicle.

In this Research Topic we gathered recent discoveries on the effects of predation risk on prey, from behavioral responses at the individual level to transgenerational effects and cascading effects at population and community levels. These articles open new avenues for investigating predator-prey interactions, which is crucial to better understand the functional role of predators in rapidly changing ecosystems.

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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Mismatch Between Risk and Response May Amplify Lethal and Non-lethal Effects of Humans on Wild Animal Populations

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Human activity has rapidly transformed the planet, leading to declines of animal populations around the world through a range of direct and indirect pathways. Humans have strong numerical effects on wild animal populations, as highly efficient hunters and through unintentional impacts of human activity and development. Human disturbance also induces costly non-lethal effects by changing the behavior of risk-averse animals. Here, we suggest that the unique strength of these lethal and non-lethal effects is amplified by mismatches between the nature of risk associated with anthropogenic stimuli and the corresponding response by wild animals. We discuss the unique characteristics of cues associated with anthropogenic stimuli in the context of animal ecology and evolutionary history to explore why and when animals fail to appropriately (a) detect, (b) assess, and (c) respond to both benign and lethal stimuli. We then explore the costs of over-response to a benign stimulus (Type I error) and under-response to a lethal stimulus (Type II error), which can scale up to affect individual fitness and ultimately drive population dynamics and shape ecological interactions. Finally, we highlight avenues for future research and discuss conservation measures that can better align animal perception and response with risk to mitigate unintended consequences of human disturbance.

Keywords: anthropogenic disturbance, antipredator behavior, conservation behavior, harvest, human-induced fear, predation risk, recreation, risk effects

INTRODUCTION

Humans often fill an outsized role in ecological communities, exerting strong lethal and non-lethal effects on animal populations. People around the world rely on wild animal products (Ripple et al., 2016) and harvest rates by humans far exceed predation rates of wild predators globally (Darimont et al., 2015). Furthermore, many aspects of the built environment (e.g., roads, buildings, and energy infrastructure) or introduced materials (e.g., poisons and plastics) pose an incidental lethal threat to wild animals (Horn et al., 2008; Smallwood, 2013; Loss et al., 2015; Mccardle and Fontenot, 2016). Even where human activity is non-lethal, the impacts of human disturbance can be analogous to the risk effects of predation (Frid and Dill, 2002). Disturbance can induce costly antipredator behaviors (Smith et al., 2017; Suraci et al., 2019) that are known to compromise individual fitness and influence population dynamics in other predator-prey systems, with implications for entire ecosystems (Peckarsky et al., 1993; Peacor et al., 2011; Cherry et al., 2016). Many recent studies have demonstrated that animals perceive and respond to risk associated with human activity and infrastructure, even in the absence of a true threat (Larson et al., 2016; Gaynor et al., 2018). As the human footprint expands across the planet, both the lethal and non-lethal impacts of human disturbance have important implications for global biodiversity conservation.

Human activity is driving global declines of wild animal populations (Dirzo et al., 2014), in part due to the inability of animals to effectively respond to pervasive, rapid environmental change (Sih, 2013). Although humans have co-evolved and interacted with many species for millennia (Bird and Nimmo, 2018) and many animals do effectively navigate the risks posed by people (Oriol-Cotterill et al., 2015; Thurfjell et al., 2017; Ditmer et al., 2018), modern human cues can be especially novel, unreliable, and/or pervasive, leading to mismatches between exhibited behaviors and optimal behavioral responses (Trimmer et al., 2017a). Anti-predator behavior and other risk mitigation strategies, which reduce an individual animal's risk of mortality from predation or other environmental threats, can have high-stakes fitness consequences if over- or under-employed. Furthermore, human-induced changes in animal behavior can potentially alter ecosystems through many pathways, though many have yet to be documented and it remains an important area for future research (Wilson et al., 2020). Risk-response mismatch in human-modified environments is therefore of particular conservation concern (Saul and Jeschke, 2015; Owen et al., 2017; Guiden et al., 2019). In the modern world, many animal populations are exposed to diverse anthropogenic threats while also inhabiting environments saturated with benign anthropogenic stimuli. A failure to respond appropriately to both benign and lethal anthropogenic stimuli can cause or exacerbate population declines.

We contend that the mismatches between risk and response can amplify the unique strength of anthropogenic lethal and non-lethal effects on animal populations (**Figure 1**). A Type I error, in which animals perceive risk from a benign stimulus, can lead to an over-response that amplifies the non-lethal effects of human disturbance. A Type II error, in which animals fail to perceive or respond appropriately to risk from a stimulus that poses a threat, can lead to an under-response that accentuates the lethal effects of human disturbance. Here we discuss how the unique characteristics of anthropogenic cues can drive mismatches between the actual level of risk and animal response, with the potential for population-level consequences. We then argue that, given the pervasiveness of human activity, such mismatches between actual and perceived anthropogenic risk are widespread and may contribute to species declines globally, exacerbating the myriad impacts of humans on wild animal populations. Finally, we suggest avenues for future research to better integrate behavioral risk mismatches into conservation and management strategies.

THE UNIQUE NATURE OF ANTHROPOGENIC RISK CUES

Over evolutionary and ecological time scales, animals develop a set of decision rules for distinguishing between safe and risky situations based on sensory cues (Blumstein and Bouskila, 1996; Welton et al., 2003). When faced with novel anthropogenic stimuli, animals are sometimes able to rely on these decision rules, accurately assess the level of threat, and respond accordingly for an optimal fitness outcome (Sih, 2013). When sensory cues are reliable, animals may also be able to draw on past experience and rapidly develop appropriate responses to changes in their environment (e.g., habituating to a new but benign stimulus; Trimmer et al., 2017b). However, as outlined below, such decision rules and opportunities for learning may break down when animals are faced with the novelty, diversity, and unreliability of anthropogenic stimuli and associated sensory cues, or with the emergence of confounding ecological traps, all of which may limit an animal's ability to appropriately detect, assess, or respond to human activity (Sih et al., 2011; Sih, 2013; Dominoni et al., 2020; **Figure 1**). We propose three alternative hypotheses that may explain the mismatch between risk and response to anthropogenic stimuli in a given situation (**Table 1**):

1. Detection Mismatch Hypothesis: Lethal anthropogenic stimuli produce cues that are not detectable by the animal at the relevant scale of the threat, leading to Type II error.
2. Assessment Mismatch Hypothesis: Non-lethal or lethal anthropogenic stimuli produce detectable cues, but the associated level of risk is not accurately assessed by the animal, leading to Type I or II error.
3. Response Mismatch Hypothesis: Lethal anthropogenic stimuli produce cues that are accurately assessed as risky, but the animal's response is inappropriate or inadequate to reduce the associated threat, leading to Type II error.

Observation of Type I errors (over-response) provide support for the Assessment Mismatch Hypothesis, largely occurring as a result of animals incorrectly assessing a benign anthropogenic stimulus as threatening due to novelty or similarity to predator cues (Frid and Dill, 2002). Observation of Type II errors (under-response) may support any of the above hypotheses (Detection, Assessment, or Response Mismatch) and occur when animals are presented with novel threats to which their sensory capabilities or behavioral decision rules are not well adapted. Each hypothesis also predicts different lethal or non-lethal outcomes. Detection and response mismatch can result in injury or death due to under-response to lethal stimuli, while assessment mismatch may result in either sub-lethal fitness costs (in the case of over-responding to benign stimuli) or death (following under-response to lethal stimuli). Quantifying support for our proposed hypotheses can aid in determining the presence, mechanism, and outcome of risk-response mismatches in animal populations. Below, we discuss how specific characteristics of human cues may cause detection, assessment, and response mismatches in animal populations.

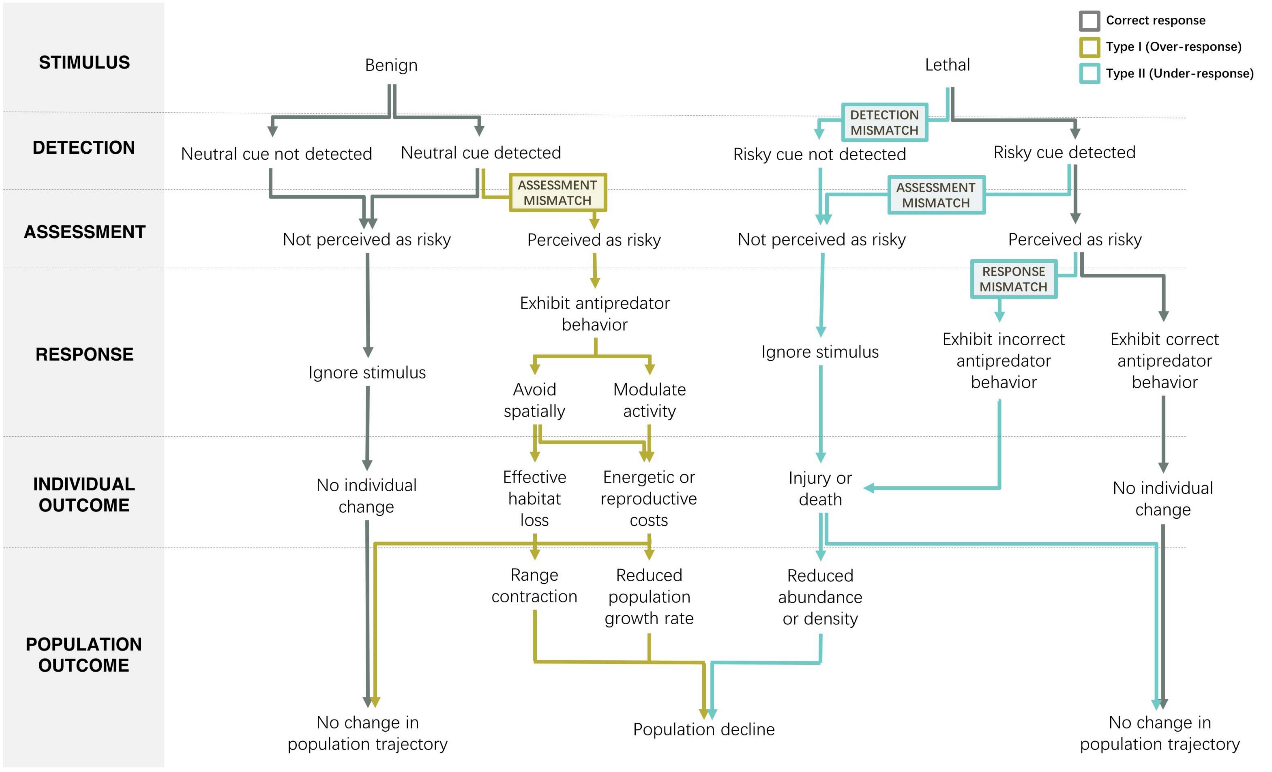


FIGURE 1 | Mismatch in anthropogenic stimuli and animal risk perception and response can lead to population declines. Due to the novel, widespread, and/or unreliable nature of modern anthropogenic cues, animals often fail to align their behavior with the level of risk associated with anthropogenic stimuli. Mismatched risk responses may represent important pathways through which human activity is driving animal declines worldwide. For benign anthropogenic stimuli that pose no threat to the animal, there is the potential for Type I error to occur in the assessment stage if an animal perceives the benign stimulus as risky and responds with unwarranted risk mitigation behavior. Avoiding a benign anthropogenic stimulus in space can reduce habitat utilization, leading to range contraction, and anti-predator responses can also have energetic and reproductive costs that decrease fitness and population growth rates. For lethal anthropogenic stimuli that pose either an intentional or unintentional threat to the animal, the optimal responses pathway may break down at three stages: Type II errors can arise from a failure to detect cues, to assess them as risky, or to respond appropriately. In all cases, an under-response to lethal stimuli can potentially result in injury or death, which may scale up to reduce population abundance and amplify the lethal effects of humans.

Detection Mismatch Hypothesis: Inadequate Detection of Risky Anthropogenic Cues (Type II Error)

Inability to Detect Sensory Stimuli

Some anthropogenic threats may not be detectable due to mismatch between all associated cues and the sensory modalities of the animal (Fisher et al., 2006; Serieys et al., 2015), leading to an unavoidable under-response to risk. This failure to detect risk is responsible for birds colliding with windows, fish consuming toxic plastics, and a range of other unintended phenomena (Loss et al., 2012). In other cases, anthropogenic threats are designed to be undetectable: advances in hunting technology have not only made humans dramatically more lethal than non-human predators (Darimont et al., 2015), but in many cases also serve to minimize the prey's ability to detect the hunter. For instance, while fished populations can quickly learn to avoid spear fishers, which generate many of the cues associated with non-human predators (Tran et al., 2016), large-scale fishing is done remotely using gear like monofilament lines and nets that are effectively invisible to fish (Meekan et al., 2018).

Cue and Risk Separated in Time and Space

Some anthropogenic threats may generate perceptible cues that are disconnected from the threat itself in space and/or time, disrupting an animal's ability to detect the relevant risk cues. For instance, retaliatory killing in response to livestock predation or crop raiding is a major threat to many wildlife populations, but the stimuli that trigger these behaviors, i.e., livestock pens or agricultural fields, may be dissociated from cues of human risk if depredating animals are killed in another place or time (Thapa, 2015; Loveridge et al., 2017). Similarly, animals that are hunted using long range rifles from hundreds of meters away may exhibit less change in habitat use than those exposed to bowhunters who must hunt at much closer range (Thurfjell et al., 2017).

Assessment Mismatch Hypothesis: Incorrect Assessment of Risky or Neutral Anthropogenic Cues (Type I or II Error)

Unreliability of Cues

Many anthropogenic cues are only associated with a threat in certain contexts, making them unreliable indicators of

TABLE 1 | Predicted responses to anthropogenic stimuli associated with three alternative hypotheses for risk-response mismatch.

Hypothesis	Conditions leading to mismatch	Observable outcomes	Examples
Detection mismatch	• Limited sensory capacity of organism (morphological or physiological constraints) • Cue is decoupled from threat in space or time	Type II error • No discernable response to stimulus	• Animal collides with window glass (Loss et al., 2012) • Animal consumes poisons/toxins (Serieys et al., 2015) • Animal does not detect boat cue decoupled in space from fishing equipment (Meekan et al., 2018) • Animal does not substantially avoid or anticipate retaliatory killing (Loveridge et al., 2017)
Assessment mismatch	• Unreliability of cue; may be associated with both lethal and non-lethal human activity • Novelty of cue; does not resemble risk cues encountered in evolutionary history • Cue is also associated with benefit	Type I error • Avoidance of stimulus in time or space • Changes in activity budget, movement, foraging in proximity to stimulus Type II error • Animal may examine or acknowledge stimulus, but exhibits no risk-avoidance response (and may even show attraction to it)	Type I error • Animal avoids non-lethal human infrastructure (Sawyer et al., 2017) • Animal avoids habitats with greater non-lethal recreation (Rösner et al., 2014) Type II error • Animal does not avoid hunters or fishing gear (Thouless et al., 1991; Meekan et al., 2018) • Animal is attracted to resource subsidies increases vulnerability to harvest or accidental death (Johnson et al., 2020) • Toxins present olfactory trap for a foraging animal (Savoca et al., 2017)
Response mismatch	• Novelty of cue; does not resemble risk cues encountered in evolutionary history • Animal exhibits generalized response to all risk cues • Animal has morphological or physiological constraints that limit degree of response	Type II error • Animal exhibits inappropriate response that does not reduce risk • Animal exhibits response of inadequate magnitude	Type II error • Animal freezes in front of vehicle, leading to collision (Mazerolle et al., 2005) • Animal flees vehicle at inadequate speed (Bernhardt et al., 2010) • Animal flees harvest equipment to inadequate distance (Suuronen et al., 1997)

risk. This unreliability makes it difficult for animals to learn how to appropriately assess these cues, or to correctly apply evolved decision rules. Although some species do appear able to differentiate between lethal and non-lethal human activity (McComb et al., 2014; Tran et al., 2016), cue overlap between risky and non-risky humans—for example, the sound of a human voice—may make this distinction difficult for many species and populations, particularly in places where hunting and non-lethal recreation co-occur (Kays et al., 2017). Generalized responses will therefore be associated with an "incorrect" risk perception some of the time: if an animal's generalized response to humans is to avoid or flee, there will be more Type I error (over-response) as a result of benign human activities, but if an animal's generalized response is to ignore people, there will be more Type II error (under-response) when exposed to intentional take. The degree to which these generalized responses scale up to have a net impact on populations will depend on the relative frequency of lethal and benign stimuli in the environment.

Habituation to neutral cues or sensitization to risky cues can potentially reduce erroneous responses to human activity as animals learn from previous experiences with humans. However, when cues are unreliable, habituation can lead to more Type II error (under-response) and sensitization to more Type I error (over-response), furthering the mismatch between underlying risk and response. For instance, DeVault et al. (2016) showed that rock doves (*Columba livia*) that have habituated to the cues of passing vehicles (a benign stimulus when animals are not directly on the road) failed to escape effectively in simulated collision scenarios, suggesting that the subtle difference in context between lethal and benign vehicles (i.e., animal on or off the road) may be difficult for some species to distinguish.

Novelty of Risks and Anthropogenic Disturbances

In addition to being unreliable signals of risk, anthropogenic stimuli are often novel. Even in animal populations that have been long exposed to human activity, the continuous introduction of new technologies and features of the built environment generates cues that have no evolutionary analog (Darimont et al., 2015; Nyhus, 2016). As is the case with invasive predators (Sih et al., 2010; Carthey and Blumstein, 2017), the similarity of novel anthropogenic cues to predator cues and the neophobia of the animal should govern the nature of an animal's response to the novel cue and the most likely form of error. Where novel cues bear little resemblance to familiar stimuli, the likelihood than an animal will assess these cues as risky and respond accordingly may depend on the amount and diversity of predation risk the population has experienced in the past (Cox and Lima, 2006; Ferrari et al., 2015; Ehlman et al., 2019).

Additionally, the risk disturbance hypothesis (Lima and Bednekoff, 1999; Frid and Dill, 2002) suggests that many features of humans and our associated technologies and infrastructure—loud noises, large body size, rapidly approaching objects—do overlap with those of predators and thus elicit responses to generalized predation risk, even where activity is benign (Sih et al., 2010; Trimmer et al., 2017b). However, an evolved fear response to large or fast objects may not adequately prepare animals to appropriately assess deadly anthropogenic cues that are not associated with an approaching human form. Furthermore, in populations that have not experienced predation in their recent evolutionary history, animals may not exhibit any generalized risk response at all, as suggested by the "island tameness hypothesis" (Cooper et al., 2014). In these cases, both ontogenetic and evolutionary naiveté to risky cues contribute to Type II error (Cox and Lima, 2006). Additionally, because humans and associated threats are often highly lethal (Darimont et al., 2015), there may be fewer failed "predation attempts" by hunters, vehicles, or other anthropogenic infrastructure, from which an animal might otherwise learn to associate these novel cues with risk.

Ecological and Evolutionary Traps

Not only do animals often fail to accurately associate risk with anthropogenic stimuli; they may also assign perceived benefit to stimuli that are, in fact, risky (e.g., Johnson et al., 2020). Snakes are attracted to the heat of asphalt on roadways, putting them at risk for vehicle collisions (Mccardle and Fontenot, 2016). In other cases, hunters or fishers will intentionally pair risky stimuli like traps and nets with attractive cues like baits (Carroll, 2007; Mandelman et al., 2008). These maladaptive responses to so-called ecological or evolutionary traps can increase the likelihood of a Type II error, or under-response to risk, due to incorrect assessment of risky cues or miscalibration of resource-safety trade-offs (Robertson et al., 2013).

Response Mismatch Hypothesis: Inappropriate Response to Risky Anthropogenic Cues (Type II Error)

Even in the case of accurate risk detection and assessment, animals may not respond appropriately to anthropogenic threats. In certain situations, an animal's generalized anti-predator response may not reduce anthropogenic risk because the stimulus is novel in the context of a species' evolutionary history. The "deer in headlights" is a clear example of a counterproductive response: by freezing to avoid "predator" detection in response to an oncoming vehicle, animals actually increase their risk of collision (Mazerolle et al., 2005). In other cases, an animal's response may be appropriate but inadequate (Bernhardt et al., 2010), given that the lethality of anthropogenic threats often necessitates extreme responses to risky cues to avoid death. Animals may be limited both behaviorally and physiologically in their responses to risks; for example, the generalized response of many birds to volant predators is to dodge the predator once it is at close range – a response that is ineffective with airplanes (Bernhardt et al., 2010) and vehicles (DeVault et al., 2015). Response mismatches can also occur when humans are intentionally lethal, such as when game species are chased into trees using hound dogs (Bryce et al., 2017) where they can be shot by hunters, or when narwhals exhibit maladaptive physiological responses from net entanglement (Williams et al., 2017).

INDIVIDUAL- AND POPULATION-LEVEL COSTS OF MISMATCH

The overexploitation of wild animals by humans and other forms of incidental killing have clear numerical top-down effects on animal populations, but the behaviorally mediated effects of lethal and non-lethal human activity have received less attention. These behavioral mechanisms and resulting population-level consequences are difficult to measure, but may represent important pathways through which human activity contributes to population declines. Inappropriate behavioral strategies to confront anthropogenic risks can be key impediments to the long-term viability of populations. The incorrect detection, assessment, and response to risky cues can lead to population declines either directly through death (Type II error) or indirectly through stress effects or functional habitat loss (Type I error; **Figure 1**). In order to predict and mitigate population-level consequences and subsequent species declines from anthropogenic stimuli, it is necessary to test: (1) if mismatch is occurring; (2) at which stage mismatch occurs (detection, assessment, or response); and (3) why the mismatch occurs.

Costs of Type I Errors

The Assessment Mismatch Hypothesis predicts that the unreliability or novelty of human cues can result in a generalized risk response to benign anthropogenic stimuli. Signal detection and error management theory show that, in general, animals tend to overrespond to potentially risky stimuli (Johnson et al., 2013; Orrock et al., 2015), given that the possibly lethal cost of Type II error (under-response) is greater than the energetic and opportunity costs of Type I error (over-response; Bouskila and Blumstein, 1992). However, given the many neutral anthropogenic cues that animals may associate with some degree of risk, this "erring on the side of caution" can be costly (Fardell et al., 2020). Mismatch can thus amplify the risk or non-lethal effects of anthropogenic stimuli, which can have a far greater cost than those from predation given the widespread nature of human disturbance (Venter et al., 2016) and the maladaptive nature of Type I errors (Carrete and Tella, 2013; Møller et al., 2014). For populations prone to neophobia or generalized risk responses, the costs of responding to pervasive and continuously changing human stimuli may quickly reach unsustainable levels.

In response to perceived risk from people, animals may avoid areas of higher human use (Rösner et al., 2014; Patten and Burger, 2018; Wisdom et al., 2018) or times of day when people are most active (Gaynor et al., 2018; Nickel et al., 2020), preventing them from accessing resources (Kerley et al., 2002; Smith et al., 2015). While opportunity costs on individual fitness can be difficult to quantify, avoidance of human activity has been linked to range contraction and reduced habitat availability for several populations (Merenlender et al., 2009; Rogala et al., 2011; Heinemeyer et al., 2019). This functional habitat loss, in addition to physical habitat loss, is rarely quantified when identifying available habitat for populations (Northrup et al., 2015). Avoidance of anthropogenic structures dramatically reduces the habitat and resources available to animals through the act of over-response to human activity (Lesmerises et al., 2018; Dwinnell et al., 2019).

Over-response to anthropogenic stimuli can also have direct energetic, reproductive, or physiological costs for wild animals. In the presence of humans, increased vigilance and reduced feeding time can lead to lower energetic intake (Stockwell et al., 1991; Fernández-Juricic et al., 2003; Blumstein et al., 2005; Smith et al., 2015, 2017). Energy expenditure can increase as a result of flight in the presence of people or increased movement in areas with higher human disturbance (Bradshaw et al., 1998; Stankowich, 2008). A heightened perception of risk in response to people can also increase stress levels (Creel et al., 2002; Müllner et al., 2004; Amo and López, 2006; Zbyryt et al., 2017). Furthermore, human disturbance has been found to interfere with reproductive activities: for example, ecotourism has been shown to negatively influence ruddy turnstone (*Arenaria interpres*) nesting success (Beale and Monaghan, 2004), hoatzin (*Opisthocomus hoazin*) chick survival (Müllner et al., 2004), yellow-eyed penguin (*Megadyptes antipodes*) fledgling weight (McClung et al., 2004), and California sea lion (*Zalophus californianus*) population growth rates (French et al., 2011).

Costs of Type II Errors

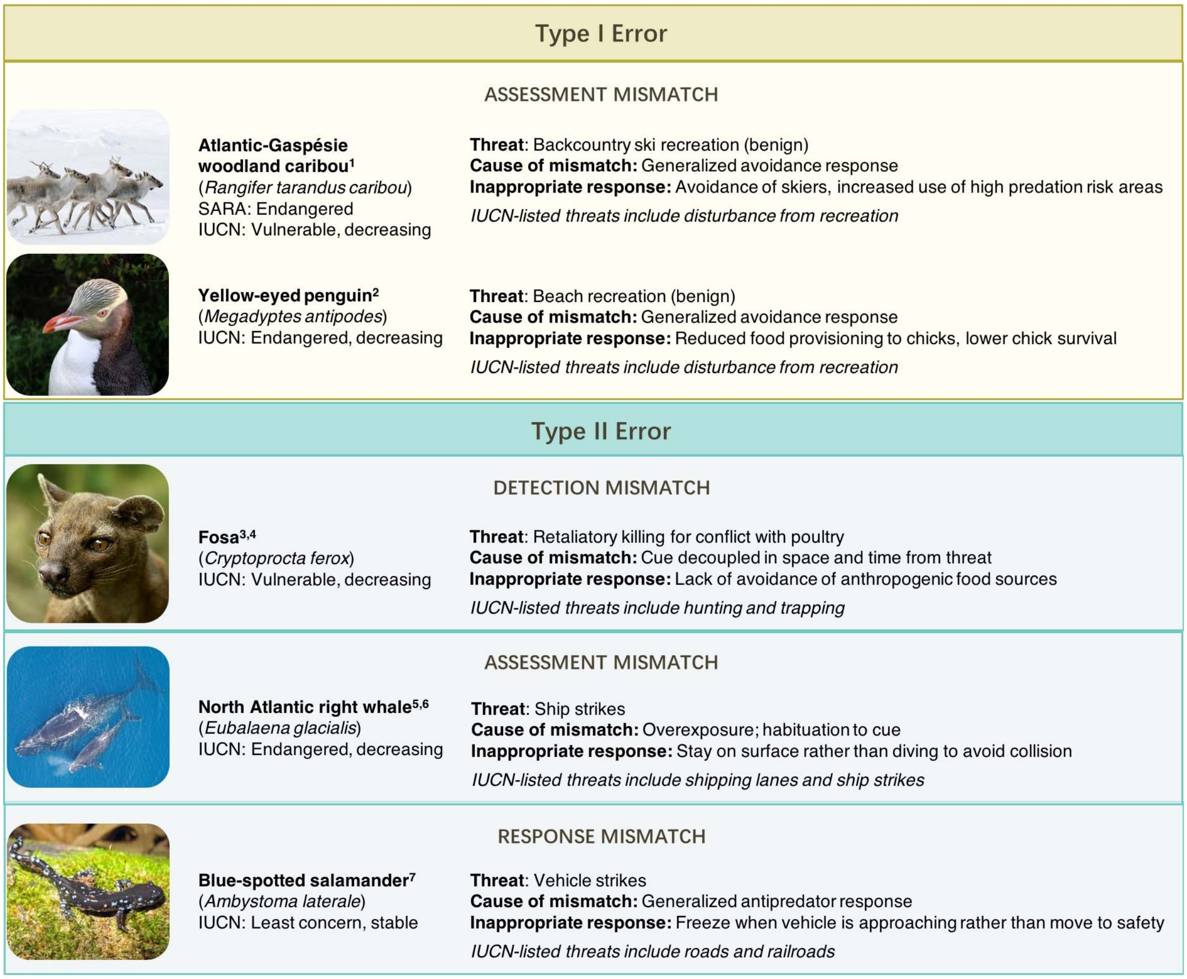
The costs of a Type II error, in which an animal perceives a risky stimulus as safe, are more straightforward to quantify, as under-responding to lethal stimuli often results in death or injury. These lethal impacts of humans have been well-documented (Darimont et al., 2015). While the magnitude and effectiveness of human hunting can be largely attributed to technology and demand, it is also likely that the numerical effects of hunting are amplified by the failure of animals to accurately associate cues with risk for the reasons discussed above. Animals are easier to encounter, detect, and capture if they do not associate risk with stimuli such as vehicles, boats, radar, scopes, guns, or traps, or with humans themselves (e.g., Donadio and Buskirk, 2006). For example, during the late Pleistocene extinctions in the Americas, naïve prey under-responded to human hunters, which magnified the numerical effect of harvest (Cox and Lima, 2006). Novelty, unreliability, and spatiotemporal disconnect continue to result in high rates of human caused mortality (both intentional and unintentional) and species declines. Associated under-responses to risk contributed to the recent extinction of a number of species, including the Saudi gazelle (*Gazella saudiya*; Thouless et al., 1991) and Caribbean monk seal (*Neomonachus tropicalis*; McClenachan and Cooper, 2008). Many of the unintentional causes of animal mortality are also exacerbated by the failure of individual animals to associate them with risk and avoid them accordingly, including roads, infrastructure like glass windows and powerlines, pollutants and poisons, and accidental killing (e.g., nest destruction) through agriculture and forestry (Mazerolle et al., 2005; Bernhardt et al., 2010). Ultimately, the costs of individual Type II errors likely contribute to source-sink dynamics, population declines, and local extinctions. Because Type II errors can occur at multiple stages, testing of the Detection, Assessment, and Response Mismatch Hypotheses can elucidate the mechanism of risk-response mismatch in vulnerable populations.

CONSERVATION APPROACHES TO REALIGN STIMULI AND RISK PERCEPTION

While studies directly linking over- or under-response to anthropogenic stimuli with population declines remain rare, the costs associated with inappropriate responses to human risk suggest that these mechanisms may constitute major threats to some wildlife species (**Figure 2**), contributing to heightened extinction risk. The expansion of key research areas would aid in our ability to predict where and when behavioral mismatches are likely to be of conservation concern and suggest potential mitigation strategies to alleviate the impacts of inappropriate responses to anthropogenic stimuli.

For Type I errors, the potential for habituation to neutral human cues is a primary factor determining whether short-term, sublethal costs of responding to humans (e.g., increased vigilance and changes in habitat use) will translate into long-term, population-level impacts. Some wildlife populations have been suggested to habituate relatively quickly to anthropogenic disturbances initially perceived as threatening (e.g., non-lethal recreation and ecotourism; Bateman and Fleming, 2017). Individuals in these populations are able to learn, through repeated exposures, to dissociate human cues from risk, thus reversing the risk assessment mismatch and reducing or avoiding downstream costs (**Figure 1**). In recreational areas, habituation can be facilitated by creating fencing or buffers that make human activity appear less threatening and more predictable (Weston et al., 2012). In other cases, responses to disturbance appear to be highly persistent. For instance, mule deer (*Odocoileus hemionus*) avoidance of resource extraction infrastructure has been shown to persist for at least 15 years and across generations, potentially contributing to population declines (Sawyer et al., 2017). The speed with which habituation occurs, or whether it occurs at all, will determine the magnitude of any long-term impacts on a population exposed to a benign anthropogenic stimulus. However, the drivers of variation in the likelihood and extent of habituation to human stimuli are not well understood and are likely a function of both stimulus type and ecological context (e.g., similarity between anthropogenic cues and those of actual predators).

Additionally, even habituation (e.g., reduced population-average response to human disturbance with time) may not imply an absence of Type I error costs at the individual level (Bejder et al., 2009; Blumstein, 2016). Reduced responsiveness to human disturbance at the population level may be the result of a "personality filter" (Wat et al., 2020) wherein less tolerant individuals avoid the disturbance source (thus experiencing effective habitat loss) while a subset of more human-tolerant



Type I Error

ASSESSMENT MISMATCH

Atlantic-Gaspésie woodland caribou[1]
(*Rangifer tarandus caribou*)
SARA: Endangered
IUCN: Vulnerable, decreasing

Threat: Backcountry ski recreation (benign)
Cause of mismatch: Generalized avoidance response
Inappropriate response: Avoidance of skiers, increased use of high predation risk areas
IUCN-listed threats include disturbance from recreation

Yellow-eyed penguin[2]
(*Megadyptes antipodes*)
IUCN: Endangered, decreasing

Threat: Beach recreation (benign)
Cause of mismatch: Generalized avoidance response
Inappropriate response: Reduced food provisioning to chicks, lower chick survival
IUCN-listed threats include disturbance from recreation

Type II Error

DETECTION MISMATCH

Fosa[3,4]
(*Cryptoprocta ferox*)
IUCN: Vulnerable, decreasing

Threat: Retaliatory killing for conflict with poultry
Cause of mismatch: Cue decoupled in space and time from threat
Inappropriate response: Lack of avoidance of anthropogenic food sources
IUCN-listed threats include hunting and trapping

ASSESSMENT MISMATCH

North Atlantic right whale[5,6]
(*Eubalaena glacialis*)
IUCN: Endangered, decreasing

Threat: Ship strikes
Cause of mismatch: Overexposure; habituation to cue
Inappropriate response: Stay on surface rather than diving to avoid collision
IUCN-listed threats include shipping lanes and ship strikes

RESPONSE MISMATCH

Blue-spotted salamander[7]
(*Ambystoma laterale*)
IUCN: Least concern, stable

Threat: Vehicle strikes
Cause of mismatch: Generalized antipredator response
Inappropriate response: Freeze when vehicle is approaching rather than move to safety
IUCN-listed threats include roads and railroads

1. Lesmerises et al. (2018). 2. McClung et al. (2004). 3. Kotschwar et al. (2015). 4. Merson et al. (2019). 5. Nowacek et al. (2004). 6. Parks et al. (2011). 7. Mazerolle et al. (2005).

FIGURE 2 | Conservation implications of risk-response mismatch. Examples of species whose declines may be accelerated as a result of Type I and Type II errors in anthropogenic risk detection, assessment, and response. Global species status shown from the International Union of Concerned Scientists (IUCN); Canadian population status shown for Atlantic-Gaspésie woodland caribou from the Species at Risk Act (SARA).

individuals continues to use disturbed areas (Samia et al., 2015). Animals that fail to avoid human disturbance may also be restricted in their ability to leave disturbed areas due to a lack of suitable habitat elsewhere (Blumstein, 2016), despite experiencing persistent sub-lethal costs from fear of humans. Human disturbance can act as a selection pressure, shifting the population toward bolder individuals that are more tolerant of benign human stimuli (Arroyo et al., 2017). However, increased boldness may have other indirect costs such as decreased breeding success (through reduced parental care) and increased susceptibility to predation from non-human predators (Geffroy et al., 2015).

Mitigation of the conditions that promote Type I and II errors can be achieved when mechanisms of risk and response mismatches are better understood (**Figure 3**). Novel cues, such as those produced by many human activities, may either elicit a fear response upon first encounter (i.e., neophobia; Crane et al., 2019) or may require learning to be recognized as risky (Griffin, 2004). Theory suggests that the likelihood of a species responding to a novel predator cue will increase with background levels of risk in the animal's environment and the diversity of predators with which the species has experience (Blumstein, 2006; Ehlman et al., 2019). Empirical work examining the generality of these predictions for wildlife responses to human stimuli (e.g., whether greater predator diversity leads to a higher probability of Type I responses to neutral human cues) will improve our ability to anticipate where deleterious behavioral responses to benign human stimuli are likely to occur. In other cases, a lack of overlap between the risky cues of lethal human stimuli and those of predators with which a species is familiar may necessitate learning to avoid Type II misclassification of such cues as neutral. In an analogous scenario – the exposure of naïve prey to a novel predator through invasion or reintroduction – predator training programs have been used to help prey learn an appropriate fear response to the novel predator by pairing predator cues with aversive stimuli (e.g., Van Heezik et al., 1999). Such programs

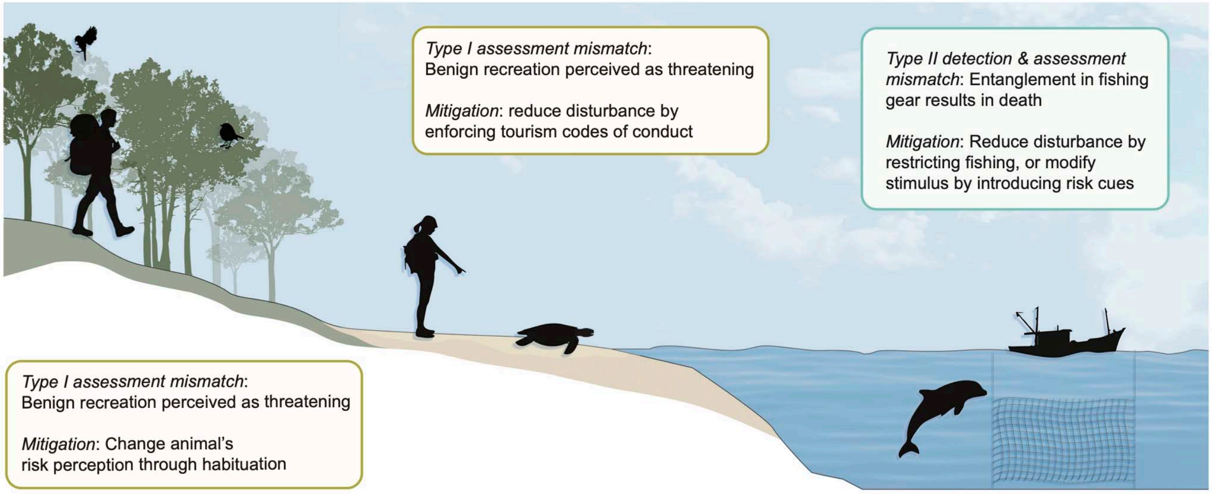


FIGURE 3 | Examples of mitigation measures for reducing risk-response mismatch in wild animals. Managers can employ several strategies to mitigate costly consequences of mismatch between anthropogenic risk and response in wild animal populations. The design of interventions should be informed by the type of mismatch (detection, assessment, or response, and Type I or II error), the nature of the anthropogenic stimulus, and the behavioral plasticity of the target species. One mitigation approach for Type II assessment errors is to gradually habituate animals to a benign stimulus, such as non-consumptive outdoor recreationists. Another strategy, which can be employed for all types of mismatch, is to reduce animal encounters with the disturbance stimulus by minimizing the disturbance (e.g., mitigating ecotourism-induced disturbances). The stimulus itself can also be modified to be more detectable (for Type II detection error) or more fear-inducing (for Type I or II assessment error) – these approaches can be employed to minimize fisheries bycatch.

have been met with mixed success (Blumstein et al., 2019), but may be valuable for efforts to reintroduce species susceptible to novel anthropogenic threats. For instance, using aversive conditioning to train "problematic" carnivores or reintroduced herbivores to associate humans and their settlements with risk may aid in reducing human-wildlife conflict and retaliatory killings (Petracca et al., 2019; Young et al., 2019). This approach may be particularly effective in species where fear responses are culturally transmitted from older to younger individuals (McComb et al., 2014).

Fear conditioning to human stimuli represents one example of a broader set of potential conservation strategies for mitigating the impacts of behavioral mismatch, which can be designed to realign risk perception with the actual level of threat posed by human stimuli. Efforts to match risk and response can either increase the risk associated with lethal stimuli or decrease perceived risk of benign stimuli. For animals in which detection mismatch is the source of increased risk, conservation interventions can focus on introducing perceptible cues (e.g., bird-safe glass) or removing the threat (e.g., proper disposal of plastics). When Type I assessment mismatch occurs, repeated exposure of young individuals to non-threatening human encounters can be employed to intentionally habituate wildlife to benign recreational activity (Blumstein, 2016). Type II assessment mismatches can be managed to increase animal responses to risky human cues; recent proposals have called for amplifying predation risk associated with human hunters by increasing hunter cue intensity, which may help to reduce human-wildlife conflict (Cromsigt et al., 2013). Moving forward, we suggest that the potential for mismatched behavioral responses to human cues must be a key consideration when designing species recovery and reintroduction programs or when opening otherwise low disturbance areas to increased recreational or development activity. Indeed, some conservation and management programs have already begun to incorporate information on potential mismatches between anthropogenic risk and animal responses, confirming the importance of such approaches for effective wildlife management. Here we highlight three conservation scenarios in which managing for potential risk-responses mismatches shows particular promise (**Figure 3**).

Mitigating Non-lethal Impacts of Recreation Through Habituation

Outdoor recreation (e.g., hiking) can substantially impact animals if hikers are perceived as risky, leading to costly changes in time allocation and effective habitat loss (Taylor and Knight, 2003; Patten and Burger, 2018). Here, the costs of Type I over-response to humans stem from a mis-assessment of passive recreation activities as risky, suggesting that habituating animals to hikers may help reconcile outdoor recreation with wildlife conservation (Larson et al., 2016). The predictability of recreational activity can facilitate such habituation; red deer (*Cervus elaphus*) exhibit minimal responses to hikers on well-used trails while strongly avoiding off-trail hikers (Westekemper et al., 2018) and northern cardinals (*Cardinalis cardinalis*) are less likely to flush when people remain on trails (Smith-Castro and Rodewald, 2010). Increasing predictability of human activity by enforcing "stay on trail" rules in protected areas may therefore decrease recreation impacts on wildlife by accelerating habituation (**Figure 3**). Intentional habituation (e.g., intensive experimental trail use or presentation of human cues along trail networks) could be valuable in protected areas transitioning to increased public access (Blumstein, 2016).

Addressing the Costs of Ecotourism-Induced Assessment Mismatches

Ecotourism activities that provide opportunities to view wildlife at close range can bring in financial resources to support wildlife conservation (Krüger, 2005). However, these efforts come at a cost to wildlife if resulting behavioral modifications have energetic or demographic consequences (Ellenberg, 2017; Geffroy et al., 2017). Deleterious effects of ecotourism are enhanced for wildlife that are large, conspicuous, predictable, and found in open habitats (Green and Higginbottom, 2000). Efforts to examine the fitness consequences of ecotourism-induced risk-response mismatches in conspicuous wildlife are necessary to meaningfully improve wildlife watching practices. In sea turtles, for example, ecotourism to watch nesting females lay their eggs has particularly strong effects on nesting behaviors, including reduced time spent covering the recently laid eggs (Smith et al., in press). However, behavior changes induced by these guided "turtle watch groups" that follow evidence-based guidelines, such as staying outside of the field of view of the turtle, have not been found to result in measurable consequences for reproductive behaviors including clutch size, hatching success, or emergence success (Johnson et al., 1996; Smith et al., in press). The important disconnect between ecotourism-induced behavior change and reproductive success in nesting sea turtles [and minke whales, (*Balaenoptera acutorostrata*); Christiansen and Lusseau, 2015] indicates that assessment mismatch doesn't intrinsically lead to individual-level consequences. However, ecotourists that do not comply with turtle-viewing codes of conduct overwhelmingly cause sea turtles to abort their nesting attempts (Waayers et al., 2006). In this case, tools to reduce demographic impacts of sea turtle ecotourism are already being studied and implemented (Smith et al., in press), and simply enforcing conservative codes of conduct should reduce Type I assessment errors made by nesting females exposed to ecotourist groups (**Figure 3**). In ecotourism systems that do not yet have institutional or established codes of conduct, research on the fitness consequences of behavior change is essential to develop protocols that mitigate the impact of potential assessment mismatches.

Reducing Bycatch in Fisheries

Fisheries bycatch of non-target species has contributed to the declines of many species of conservation concern (Lewison et al., 2004). Animals that are unable to detect intentionally invisible fishing equipment (detection mismatch) or do not associate equipment with risk (assessment mismatch) can under-respond and face capture at high rates (Werner et al., 2015). Although many attempts to reduce bycatch have been pursued through mechanical innovations (e.g., bycatch reduction devices; Vasapollo et al., 2019), behavioral approaches are being increasingly developed and implemented. Modifications to fishing equipment that better align risk and assessment in non-target animals have been introduced to minimize bycatch (Gilman, 2011), including introduction of risk cues alongside fishing equipment so that the gear is detected, perceived as risky, and avoided by bycatch species (Southwood et al., 2008; Jordan et al., 2013; **Figure 3**). Specific sensory tools include acoustic deterrents (Southwood et al., 2008; Waples et al., 2013) olfactory or electrosensory cues (Jordan et al., 2013), LED lights (Bielli et al., 2020), or predator decoys (Bostwick et al., 2014). For species that are not consistently excluded using bycatch reduction devices [e.g., smalleyed round stingray (*Urotrygon microphthalmum*); Willems et al., 2016], innovations in behavioral deterrents may be necessary to reduce capture. Further study is needed to understand the long-term effectiveness of behavioral bycatch reduction tools, the cost and durability for their large-scale application, and the potential loss of target catch.

CHALLENGES IN UNDERSTANDING ANIMAL PERCEPTION OF RISK

Despite many examples of apparent risk-response mismatch in wildlife, it is often challenging, if not impossible, for scientific observers to concretely assess perception of risk among free-ranging animals (Gaynor et al., 2019). In many instances of risk-response mismatch, it may be the case that animals do correctly detect and assess lethal anthropogenic risk but choose not to respond, perhaps because they perceive other threats (e.g., conspecifics, competitors, starvation, or thermal conditions) to be greater than the risk posed by humans. For instance, female moose with calves show increased use of roadways in Yellowstone National Park, United States, despite the potential risk of vehicle mortality, presumably because moose calf predators (i.e., large carnivores) tend to avoid high human presence around roads (Berger, 2007). Animals may also determine that the benefits associated with human activity outweigh the risks, as in the case of brown bears in Banff National Park, Canada, which are attracted to spilled grain on railway tracks despite risk of train collision (St. Clair et al., 2019). Because human activities often alter many aspects of an animal's environment – including creating heat islands, benefiting synanthropic competitors, and modifying food availability – the context- and state-dependence of animal decision-making may result in an adaptive behavior that appears to researchers to be a risk-response mismatch. Similarly, an animal may perceive relatively low risk from benign human activities, yet it might assess that the cost of responding is minimal and therefore exhibit anti-predator behavior. Complex risk trade-offs undermine the ability of researchers to fully understand the role of animal perception in influencing responses to predation risk, even in predator-prey interactions with a long evolutionary history. The many environmental and ecological changes associated with anthropogenic activities (e.g., resource subsidies, altered competition and predation regimes, and cue inundation) further complicate the assessment of risk perception in animals. Experimental exposure of anthropogenic cues to animals across disturbance and environmental gradients can be used to better tease apart the mechanisms of behavioral responses and to explore the contexts in which risk-response mismatches occur (Smith et al., 2020).

CONCLUSION

Human activity has rapidly and dramatically reshaped ecosystems worldwide, and many species have been unable to adapt to the novelty and diversity of both lethal and non-lethal disturbances. Animals cannot always rely on existing sensory mechanisms and decision rules to accurately detect, assess, and respond to lethal and benign anthropogenic stimuli due to the unique nature of human cues. The resulting over-responses to benign stimuli (Type I error) and under-responses to lethal stimuli (Type II error) can be costly, and likely contribute to the outsized lethal and non-lethal effects of humans. Mismatches between anthropogenic risk and animal response may therefore represent an important but often-overlooked pathway linking anthropogenic disturbance to global defaunation and animal declines. Beyond the individual- and population-level consequences discussed here, risk-response mismatch in animals also likely affect species interactions and community-level biodiversity by changing the behavior, density, or distribution of important predators, competitors, or prey (Wilson et al., 2020). Future research is needed to understand the contribution of animal physiology and evolved behavioral strategies to errors in risk response, the processes by which errors scale up to negative consequences at the population level, and which conservation approaches can best alleviate unintended mismatches in risk response. Our hypotheses regarding the underlying drivers of mismatch can serve as a starting point to addressing these critical questions. By understanding how animals adapt (or fail to adapt) to novel anthropogenic stimuli, either through learning or natural selection, we can better design management interventions to mitigate unintended consequences of human activity and conserve vulnerable species.

AUTHOR CONTRIBUTIONS

All authors contributed equally to the conception of this work and to the writing, and approved it for publication.

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Nonconsumptive Predator Effects on Prey Demography: Recent Advances Using Intertidal Invertebrates

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Predators influence prey demography through consumption, but the mere presence of predators may trigger behavioural changes in prey that, if persistent or intense, may also influence prey demography. A tractable system to study such nonconsumptive effects (NCEs) of predators involves intertidal invertebrates. This mini review summarises recent research using barnacles and mussels as prey and dogwhelks as predators. The field manipulation of dogwhelk density revealed that pelagic barnacle larvae avoid benthic settlement near dogwhelks, which limits barnacle recruitment, a relevant outcome because recruitment is the only source of population replenishment for barnacles, as they are sessile. This avoidance behaviour is likely triggered by waterborne dogwhelk cues and may have evolved to limit future predation risk. Increasing densities of barnacle recruits and adults can prevent such NCEs from occurring, seemingly because benthic barnacles attract conspecific larvae through chemical cues. Barnacle recruit density increased with the abundance of coastal phytoplankton (food for barnacle larvae and recruits), so barnacle food supply seems to indirectly limit dogwhelk NCEs. By inhibiting barnacle feeding, dogwhelk cues also limited barnacle growth and reproductive output. Wave action weakens dogwhelk NCEs likely through hydrodynamic influences. Dogwhelk cues also limit mussel recruitment, as mussel larvae also exhibit predator avoidance behaviour. The NCEs on recruitment are weaker for mussels than for barnacles, possibly because mussel larvae can detach themselves after initial settlement, an ability that barnacle larvae lack. Overall, these field experiments provide evidence of predator NCEs on prey demography for coastal marine systems.

Keywords: barnacle, demography, mussel, *Mytilus*, *Nucella*, predation risk, *Semibalanus*, whelk

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INTRODUCTION

Predators influence the demography of prey through the consumption of organisms. The mere presence of predators, however, may trigger behavioural changes in prey that may ultimately also influence prey demography. For example, when detecting predator cues, prey can move away (Werner and Peacor, 2003; Keppel and Scrosati, 2004; Metaxas and Burdett-Coutts, 2006;

Zanette and Clinchy, 2019) or reduce feeding activities (Peacor and Werner, 2000; Schmitz et al., 2008; Hermann and Thaler, 2014; Urban and Richardson, 2015; Boudreau et al., 2018) to limit predation risk. Depending on the magnitude and persistence of such behavioural responses, demographic consequences may result. These consequences can be referred to as nonconsumptive effects (NCEs) of predators on prey demography.

Predator effects on prey behaviour typically occur soon after predator cues are detected by prey, so they have been studied for many terrestrial and aquatic species (Peacor et al., 2020). However, due to their inherent complexity and longer times to be expressed, predator NCEs on prey demography have historically been less studied. As they are seemingly widespread, however (Peckarsky et al., 2008), their study has been gaining traction in recent years. Thus, for example, negative predator NCEs on prey reproduction (Creel et al., 2011; Zanette et al., 2011; Mukherjee et al., 2014; Dulude-de Broin et al., 2020), recruitment (Benkwitt, 2017), and survival (MacLeod et al., 2018) have been described for terrestrial and aquatic vertebrates and on prey survival for freshwater invertebrates (McCauley et al., 2011; Siepielski et al., 2014).

Given the large animal diversity on Earth, it is worth examining how predator NCEs on prey demography may take place in organisms with different life histories and living in different environments. Such an approach will enrich our understanding of the array of responses and underlying mechanisms that can be found in nature. This mini review focuses on intertidal sessile organisms as prey. In rocky intertidal habitats (those between the highest and lowest tides on marine rocky shores), sessile filter-feeders are often abundant, especially barnacles and mussels (Menge and Menge, 2013; Valdivia et al., 2015; Scrosati and Ellrich, 2018). Because sessile organisms remain attached to the substrate, monitoring their demography can be easily done, especially during low tides when such habitats can be safely accessed on foot. In addition, their main predators are often benthic invertebrates (e.g., snails) that move slowly across the substrate, which facilitates their field manipulation. Therefore, in recent years, studies have used these organisms to enrich our knowledge on how predator NCEs on prey demography can take place. This mini review summarises the main findings of such studies.

MODEL SPECIES AND RELEVANT LIFE-HISTORY TRAITS

A convenient model prey species is the barnacle *Semibalanus balanoides* (**Figure 1**), which is often abundant in North Atlantic rocky intertidal communities (Jenkins et al., 2000; Scrosati and Heaven, 2007). Adults are benthic and live permanently attached to the rocky substrate. They reproduce through pelagic larvae that undergo various nauplius stages for 5–6 weeks in coastal waters (Bousfield, 1954) until reaching the final stage (cyprid), which lives on its own reserves without feeding and seeks benthic settlement (Minchinton and Scheibling, 1991). Soon after a cyprid settles on a substrate, it metamorphoses into a recruit, which looks as a typical barnacle but is small (**Figure 1**). For barnacles, then, settlement refers to the permanent contact that pelagic cyprid larvae establish with the substrate, while recruitment is the appearance of new benthic organisms on the substrate as a result of the metamorphosis of settled cyprid larvae.

Recruitment is a key demographic step for barnacles because it is the only source of population replenishment, as adult migration is impossible because of their sessile nature. To find suitable substrate for settlement, cyprids of *S. balanoides* follow chemical cues produced by benthic conspecifics (Gabbott and Larman, 1987; Crisp, 1990; Hills and Thomason, 1998). In contrast, cyprids of this barnacle species are repelled by chemical cues from its main benthic predator, the dogwhelk *Nucella lapillus* (**Figure 1**; Ellrich et al., 2015a). Thus, the field manipulation of dogwhelk density has been useful to understand dogwhelk NCEs on barnacle recruitment. NCE intensity is in turn modulated by biotic and abiotic factors. Ultimately, by inhibiting barnacle feeding, dogwhelk cues affect barnacle reproduction, another key demographic rate. These findings are discussed below.

Blue mussels (*Mytilus* spp.; **Figure 1**) are also convenient model prey species for NCE research, as they are also sessile organisms with pelagic larvae and also frequently occur on North Atlantic rocky intertidal communities (Hunt and Scheibling, 2002; Tam and Scrosati, 2011; Scrosati and Ellrich, 2018). Intertidal blue mussels are also commonly preyed upon by *N. lapillus* (Crothers, 1985; Sherker et al., 2017). Thus, dogwhelk NCEs on mussel recruitment have also been investigated, which is also discussed below.

DOGWHELK NCEs ON BARNACLE SETTLEMENT AND RECRUITMENT

Predator NCEs on barnacle settlement and recruitment were studied by manipulating dogwhelk density in rocky intertidal habitats in Nova Scotia, Canada, that experience a moderate degree of wave exposure. An experimental unit was a cage divided with mesh in a central compartment and a peripheral compartment (see photos in Ellrich et al., 2015a). The central compartment hosted a plate covered by a tape with a sandpaper texture (Permastik anti-skid safety tread, RCR International, Boucherville, QC, Canada) that provided a settlement substrate for cyprids similar to the natural rocky substrate (Ellrich et al., 2016b). Cyprids were free to access the central compartment during high tides through the cage's mesh. The used mesh type was found not to alter water flow in caging experiments done in intertidal habitats (Beermann et al., 2013). The peripheral compartment surrounded the central compartment and had either no dogwhelks or dogwhelks at natural densities. The caged dogwhelks were unable to access the central compartment, but their waterborne chemical cues could reach it during high tides.

In Atlantic Canada, cyprids of *S. balanoides* settle on intertidal substrates in May and June, which is thus the recruitment season for this species (Scrosati, 2020). Thus, to investigate dogwhelk NCEs, cages of both treatments were installed at the intertidal zone in late April. Barnacle settlement (density of settled cyprids) was measured in mid-May, while barnacle recruitment (recruit density) was measured in late June, once new recruits no longer appeared on the substrate. All macroalgae and sessile invertebrates were previously removed from the vicinity

FIGURE 1 | (A) Barnacles (*Semibalanus balanoides*), including (a) adults and (r) recruits (recruits being 1–2 mm in shell diameter), **(B)** blue mussels (*Mytilus* spp.; shell length of up to a few cm), and **(C)** dogwhelk (*Nucella lapillus*; shell length typically of up to a few cm) from rocky intertidal habitats on the Nova Scotia coast, in Atlantic Canada. Photographs taken at low tide by the author.

of the cages to eliminate their possible influences on barnacle recruitment (Jenkins et al., 1999; Beermann et al., 2013). The caged dogwhelks were not fed during the experiments but, to prevent starvation, they were replaced every 2 weeks with new dogwhelks. More details on methods are provided in Ellrich et al. (2015a, 2016a).

Dogwhelk presence decreased barnacle larval settlement by an average of 69% (Ellrich et al., 2016a) and barnacle recruitment by experimentwise averages of 51–83% (Ellrich et al., 2015a). These results suggest that cyprids exhibited an avoidance behaviour in the presence of waterborne dogwhelk cues, ultimately decreasing benthic recruitment. Such a decrease should be demographically relevant because recruits are the only source of barnacle population replenishment, as these are sessile organisms. A lower recruitment might also limit reproduction because, barnacles being internal cross-fertilisers, reproductive success depends on the proximity to neighbours (Anderson, 1993). On the Japanese Pacific coast, dogwhelks (*Nucella lima*) were also found to exert negative NCEs on barnacle (*Balanus glandula* and *Chthamalus dalli*) recruitment (Yorisue et al., 2019).

BIOTIC DRIVERS OF NCE INTENSITY: DOGWHELK DENSITY

The value of dogwhelk density used in the cages referred to above (3 dogwhelks dm^{-2}) was common on the shore. A separate field experiment showed that lower densities cause either a weaker limitation of barnacle recruitment or, if too low, no limitation at all (Ellrich et al., 2015b). This finding is consistent with increases in predator density increasing levels of waterborne predator cues (Loose and Dawidowicz, 1994; von Elert and Ponert, 2000; Kesavaraju et al., 2007; Ferland-Raymond et al., 2010).

BIOTIC DRIVERS OF NCE INTENSITY: BARNACLE DENSITY AND FOOD SUPPLY

Chemical cues from adult barnacles attract conspecific cyprids that are seeking settlement (Gabbott and Larman, 1987; Prendergast et al., 2008). This is thought to allow cyprids to find suitable habitat for benthic development (Clare, 2011), a critical choice because recruits cannot move away after metamorphosis from a settled cyprid. Therefore, a field experiment found that the presence of adult barnacles can prevent the occurrence of dogwhelk NCEs on barnacle recruitment (Ellrich et al., 2016b). Barnacle recruit density has similar effects. Under recruit densities of up to experimentwise averages of 200 recruits dm^{-2}, dogwhelk cues (from 3 dogwhelks dm^{-2}) limited barnacle recruitment by 51–83% (Ellrich et al., 2015a), but no NCEs occurred under recruit densities averaging 300 recruits dm^{-2} (Ellrich et al., 2015a). The absence of NCEs at high recruit densities may have resulted from an abundance of cyprid settlement cues produced by the quickly accumulating recruits (Shanks, 2009) and by more abundant chemical footprints left by cyprids exploring the substrate for settlement, which also attract conspecific cyprids (Yule and Walker, 1985; Phang et al., 2008). Settling cyprids might also become less selective themselves under high densities. Ultimately, food supply may have been critical for the occurrence of the high recruit densities that prevented dogwhelk NCEs from happening. In barnacles, the pre-cyprid larval stages (nauplii) and the recruits feed on phytoplankton (Anderson, 1993). The high recruit densities noted above occurred under a high coastal phytoplankton abundance (Ellrich et al., 2015a), which may have enhanced the survival of larvae and recruits (Scrosati and Ellrich, 2016, 2018), thus increasing their density.

DOGWHELK NCEs ON BARNACLE GROWTH AND REPRODUCTIVE OUTPUT

A laboratory experiment showed that waterborne cues from *N. lapillus* limit feeding activity in adult *S. balanoides* (Johnston et al., 2012), presumably because the cirral swipes that barnacles make to harvest plankton can also disperse metabolites that attract dogwhelks (Barnes, 1999). Correspondingly, a field experiment showed that dogwhelks have negative NCEs on barnacle growth from spring to fall. As body size is related to reproductive output in barnacles (Wethey, 1984), dogwhelk cues also limited egg production per barnacle in the fall (Ellrich et al., 2016a). Although not measured, such NCEs may have resulted in a lower larval production in the following spring.

ABIOTIC DRIVERS OF NCE INTENSITY: WAVE EXPOSURE

The experiments discussed above were done in habitats subjected to a moderate wave action. A field experiment using the same cage design found that a higher degree of wave exposure (in habitats where dogwhelks also occur) prevented the occurrence of dogwhelk NCEs on barnacle recruitment (Ellrich and Scrosati, 2016). This result is consistent with a pattern of predator cue dilution under increased water velocities and with the notion that turbulent conditions decrease the ability of mobile organisms (such as cyprids) to locate cue sources (Finelli et al., 2000; Large et al., 2011; Robinson et al., 2011; Pruett and Weissburg, 2019, 2021).

DOGWHELK NCEs ON MUSSEL RECRUITMENT

Through an experiment done in the same sheltered habitats where dogwhelk NCEs on barnacle recruitment were revealed (Ellrich et al., 2015b, 2016b; Ellrich and Scrosati, 2016), dogwhelk NCEs on mussel recruitment were also evaluated (Ehlers et al., 2018). Two intertidal mussel species (*Mytilus edulis* and *Mytilus trossulus*) occur on the studied rocky shores (Tam and Scrosati, 2011, 2014), both of which are preyed upon by *N. lapillus* (Sherker et al., 2017). Because of morphological similarities (Innes and Bates, 1999) and hybridisation (Riginos and Cunningham, 2005), their visual identification is difficult, so mussel recruits were counted as *Mytilus* spp., as commonly done in field studies with these species (Cusson and Bourget, 2005; Le Corre et al., 2013). The same cage design was used to manipulate dogwhelk density but, instead of a plate, the central compartment of the cages hosted a plastic mesh scourer (Our Compliments Pot Scrubber, Mississauga, ON, Canada; see a picture in Ehlers et al., 2018). Mesh scourers are often used to quantify intertidal mussel recruitment because they resemble preferential habitat (filamentous algae or byssal mussel threads) for mussel larval settlement (Menge and Menge, 2013; South, 2016). This experiment ran between late May and late July and found that dogwhelk cues limited mussel recruitment, but only by 13% on average (Ehlers et al., 2018).

The weaker dogwhelk NCEs on mussel recruitment than on barnacle recruitment suggest that prey life history traits may help to predict NCE intensity. While barnacles cannot move away after recruitment, mussels can relocate, albeit limitedly, across the substrate after recruitment (Bayne, 1964; Hunt and Scheibling, 2002). Mussel adults can also immobilise dogwhelks with byssus (Farrell and Crowe, 2007). Overall, these abilities allow mussels to avoid predation through mechanisms that barnacles lack. Such differences might explain why mussel recruitment was less responsive to dogwhelk cues than barnacle recruitment. It will be interesting to evaluate if actively mobile benthic prey (e.g., herbivore snails) have even weaker responses to dogwhelk presence.

CONCLUDING REMARKS

Overall, this mini review summarises recent studies with intertidal invertebrates that have revealed predator NCEs on prey demographic traits and external factors modulating such effects (**Figure 2**). Barnacles and mussels have demonstrated to be good model species to monitor demographic responses in prey, as counts can be accurately done because they are sessile organisms. The acquired body of knowledge is valuable because it resulted from field experiments, done under complex natural conditions that laboratory experiments cannot fully reproduce, as noted by other researchers (Weissburg et al., 2014; Babarro et al., 2016; Wiggins et al., 2018).

The predator avoidance behaviour shown by barnacle and mussel larvae when seeking settlement may have evolved to limit predation risk for the subsequent benthic stages. This could be so because adult movements across the substrate are impossible for barnacles and limited for mussels. Although predators could eventually reach an area where they were absent at the time of prey settlement, the avoidance of predators by settling prey larvae does reduce future predation risk to an extent. Ultimately, the occurrence of negative predator NCEs on the recruitment of barnacles and mussels should locally limit population density for these organisms because of their sessile nature. For barnacles, this could be detrimental for reproduction because they need nearby neighbours to cross-fertilise.

The presence of barnacle adults and high recruit densities prevented the occurrence of dogwhelk NCEs on barnacle recruitment. Benthic barnacles attract conspecific cyprids through chemical cues, which is thought to aid cyprids find favourable habitats to settle. It appears that an abundance of conspecific settlement-inducing cues would thus neutralise the effects that dogwhelk cues would otherwise exert on cyprids seeking settlement. However, the occurrence of too many adult barnacles on the substrate might limit conspecific recruitment, as high adult densities may indicate cyprids the potential for strong intraspecific competition after recruitment (Scrosati and Ellrich, 2017). Therefore, for a given dogwhelk density, NCE intensity may have a non-linear dependence on adult barnacle density. On the other hand, a very high supply of cyprids from the water column (favoured by a high phytoplanktonic food supply) could swamp the shore with settlers, making benthic recruitment less responsive to dogwhelk cues. Factorial field experiments manipulating these variables could clarify these possible interactions. It could also be of interest to obtain more realistic estimates of NCE intensity given that dogwhelks move across the substrate (which cages do not allow to happen). The main goal of the field experiments hereby described was to demonstrate that NCEs on prey demographic traits can occur. Measures of NCEs on demography could thus be refined by manipulating dogwhelk density over time to mimic natural dogwhelk movements across the substrate.

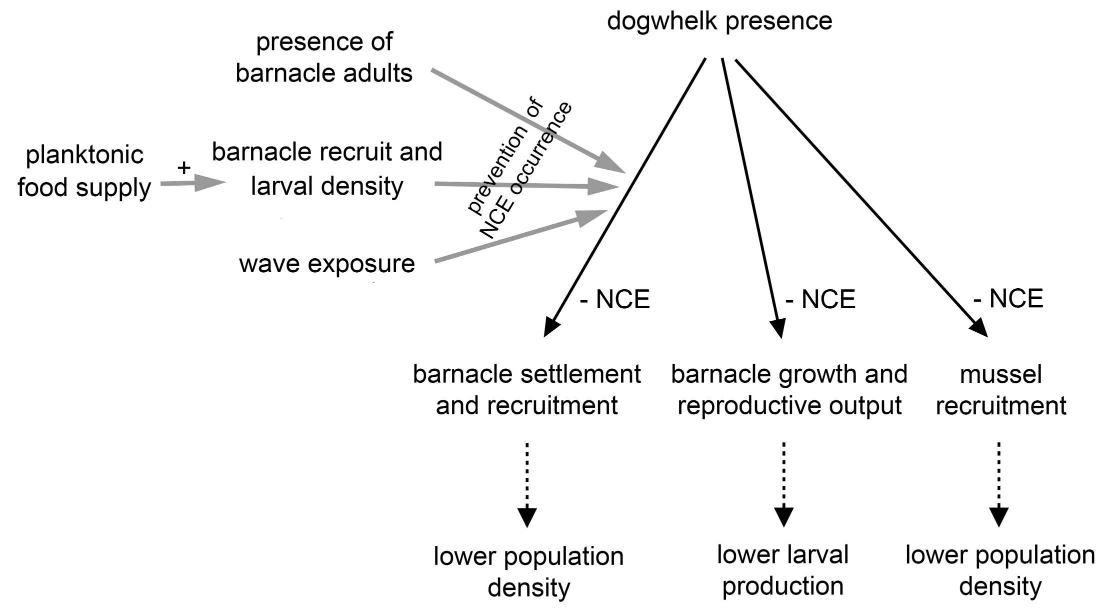

FIGURE 2 | Diagram summarising the current evidence on dogwhelk NCEs on barnacle and mussel demography supported by field experiments (solid black lines). The factors listed on the left have experimentally been shown to prevent the occurrence of such NCEs (solid grey lines). The three items depicted at the bottom represent possible additional outcomes (dotted black lines).

The chemical nature of the dogwhelk cues that trigger the observed NCEs on barnacle and mussel demography is not known with certainty. Based on studies for other aquatic predator–prey systems (Poulin et al., 2018; Puglisi et al., 2019), such cues could be constitutive and/or related to the dogwhelks' diet. Identifying their chemical nature should thus help to understand the physiological constraints affecting dogwhelks that can ultimately influence their remote detection by prey.

It is worth noting that predation risk can trigger morphological and physiological responses in prey besides behavioural responses (Hawlena and Schmitz, 2010). For brevity and consistency, this mini review has focussed on behavioural responses influencing prey demography. Through a field experiment, dogwhelk cues were also found to trigger shell thickening in mussels, which was experimentally shown to increase handling times of mussels by dogwhelks during attacks (Sherker et al., 2017). Whether that outcome decreases mortality rates in populations remains untested, but it is possible because longer handling times may limit predation success. Thus, investigating demographic influences of prey responses to predation risk other than behavioural might also be interesting using intertidal invertebrates.

It also worth emphasising that this mini review was aimed at summarising the current evidence of predator NCEs on prey demography using intertidal invertebrates. Its goals did not include aspects of NCE research that are more common in the literature, such as comparisons of consumptive versus nonconsumptive effects of predators (Peckarsky et al., 2008; Weissburg et al., 2014; Peacor et al., 2020). Such studies could be done using intertidal invertebrates by, for example, manipulating the ability of dogwhelks to consume barnacle and mussel recruits in addition to manipulating dogwhelk presence to evaluate their NCEs.

Finally, as for all interspecific interactions (Menge and Sutherland, 1987; Kondoh, 2001; Silliman and He, 2018), the intensity of predator NCEs on prey demography likely depends greatly on the abiotic context and food supply (Kimbro et al., 2020; Wirsing et al., 2021). In fact, as noted above, the intensity of dogwhelk NCEs on barnacle recruitment was found to depend on wave exposure and prey food supply. These factors, in turn, depend on coastal oceanography and climate (Ardhuin et al., 2019; Menge and Menge, 2019; Shanks and Morgan, 2019). Thus, for predator–prey systems in general, future research could aim to understand environmental influences on predator NCEs on prey demography.

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RAS is the single author of this manuscript.

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The Ghost of the Hawk: Top Predator Shaping Bird Communities in Space and Time

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Despite the wide recognition that strongly interacting species can influence distributions of other species, species interactions are often disregarded when assessing or projecting biodiversity distributions. In particular, it remains largely uncharted the extent to which the disappearance of a keystone species cast repercussions in the species composition of future communities. We tested whether an avian top predator can exert both positive and negative effects on spatial distribution of other species, and if these effects persist even after the predator disappeared. We acquired bird count data at different distances from occupied and non-occupied nests of Northern goshawks *Accipiter gentilis*. Using a Bayesian joint species distribution model, we found that large bird species (preferred prey) are less abundant in the proximity of nests occupied by goshawks, whereas smaller species –expected to get protection from subordinate predators displaced by goshawks– more often showed an opposite association. These spatial differences level off gradually, but still persist for years after the goshawks have disappeared. This indicates that the composition of local bird populations and communities might be conditional on past species interactions. Therefore, endeavors centered around species distributions could largely benefit from acknowledging the local extinction of keystone species.

Keywords: Bayesian community-model, ecological legacy, species distribution, predator-prey interactions, keystone species, heterospecific attraction

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INTRODUCTION

Predators have had a central role in the concept of keystone species (Paine, 1966; Power et al., 1996) not only because of the direct negative pressure they impose on prey, but also due to the indirect effects they cause that may cascade through the entire community (Ripple et al., 2014). Although being a largely overlooked phenomena, keystone species can attract other species. Habitat selection theory predicts that animals prefer locations that maximize their fitness (Fretwell and Lucas, 1969; Rosenzweig, 1981; Morris, 2003). Predation risk is an important feature that defines habitat quality, sometimes driving prey to patches of lower relative quality but with lower predation risk (Lima and Dill, 1990; Lima and Bednekoff, 1999; Lind, 2005). Interestingly, because apex predators can also displace other competing predators (Sergio and Hiraldo, 2008), prey species that are hunted by subordinate predators should benefit from the presence of a top predator (Crooks and Soulé, 1999; Sergio et al., 2004; Ritchie and Johnson, 2009). Several studies have shown the presence of heterospecific attraction (Mönkkönen et al., 1999) for prey seeking shelter under protector species (see reviews by Caro, 2005; Quinn and Ueta, 2008; Lima, 2009). However, studies investigating attraction to protector species have, so far, mainly focused on pairs of species while not accounting for general consequences on the communities (but see Forsman et al., 2001). Additionally, the concept of keystone species has been considered mainly for mammalian carnivores (Sergio et al., 2008; Caro, 2010) while avian predators have been investigated but only to a smaller extent (Thomson et al., 2006; Mönkkönen et al., 2007).

It is well-established that effects of past land-use on ecosystems can persist for long periods of time (Koerner et al., 1997; Knick and Rotenberry, 2000; Hermy and Verheyen, 2007; Cuddington, 2011). Much less attention has been paid to how observed patterns of species distribution are a result of past species interactions, with no studies investigating how the extinction of a keystone species may leave a community footprint that persists in time. The capacity and speed by which single species, and species assemblages in general, adjust to new environmental conditions are associated with landscape connectivity, mobility and interactions among the species in the community (Pimm, 1984; Hanski, 1998; Peterson et al., 1998). It is thus expected that in a continuous landscape, highly mobile taxa (e.g., birds or large mammals) would rapidly respond to local perturbations. However, cues used to assess habitat quality may persist in time (Seppänen et al., 2007), arguably causing a “memory effect” in how animal assemblages react to changes. Being able to assess the relevance of past interactions is thus relevant for studies aiming to forecast ecosystem restoration or to better assess true habitat requirements of species.

In this study, we investigate the impact of an avian top predator, the northern goshawk *Accipiter gentilis*, on the bird community in space and time. We hypothesized that (i) the top predator can have both positive and negative effects on the spatial distribution of other species, and that (ii) these effects may persist even after the top predator has disappeared. We further hypothesized that these effects are driven by (iii) the displacement of prey species due to predator-prey interactions, and (iv) the attraction of species that benefit from the top predator displacing subordinate predators. We therefore expected that (i) bird assemblage composition is conditional on distance from goshawk nests and that (ii) this effect gradually diminishes in time after the goshawk’s disappearance. While (iii) prey species are expected to be less common nearby occupied nests, (iv) species that benefit from protection by the top predator are expected to be more common near the nest when the top predator is present.

MATERIALS AND METHODS

Study Species, Study Area, and Sampling Design

To investigate the effect of top predators on avian assemblages, we used the widely distributed northern goshawk *Accipiter gentilis* (henceforth goshawk) as a top predator model species. The goshawk is a forest-dwelling species that primarily preys on middle-sized birds in Europe (Kenward, 2006). In Finland, the goshawk is usually found in mature Norwegian spruce *Picea abies* forest (Tornberg et al., 2006). During the breeding season, from March to August, goshawks concentrate their activity within a few kilometers of their nest and expand their range or move to other areas after the breeding season (Kenward, 2006; Tornberg et al., 2006).

Through an ongoing long-term goshawk population monitoring (e.g., Byholm and Nikula, 2007; Byholm et al., 2007, 2011; Burgas et al., 2016), we inspected goshawk territories on the west coast of Finland (latitude 62°00′–62°55′N, longitude 21°05′–22°40′E) from the second week of May to the first week of June in 2007. For each territory, we counted individuals of all bird species found at 12 sample sites around the goshawk nest; at 50, 250, and 500 m in each cardinal direction. In total, we surveyed 708 sample sites. The distances were chosen to investigate how distance from the goshawk nest influences the bird community. Because goshawk activity concentrates in forest habitats, we focused on forest bird species (*sensu* Solonen, 1994). Bird abundances were recorded for 7 min both from vocalizations and visual observations in every site within the first 4 h since sunrise. Birds flying over a site were not recorded. At the time of the bird surveys, goshawks were breeding in 29 of the 59 nests included in this study. Among the remaining 30 nests, goshawks were known to have been breeding in the nest the previous year in 14 nests, 2 years earlier in 6 nests, 3 years earlier in 6 nests, 4 years earlier in 2 nests, and 5 years earlier in 2 nests. All non-breeding territories were inspected to confirm that goshawks were not nesting in an alternative location within that same territory. The habitat composition of each sample site was classified in the field to the closest 10% among the following five categories: pine *Pinus sylvestris* forests, spruce forests, young forest plantations, pine fens, and open habitats.

We expected that while some species should be negatively associated with the goshawk (e.g., prey species, subordinate raptor species), other species that are not prevalent in the diet of the goshawk and/or that do not compete for resources should be either unaffected by the goshawk presence or show positive association if the goshawk offers shelter from other predators. To determine the diverging effect of the goshawk on the bird community, we categorized bird species into two groups according to body mass (Mönkkönen et al., 2007). We regarded birds with a body mass equal or higher than the redwing *Turdus iliacus* (i.e., 60 g) as a proxy for goshawk prey (hereafter large birds). Conversely, we presumptively denoted as non-susceptible to predation all bird species with body mass smaller than 60 g (hereafter small birds). This categorization is in line with the fact that goshawks rarely prey on small birds, even though these are the most abundant species in the forest (Møller et al., 2012).

Statistical Analyses

Habitat Composition

To assess differences in habitat representation as a function of distance from the goshawk nests and time since the nests were last occupied and the interaction of distance and time, we carried out a Dirichlet regression model in R using function DirichletReg in package DiricReg (Maier, 2014). Dirichlet models are suited to analyze compositional data, where dependent variables are subject to a sum constrain.

Model for Species Abundances in the Presence and Absence of Goshawk

We applied a joint distribution model (Warton et al., 2015; Ovaskainen et al., 2017; Ovaskainen and Abrego, 2020) to analyze the data simultaneously for all species. We model the abundance y_{ijk} of species i found at site k within territory j by assuming that y_{ijk} follows a Poisson distribution where the linear predictor is modeled as

$$\begin{aligned} L_{ijk} &= \beta_{i1} + \beta_{i2} D_{jk} + \beta_{i3} D_{jk}^2 \\ &\quad + \left(\beta_{i4} + \beta_{i5} D_{jk} + \beta_{i6} D_{jk}^2\right) e^{-\frac{T_j}{\alpha_i}} + \beta_{i7} H_{jk} + \varepsilon_{ij}. \end{aligned}$$

Here D_{jk} is the distance of site k in the goshawk territory j, T_j is the time since the goshawk left its nest in territory j, and H_{jk} is the forest habitat cover (i.e., proportion of pine and spruce forest together) of site k in territory j. The model involves a quadratic function of the distance from the nest to allow the model to fit species that peak in abundance at an intermediate distance from the goshawk nest (Mönkkönen et al., 2007). The model estimates one response curve (as a function of distance to the nest) for cases where the goshawk is presently at the nest, and another response curve for nests that have been occupied by the goshawk long ago, and assumes an exponential transition between these responses with a characteristic time scale α_i for species i. The territory-level random effects (ε_{ij}) were assumed to be distributed independently among the species and territories as $\varepsilon_{ij} \sim N(0, \sigma_i^2)$, where σ_i^2 the amount of random variation among territories for species i.

To facilitate the estimation of model parameters for rare species, we used the hierarchical modeling framework modified from that of Ovaskainen and Soininen (2011). We denoted by β_i the vector of parameters to be estimated for species i, $\beta_i = (\beta_{i1}, \beta_{i2}, \beta_{i3}, \beta_{i4}, \beta_{i5}, \beta_{i6}, \beta_{i7}, \beta_{i8})$, where $\beta_{i8} = \log(\alpha_i)$. We assumed that the parameter vectors β_i are distributed (independently among the species) according to a multivariate normal distribution with mean μ (a vector of length 8) and a variance-covariance matrix Σ (a 8×8 matrix), $\beta_i \sim N(\mu, \Sigma)$. The vector μ models the responses of a "typical" species, whereas the matrix Σ measures how species vary in their responses to the explanatory variables (diagonal of Σ) and to pairs of explanatory variables (off-diagonal of Σ).

The parameters of the model were estimated using a Bayesian approach. As priors, we assumed for each component of μ a normal distribution with mean zero and variance one, for Σ an inverse Wishart distribution with 8 degree of freedom and an identity matrix as variance-covariance parameter, and for each σ_i^2 a Gamma distribution with shape equaling 0.5 and rate equaling 0.5. We sampled the posterior distributions with a Monte Carlo Markov Chain (MCMC) method [modified from Ovaskainen and Soininen (2011) to account for count data], which we ran for 70,000 iterations, out of which the proposal distributions were adapted during the initial burn-in of 20,000 iterations. We thinned the samples by 10, resulting in 500 posterior samples, and assessed MCMC convergence by computing an effective number of samples (results shown in **Supplementary Figure 1**).

RESULTS

Habitat Composition

Habitat composition at the sample site level changed substantially when moving away from the goshawk nests, with spruce forest—the preferred nesting habitat of the goshawk—representing 75% of the land cover surrounding the nest while it covered approximately 30% of the sample sites at 500 m from the nests (Dirichlet regression P-value for spruce forest < 0.001, for other land cover types > 0.05; (**Supplementary Table 1** and **Supplementary Figure 2**). Habitat composition did not vary as a function of the number of years since goshawks abandoned the nests, neither as the interaction between time and distance in any of the five habitat classes (**Supplementary Table 1** and **Supplementary Figure 2**).

Species Abundances in the Presence and Absence of Goshawk

We recorded 25 large and 31 small bird species and included in the community-level model also those 15 large and 8 small bird species that were not detected in our surveys but that breed in the study area (Valkama et al., 2011). The average abundance at site level of a typical large species was eight times lower than that of a small-sized bird (0.02 vs. 0.16 individuals/site for large and small species, respectively).

Based on the community level parameters (μ) that represent the responses of a typical species, both small and large species were more common in the absence of goshawk than in its presence (**Figures 1**, **2A,D**). However, while large species increased greatly in abundance with increasing distance from the goshawk nest (**Figures 1**, **2D**), the abundance of small species varied only little with distance from the goshawk nest, the highest abundance being found at an intermediate distance (**Figures 1**, **2A**). The posterior mean estimates for the community-level averages of the characteristic time scale parameter α was 6 years (95% credibility interval 1–21 years) for small birds and 4 years (95% credibility interval 1–18 years) for large birds. Thus, both small and large birds showed a substantial delay in their responses to goshawk abandoning the nest (**Figures 2A,D**). At the community level, habitat did not show an effect that would be either positive or negative with 95% posterior probability.

The individual species showed marked variation around the expected community-level abundance (**Figures 1**, **2**). Based on the posterior mean estimates obtained for the small species, in the presence of the goshawk, 11 species achieved the highest abundance near the goshawk nest (for example, see **Figure 2B**), 6 at an intermediate distance from the nest (for example, see **Figure 2C**), and 14 far away from the nest. Among the large species, in the presence of the goshawk none of the species achieved the highest abundance near the goshawk nest, 2 at an intermediate distance from the nest, and 23 far away from the nest (for examples, see **Figures 2E,F**).

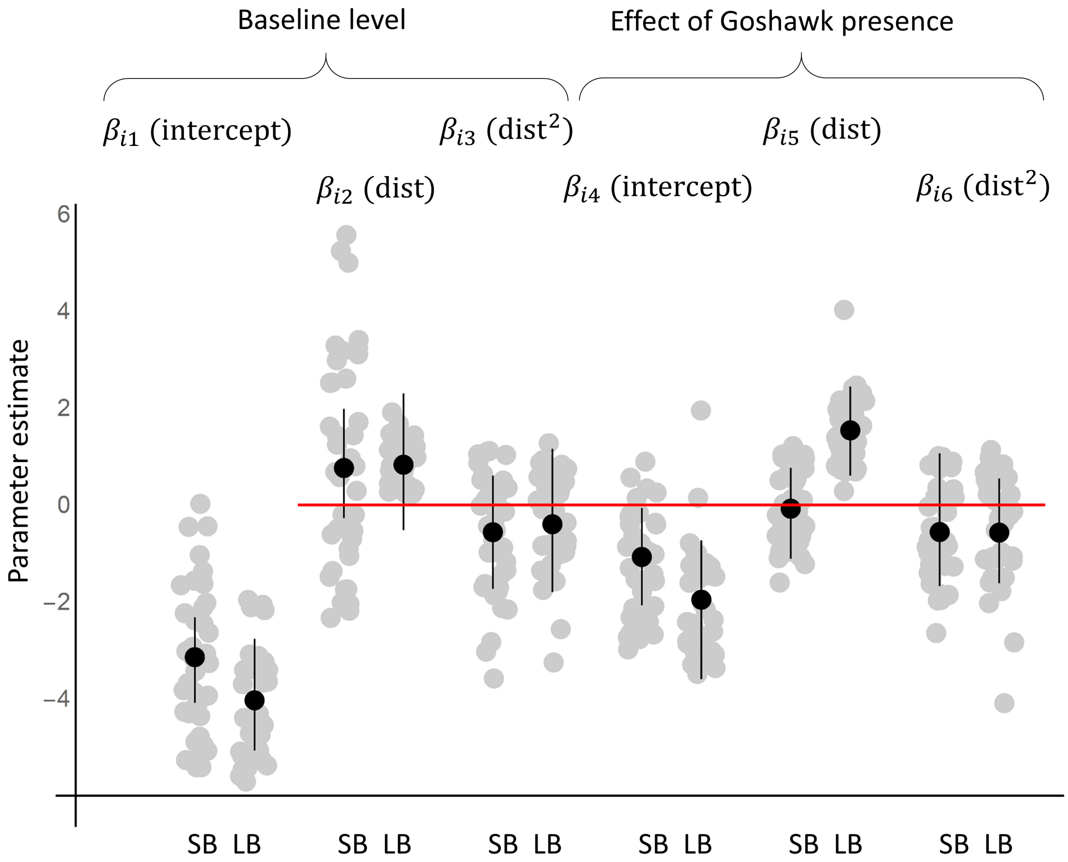

FIGURE 1 | The responses of the species to goshawk presence based on the posterior distribution of the parameters of the joint species distribution model fitted to the data. The black dots and lines show the posterior means and the 95% credibility intervals for the community level parameters (μ), whereas the gray dots show the posterior means for the species level parameters (β). The results are shown separately for the models fitted with data on small bird species (SB) and with large bird species (LB).

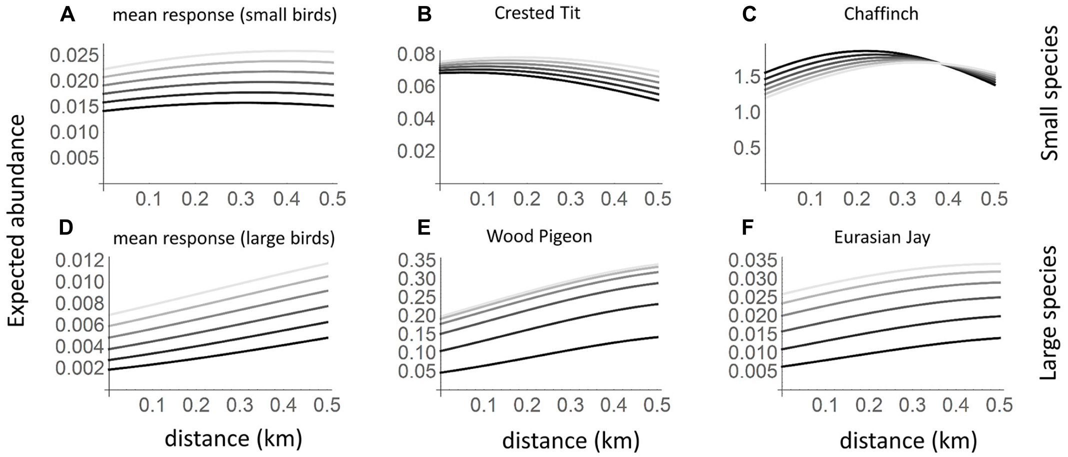

FIGURE 2 | Expected abundances of small and large bird species in relation to goshawk nest proximity and time since the goshawk abandoned the nest. The black lines correspond to the current presence of the goshawk, and the different shades of gray to situations in which goshawk abandoned the nest from one (darkest gray) to five (lightest gray) years ago. The panels correspond to a typical small species (**A**; based on community level parameters), the crested Tit *Parus cristatus* **(B)**, the chaffinch *Fringilla coelebs* **(C)**, a typical large species **(D)**, the wood pigeon *Columba palumbus* (**E**; a common prey of the goshawk) the Eurasian jay *Garrulus glandarius* (**F**; a common prey of the goshawk and an important avian nest predator). In the predictions, habitat was set to the average value over the sample sites.

DISCUSSION

Our results show that raptors, such as the goshawk, can have an important role in shaping the composition of the bird community both across space and across time. Not surprisingly, such a keystone species-effect is strongest near occupied goshawk nests, but fades gradually with distance and time after the goshawk abandons its nest. Because top predators are usually territorial with uneven presence in space and time, they can be regarded as a structuring species that creates heterogeneity in the landscape (see Thomson et al., 2006; Mönkkönen et al., 2007). As such, it is expected that, if top predators affect large portion of the communities, they should also promote higher landscape-level diversity with the same importance as structural habitat features or small patches of scarce habitats (Davidar et al., 2001; Gibbons and Lindenmayer, 2008; Timonen et al., 2010). Further research should be pursued to validate or contrast this idea.

The goshawk drove stronger impact on the assemblage of large species than on the small species assemblage. This was anticipated as predators are expected to show stronger and more direct interactions with its pool of preferred preys. The causes of community turnover can be addressed by looking at the three main patterns observed across the community. Firstly, large birds were usually more abundant far from occupied goshawk nests and increased in numbers only years after the nest was abandoned. Even though, to our knowledge, a temporal effect of keystone species on local communities has not been tested in the past, this result is in line with other studies where predators were shown to alter their prey distribution spatially by actively predating them and through behavioral response of the prey actively avoiding the predator (Norrdahl and Korpimäki, 1998; Thomson et al., 2006; Lima, 2009). Some studies have found that prey species peaked at intermediate distances from the predator (Quinn and Kokorev, 2002; Mönkkönen et al., 2007) as a compromise between the higher direct predation risk near the raptor and the higher risk of nest predation far from the raptor. In our study, this behavior was found only for the fieldfare *Turdus pilaris*. However, it is possible that our distance range (up to 500 m) was too short to reveal this kind of pattern more generally. For instance, Mönkkönen et al. (2007) found that large bird abundance in goshawk territories peaked at approximately 2 km from goshawk nests.

Secondly, small species were more abundant near occupied goshawk nests while their abundances decreased after the nest was abandoned or even showed opposite patterns far away from the goshawk nest (**Figures 2A–C**). This suggests that some of the small-bodied species may actively choose to breed in the proximity of the hawk. This supports the heterospecific attraction hypothesis (Mönkkönen et al., 1999; Haemig, 2001; Caro, 2005; Lima, 2009), and is backed by other studies showing aggregation of birds to predators (e.g., Wiklund, 1982; Bogliani et al., 1999; Mönkkönen et al., 2007). This interpretation was also endorsed by abundances of the Eurasian jay *Garrulus glandarius*, an important nest predator for small forest birds and common prey of the goshawk. Eurasian jay was extremely rare in the proximity of active goshawk nests when the goshawk was present, while its abundance increased after the goshawk's absence (**Figure 2F**).

Third, we found that several small species were more abundant at intermediate distances or even reacted in the same manner as a typical large species. Interestingly, this is against the general expectation that species outside the predator scope should, at least, not be negatively affected by it. In this respect, our results did not comply with previous findings showing that abundances of non-prey species were higher in the vicinity of predators (Norrdahl and Korpimäki, 1998; Mönkkönen et al., 2007). We see two possibilities explaining this discordancy. On the one hand, it might be that the presence of the goshawk attracts or allows the occurrence of species that have negative effects on the fitness of other species (see also Morosinotto et al., 2012). In this context, the great spotted woodpecker *Dendrocopos major*—a nest predator (Walankiewicz, 2002)—was more common in the close vicinity of the raptor nest than far away. On the other hand, because of different ecological and evolutionary history, one could expect variation in the species capability to grasp useful information. For example, given the fact that male goshawks closely resemble female Eurasian sparrowhawks *Accipiter nisus*, it is possible that some species preyed upon by sparrowhawks (which prefers small birds) are distressed by the goshawk presence if failing to clearly separate the two predators.

It took years to stabilize the community similarity and the abundances of several species after the goshawk abandoned the territory. Birds are not physically restricted in mobility, and therefore it could be expected that local abundances should adjust swiftly. There are a few reasons which can explain why the delay in the bird community was gradual. Firstly, birds are known to show breeding site fidelity (i.e., tendency of one individual to return to breed where it reproduced previously) and breeding philopatry (i.e., tendency of an individual to return to breed where it was born). This propensity may partly neutralize the importance of social cues. Because birds exhibit learning behavior and avoid returning to a location if a breeding attempt was unsuccessful (Lima and Dill, 1990), the increase of subordinated predators in subsequent years could cancel out site fidelity. Secondly, the goshawk nests are likely to remain as an indicator of the raptor's presence even after the hawk has left. Individuals are known to use indirect cues to assess the presence of other species (e.g., Forsman et al., 2012). If so, the nest of a predator gives an insight into its presence. As the goshawk nests are large structures that can persist for several years and even decades after being abandoned, it is possible that the simple presence of large empty stick nests may affect the species composition. This temporal effect resembles the delay in response of communities to habitat perturbations (Knick and Rotenberry, 2000), with the novelty that in our case the response relates to a key species in the community. Our results suggest that studies on bird community turnover (i) should be monitored during a series of years even after environmental change in order to successfully assess its effects, and that (ii) the historical co-occurrence of keystone species should be considered. Moreover, it is possible that some species preemptively avoid patches with structural habitat properties preferred by the predator (here old forest stands with widely spaced trees). Such behavior would reduce the amount of community turnover found across space and time in this study, as prey would not occupy predator-suitable sites based on habitat cues to start with. Specific study designs to study such possibility are encouraged.

Our habitat measures were coarse as compared to the actual habitat selection done by different bird species. One strength of this study is the combination of distance from the nest, with time since the nest was last time occupied separately on small and large birds. Given that we did not find a change in habitat representation as function of time neither interaction of time and distance, we find it unlikely that there would be a strong bias in forest structure that would produce spurious effects of time since goshawk nest was occupied in a different manner between small birds and large birds.

Diversity in resource limitation, heterogeneity, and interspecific differences can explain the stable coexistence of numerous competing species (Tilman, 1982, 1994) while a higher number of interactions enhances ecosystem resilience (Peterson et al., 1998; Petchey and Gaston, 2009; Tylianakis et al., 2010). Given the ubiquity of predators and the fact that they present both negative and positive interactions, it is reasonable to suggest that top predators have a positive role in sustaining biodiversity. This can have direct practical implications in conservation endeavors that devote more efforts to charismatic species like predators.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the data consists of bird counts in the field with minimal disturbance. Moreover, the field data collection was conducted in 2007, before the ethical committees for ecological field work was on place.

AUTHOR CONTRIBUTIONS

PB designed the study, secured funding, and coordinated the data collection. DB analyzed habitat composition. OO and FB run the community analyses with the input of DB and PB. DB made the first draft, on top of which all authors contributed significantly.

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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.2021.638039/full#supplementary-material

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Higher, Faster, Better: Maternal Effects Shorten Time Lags and Increase Morphological Defenses in *Daphnia lumholtzi* Offspring Generations

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Prey species can respond to the presence of predators by inducing phenotypic plastic traits which form morphological, life history or behavioral defenses. These so-called inducible defenses have evolved within a cost-benefit framework. They are only formed when they are needed, and costs associated with defenses are saved when predators are not present. However, a disadvantage compared to permanent defenses are lag phases between predator perception and the full formation of defenses. This may be especially important when the predation risk persists for longer periods, e.g., outlasts one generation and challenges prey offspring. We hypothesized that transgenerational induced phenotypic plasticity reduces lag phases in situations where hazards threaten specimens over several generations. We tested this in three generations of the freshwater crustacean *Daphnia lumholtzi* using the three-spined stickleback *Gasterosteus aculeatus* as predator. In the presence of chemical cues from fish *D. lumholtzi* expresses elongated head and tail spines. In the F0 generation defenses are constraint by a comparatively long lag phase and are not developed prior to the 3rd instar. In the F1, and F2 of induced animals this lag phase is shortened and defenses are developed upon birth. We show that induction of TGP in the mothers takes place already during the juvenile stages and transfers to the offspring generation in forms of shortened time lags and enhanced trait expression. When progeny is additionally exposed to fish cues as embryos, the addition of maternal and embryonic effects further enhances the magnitude of defense expression. Our findings detail a distinguished strategy of transgenerational phenotypic plasticity which allows to shorten lag phases of trait changes in phenotypic plasticity.

Keywords: transgenerational phenotypic plasticity, within generational phenotypic plasticity, head spines, kairomone, predator, *Daphnia*, inducible defenses

INTRODUCTION

In environments with fluctuating conditions, dedicated mechanisms that allow fast phenotypic adaptation may be crucial to improve organismal fitness (Auld et al., 2010; Holeski et al., 2012; Shama et al., 2014; Hendry, 2016; Luquet and Tariel, 2016). One such mechanism is phenotypic plasticity, which is defined as the ability of an organism with a given genotype to respond to environmental changes with an adapted phenotype (Bradshaw, 1965; Whitman and Agrawal, 2009). Very often the occurrence of phenotypic plasticity is described within one generation (within generational plasticity WGP; Ezard et al., 2014; English et al., 2015; Auge et al., 2017), but when environmental hazards are long-lasting, offspring performance can be enhanced if they are being prepared by the parents (Agrawal et al., 1999; Uller, 2008). This kind of transgenerational plasticity (TGP) is discussed to be enabled via epigenetic, cytoplasmic, somatic, nutritional and behavioral modifications from parents to offspring (Bonduriansky and Day, 2009; Harris et al., 2012).

Both, the length and the timing of the environmental cue can be decisional for transgenerational responses in the offspring (Donelson et al., 2018). A prolonged exposure to environmental cues can increase the transgenerational effect (Donelson et al., 2018). Timing is crucial as TGP may be limited to early life exposure with critical developmental windows for cue sensitivity (Hanson and Skinner, 2016; Sentis et al., 2018).

TGP has been observed in animals and plants (Mousseau and Fox, 1998; Agrawal et al., 1999) as a response to e.g., biotic stressors (Mousseau and Dingle, 1991; Kumar et al., 2015). One significant biotic stressor is predation, which frequently induces plasticity in forms of inducible defenses in prey (Tollrian and Harvell, 1999). The predation risk is often indicated by chemical cues so-called kairomones, that are unintentionally released by the predator (Dodson and Hanazato, 1995; Weiss et al., 2018). Prey species can perceive these kairomones and thereupon develop defenses ranging from behavioral, via life history, to morphological adaptations (Tollrian and Dodson, 1999; Weiss, 2019). The freshwater crustacean *Daphnia* is a prime example for developing such defenses. *Daphnia pulex,* e.g., expresses neckteeth (Krueger and Dodson, 1981; Tollrian, 1993) and *D. ambigua* expresses helmets (Hebert and Grewe, 1985) in the presence of the phantom midge larva *Chaoborus* spec. *D. atkinsoni* develops a crown of thorns (Petrusek et al., 2009) and *Daphnia magna* grows large and bulky under predation pressure of the tadpole shrimp *Triops* spec. (Rabus and Laforsch, 2011; Horstmann et al., 2018), and *D. longicephala* develops huge crests in the head region when exposed to the backswimmer *Notonecta* spec. (Grant and Bayly, 1981).

Inducible defenses should incur costs, as otherwise they likely would become permanent. These costs can be of different origin, so that they can stem from the organisms' ability of being plastic *per se.* In this case, costs, e.g., result from the maintenance of a genetic and physiological architecture to detect and adapt to predation cues. Other costs, like allocation costs, can derive from an increased energy and material demands required for the formation of the defenses. Environmental costs or external costs result from e.g., changes in swimming speed due to aberrant hydrodynamics of the defended morphotype (reviewed in Weiss and Tollrian, 2018a,b).

Besides these costs, the expression of these defenses underlies constraints. There are e.g., time lags that result from the time needed after predator perception until defense formation (Weiss and Tollrian, 2018a,b). In *Daphnia* such time lags often require at least a complete instar and are dependent on the time needed for signal processing and the change of developmental trajectories to result in adaptive morphotype expression (Weiss, 2019). Furthermore, there are developmental windows in which defenses can be expressed, so that *D. pulex* only express neckteeth in the early juvenile stages (Imai et al., 2009; Weiss et al., 2016), and *D. longicephala* express crests only in later developmental stages (Grant and Bayly, 1981; Weiss et al., 2015). Also the degree of defense expression is subject to constraints, as a larger defensive trait requires a longer time until being developed (Miner et al., 2005). One way to minimize constraints may be the shortening of time lags prior to defense expression. In line with the shortening of the time lags, the earlier onset of defense expression also a allows a stronger defense expression within a shorter time frame. Thereby defense expression can be further optimized to the predation risk. Here TGP could serve as a tool which may be especially effective when the parental environment is a proxy of the offspring's environment. This is for example the case when the predation risk overarches several generation cycles of the prey. *Daphnia* are often being preyed on by juvenile fish that have a comparatively long juvenile phase. *D. lumholtzi* is one of the few *Daphnia* species that develops morphological defenses against fish predation (Engel and Tollrian, 2009; Engel et al., 2014). A transgenerational induction of inducible defenses could further improve the cost-benefit relationship where the benefit of stronger defense expression outweighs the costs. We hypothesized that transgenerational plasticity affects (a) time lags and (b) strength of defense expression. Furthermore, we wanted to elucidate the point in time critical for the induction of TGP.

MATERIALS AND METHODS

Animal Culture

D. lumholtzi clone TE (Fairfield Reservoir, Texas, United States, kindly provided by R. Sterner) was cultured in artificial *Daphnia* medium (ADaM; Klüttgen et al., 1994) in 1 L beakers (Weck®, Germany) containing 40–50 age-synchronized individuals under 16:8 h day:night cycle at 20°C ± 1°C. Animals were fed with unlimited food conditions (1.5 g C/L) with the algae *Autodesmus obliquus*. The beakers were cleaned every 48 h to remove exuviae and excess algae. Half of the medium was exchanged weekly.

Kairomone Preparation

Fish kairomone was prepared using three-spined sticklebacks (*Gasterosteus aculeatus*). Fish were not harmed and kept under conditions complying with animal care and welfare. A maximum of 20 fish no larger than 5 cm (body length) were kept in an 80 L glass tank at 15 ± 1°C under constant 12:12 h day:night cycle.

Animals were fed *ad libitum* every 24 h with *Chironomus* larvae (Amtra, Germany). To produce kairomone enriched medium two fish (size 4–5 cm body length) were transferred into 1 L ADaM for 24 h. Subsequently, fish were removed and the water containing the kairomone was filtered (45 μm GF/C Whatman filter). To prevent bacterial degradation of the kairomone, ampicillin (10 mg/L) (Sigma Aldrich, Germany) was added as reported by Weiss et al. (2012). Kairomone was frozen at −20°C and thawed prior to use. The stock concentration of 2 fish/L was diluted to 0.2 fish/L in the bioassay. The diluted kairomone contained 5 mg/L of ampicillin. Even though ampicillin does not impact *Daphnia* development (Weiss et al., 2012), the same concentration of 5 mg/L was added to the medium of the control conditions.

Experimental Treatments

In order to determine transgenerational phenotypic plasticity in *D. lumholtzi*, we performed an experiment in which predator naïve 1st instar juvenile *D. lumholtzi* were exposed to four treatments (**Figure 1**). We performed 1.) a control treatment without predator exposure, and 2.) permanent predator exposure. With these treatments we wanted to test if *D. lumholtzi* performed TGP, and if so in what way. Therefore, we monitored these animals in the first through third juvenile instars in the F0, F1, and F2 generation. With treatment 3.) and 4.), we wanted to disentangle if induction of TGP occurs already in the mother, or if it occurs in the embryos. For that, in treatment 3.) we only exposed the mothers to kairomone. Here, we ensured that embryos did not get in contact with kairomone by removing mothers from kairomone in the 4th juvenile instar (one instar prior to ovulation) and rinsed them 3*10 min in ADaM to remove residual kairomone transferring them into control conditions until second and third clutch animals were released from the brood pouch. Neonates were then again exposed to kairomones. Consequently, in treatment 4.) we only exposed the embryos developing in naïve mothers to predator cues. For that the females were kept at control conditions until they ovulated. Subsequently, animals were exposed to kairomone until the neonates were released from the mothers' brood pouch. In treatment 3.) and 4.) we investigated defense expression in the first through third juvenile instars. All specimens of the different treatments were reared individually in 50 mL snap cap vials containing, either ADaM or kairomone enriched medium containing 20 mL medium. ADaM and kairomone enriched medium was refreshed every 48 h to ensure constant experimental conditions. The experiment was started with 5 animals per treatment group. This whole set-up was replicated 10 times.

Data Acquisition

All animals were monitored, and digital images were acquired to measure defense expression.

We used a stereomicroscope (SZX 16 Olympus; Germany) equipped with a digital camera (ColorView III, SIS Imaging Solutions Olympus; Germany) in combination with the analysis software Cellˆ D (SIS Soft Imaging System Solutions, Olympus Germany). We determined the *Daphnias*' head spine length from the tip of the head spine to the upper margin of the compound eye).

Head Spine Growth

We determined the absolute head spine growth per generation by calculating the differences of head spine length between the 3rd and 1st juvenile instar per individual animal in each generation.

Statistics

Based on a Shapiro-Wilk test, all data followed a normal distribution so that we tested for differences between treatments using a factorial ANOVA, followed by a Bonferroni *post hoc* test. We compared head spine lengths of animals obtained from the 2nd and 3rd clutch from the permanent predator exposure across the three generations in the 1st juvenile instar. Animals of the 2nd and 3rd clutch were pooled into one treatment as we did not detect clutch specific differences (again using an ANOVA). Additionally, we compared them with head spine lengths of the control treatment within each generation throughout the first three juvenile instars. Head spine lengths of maternal and embryonic predator exposure were compared to each other and the F1 control. Furthermore, we compared the increase in head spine length from the 1st to the 3rd juvenile instar across generations in the permanent predator exposure. All statistical analyses were performed with Statistica 14 (Statsoft Inc.).

RESULTS

In the F0 generation the continuous exposure to fish kairomones, significantly induced expression of morphological defenses in forms of elongated head spines in the 3rd juvenile instar. Defended *D. lumholtzi* have a median head spine length of 222.17 μm while undefended specimens have a median head spine length of 162.25 μm. In the earlier juvenile instars defenses are not expressed and predator exposed *D. lumholtzi* show a median head spine length of 160.63 μm in the first and 167.87 μm in the 2nd juvenile instar (**Figure 2A** and **Supplementary Tables 1**, **2**). In the 1st juvenile instar of the F1 and F2 generation medium head spine length reaches 190.75 and 201.64 μm in predator exposed *D. lumholtzi*. This is significantly larger than the head spine length of control *D. lumholtzi* of the equivalent generations (median 166.25 and 163.85 μm (**Figures 2B,C** and **Supplementary Tables 3**, **4**).

When comparing the strength of defense expression across generations, we find that defenses increase from the F0 to the F2 generation. Head spine lengths in the first juvenile instar are significantly larger in the F1 (190.74 μm) and F2 (201.64 μm) in comparison to the F0 (160.38 μm) generation (**Figure 2D** and **Supplementary Tables 5**, **6**). Also, between the F1 and F2 generation head spines are significantly different (**Figure 2D** and **Supplementary Tables 5**, **6**). This also holds true in the second and third juvenile instar.

Head Spine Growth Rate

We determined the head spine growth, by calculating the absolute increase in head spine length between the 3rd and the 1st juvenile

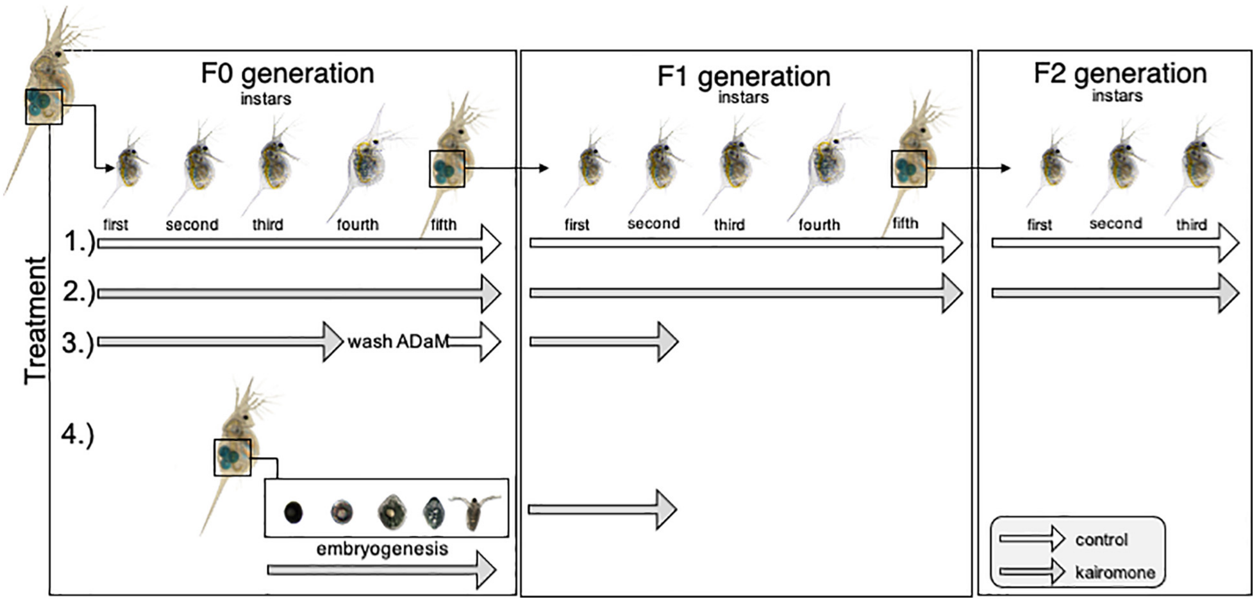

FIGURE 1 | Schematic display of kairomone exposure. 1.) In the control treatment, animals were never exposed to kairomones throughout the whole experiment. 2.) In the permanent kairomone exposure treatment individuals were permanently exposed to kairomones across all three generations. 3.) In the maternal kairomone exposure treatment animals were exposed to kairomone from the 1st until the 4th instar, then washed to remove residual kairomone, and transferred to kairomone-free medium. The 1st clutch was discarded and after being release from the brood pouch the 2nd and 3rd clutch were again exposed to fish kairomone. 4.) In the embryonic kairomone exposure treatment embryos in the mother's brood pouch were exposed to fish kairomone from ovulation until being released from the brood pouch and continuously exposed until they reached the 2nd juvenile instar. Treatments 3.) and 4.) were raised until the second juvenile instar in the F1 generation.

instar. We observed an increase in absolute head spine length across all generations. In the F1 generation, head spine growth is significantly larger than in the F0 generations. In the F2 generation head spine growth significantly exceeds this increase (**Figure 3** and **Supplementary Tables 7**, **8**).

Maternal vs. Embryonic Predator Exposure

We aimed to determine if this earlier onset and enhancement of defense expression is due to effects of fish kairomones in the maternal environment, the embryonic environment or both. Naïve offspring and animals that were exposed to predator cues during embryogenesis do not show the expression of defenses in the first two juvenile instars. When mothers were exposed to kairomones during the first four juvenile instars, their offspring develop defenses already in the 2nd juvenile instar, when exposed to kairomones upon birth. This effect is intensified, when both the maternal and embryonic environment experienced kairomones (**Figure 4** and **Supplementary Tables 9**, **10**).

DISCUSSION

Within- and transgenerational phenotypic plasticity are relevant mechanisms controlling adaptive responses to environmental changes (Donelson et al., 2018). WGP is selected when spatially and/or temporally heterogeneous cues indicate a predation risk, and costs are associated (Tollrian and Harvell, 1999; Walsh et al., 2016). If the environment experienced by the parents, serves as a proxy of the environment of the offspring, then TGP is expected to evolve (Luquet and Tariel, 2016). This has e.g., been reported in fish exposed *D. ambigua* (Walsh et al., 2016). Here, local adaptations to distinctive fluctuations in the density of the fish population were observed. If the fish population is consistently large, then TGP is selected, while WGP is favored when the fish population is changing more frequently (Walsh et al., 2016). WGP and TGP has also been reported in *Chaoborus*-exposed *D. cucullata* (Agrawal et al., 1999). *D. cucullata* express WGP in form of helmets, when exposed to predator cues short term. When the predator exposure covers generations, the offspring of *Chaoborus*-exposed mothers express significantly larger defenses than the offspring of non-exposed mothers (Agrawal et al., 1999).

Similarly, *D. lumholtzi* that are exposed to predator cues from birth display WGP in form of head spines from the 3rd juvenile instar onward. We observed TGP when *D. lumholtzi* is permanently exposed to fish kairomones. Then the daughter generations expressed significantly larger head spines than the F0 generation. Already juveniles in the first instar of the F1 and F2 show significantly larger head spines than their parents and grandparents. This increased growth is enhanced in the subsequent instars, so that also the 2nd and the 3rd juvenile instar show significantly larger head spines than the F0 generation. In the F0 generation, fish exposed *D. lumholtzi* (from fish naïve mothers) expressed increased head spines not before the 3rd juvenile instar. This means that without TGP there is a

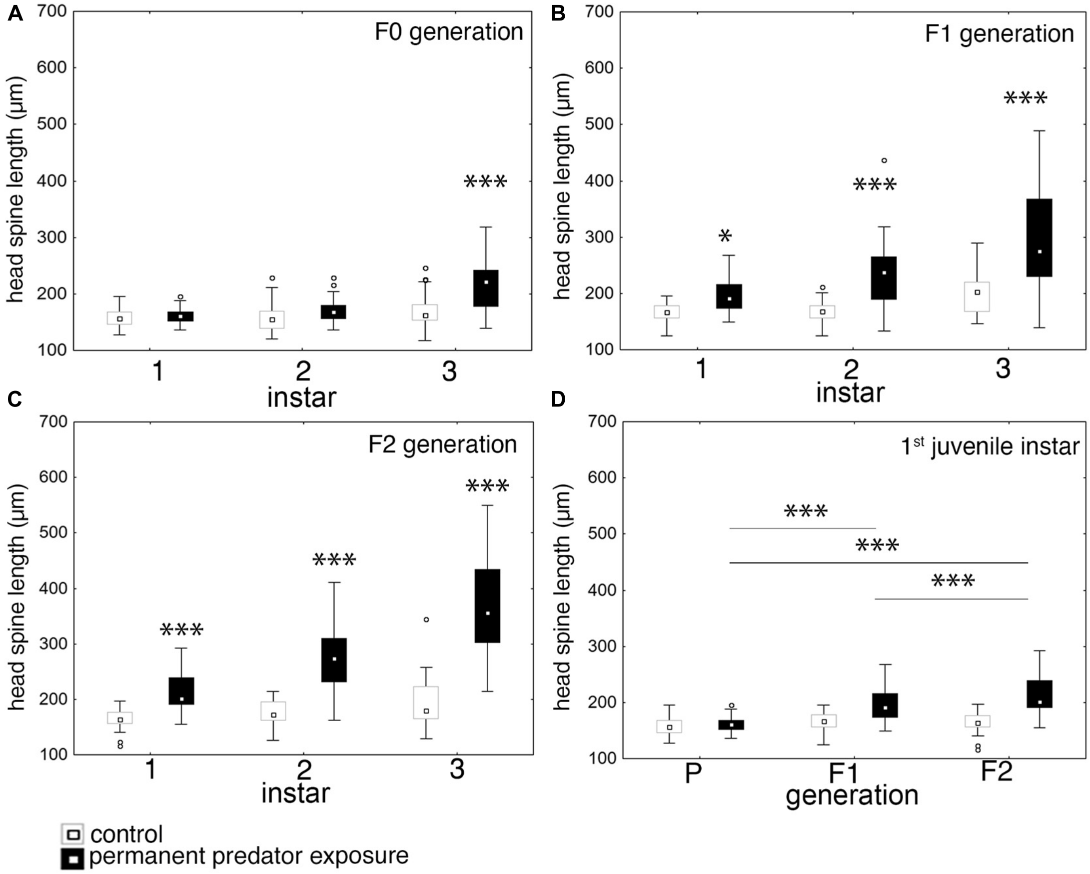

FIGURE 2 | Effect of permanent predator exposure on head spine length in *D. lumholtzi.* **(A)** F0 generation: head spine length in the first three juvenile instars in control and permanently fish exposed *D. lumholtzi*; ANOVA: $F_{(2,\ 203)} = 11.466$, $P \leq 0.001$. **(B)** F1 generation: head spine length in the first three juvenile instars in control and permanently fish exposed *D. lumholtzi*; ANOVA: $F_{(2,\ 237)} = 10.064$, $P \leq 0.001$. **(C)** F2 generation: head spine length in the first three juvenile instars in control and permanently fish exposed *D. lumholtzi*; ANOVA: $F_{(2,\ 199)} = 25.83$, $P \leq 0.001$. **(D)** Head spine length in the first juvenile instar in control and permanently fish exposed *D. lumholtzi* across the P, F1, and F2 generation. ANOVA: $F_{(2,\ 249)} = 17.77$, $P \leq 0.001$. Displayed are medians (square), midspread 50% (box) and the 25th percentile (lower whisker); 75th percentile (upper whisker), with outliers (circles). $^{*}P \leq 0.05$; $^{***}P \leq 0.001$. Bonferroni *post hoc* analysis in **Supplementary Tables 1–6**.

comparatively long lag phase, i.e., a reaction delay associated with defense expression if parents are in contact with kairomones. The offspring of these animals, however, express their defenses already upon birth. Consequently, the lag phase that initially comprised two molting cycles in the F0 generation is eliminated in the F1 and F2. Shortening the lag phase, could be a way to reduce the trade-offs associated with the defense, as during the lag phase without defenses the animals are still vulnerable to their predators and fitness levels are not yet elevated (Weiss and Tollrian, 2018a,b). Immediate defense expression may serve advantageous against predators like juvenile fish (shortly after their larval stage, i.e., 0+) that prefer smaller prey items such as juvenile *D. lumholtzi* (Kolar and Wahl, 1998).

In addition to the earlier and stronger initial expression of head spines also their growth rate is increased. When continuously exposed to predator cues, *D. lumholtzi* head spines grow faster with every subsequent generation that we tested. Such a rapid growth of the defensive structure further enhances survival chances as the defensive effect of the head spines is thereby shifted to earlier instars. The length and rigidity of spines is discussed to prevent ingestion and leads to avoidance by the predator (Engel et al., 2014). I.e., when the head spines outgrow the fishes' gape, predator handling becomes more complicated, and consumption is impaired. These head spines get stuck in fish's buccal cavity (Kolar and Wahl, 1998). The fish then shake their heads and flare their opercula attempting to dislodge their prey. The fish gain experience and then reject defended prey with higher frequency and this reduces *D. lumholtzi* mortality rates (Kolar and Wahl, 1998). Concludingly, progeny of permanently exposed *D. lumholtzi* are not only prepared by being born with a larger defense but also their defense subsequently grows faster.

Transgenerational phenotypic plasticity has been reported to dependent on a dedicated developmental window in which TGP is induced, so not only the exposure length but also the timing is decisional (Duncan et al., 2014). Very often not only the embryonic but also the parental environment was shown to impact the offsprings' phenotypes (Shea et al., 2011; Luquet and Tariel, 2016; Heckwolf et al., 2018). We therefore

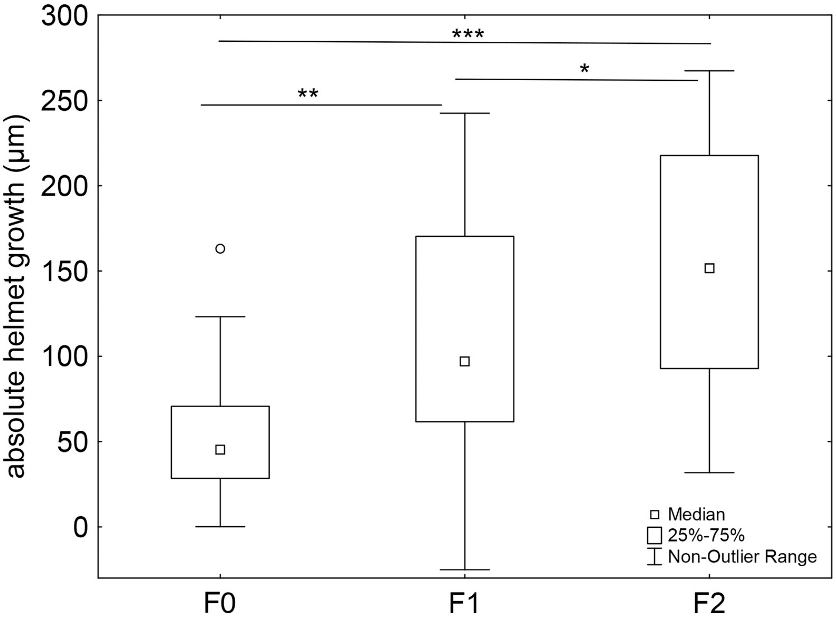

FIGURE 3 | Absolute head spine growth across generations. The proportional increase in head spine length from the 1st to the 3rd juvenile instar increases across generations when *D. lumholtzi* is permanently exposed to kairomones. F0 generation: *n* = 29, F1 generation: *n* = 37, F2 generation: *n* = 35. $F_{(2,\ 98)} = 11.008$, $P \leq 0.001$. Bonferroni *post hoc* comparison. Displayed are medians (square), midspread 50% (box) and the 25th percentile (lower whisker); 75th percentile (upper whisker), with outliers (circles). $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$. Bonferroni *post hoc* analysis in **Supplementary Tables 7**, **8**.

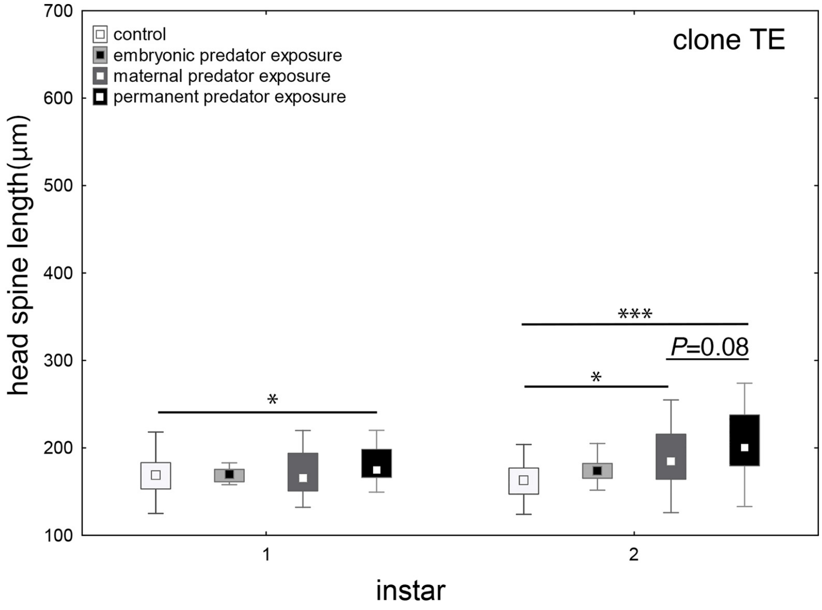

FIGURE 4 | Maternal vs. embryonic predator exposure. When *D. lumholtzi* embryos are exposed to fish kairomones, defenses are not developed in the first two juvenile instars. When the mothers are exposed to kairomones, head spines in the offspring are significantly larger in the 2nd juvenile instar. When *D. lumholtzi* is permanently exposed to kairomone, i.e., the mothers and the embryos, head spines of the offspring is significantly increased already in the 1st juvenile instar. Displayed are medians (square), midspread 50% (box) and the 25th percentile (lower whisker); 75th percentile (upper whisker), with outliers (circles). $^{*}P \leq 0.05$; $^{***}P \leq 0.001$. ANOVA and Bonferroni *post hoc* analysis in **Supplementary Tables 9**, **10**.

wanted to determine if this earlier onset and boost of defense expression is due to effects of fish kairomones in the maternal environment, the embryonic environment or both. We therefore tested two distinctive phases, i.e., prior to and during embryo development. *Daphnia* spec. have a dedicated reproductive strategy, i.e., mothers give rise to genetically identical offspring

(Smirnov, 2013). During this kind of parthenogenesis, mothers deposit subitaneous embryos in the brood pouch, that directly develop into juvenile offspring (embryogenesis) (Smirnov, 2013). When exposing naïve *D. lumholtzi* TE to kairomones during embryogenesis, this does not induce TGP; i.e., the lag phase is neither eliminated nor shortened and head spines are not boosted. This means that *D. lumholtzi* is not able to perceive and/or react to kairomones during prenatal development. Also, Laforsch and Tollrian did not detect prenatal growth of defensive structures during *D. lumholtzi* embryogenesis but found that spines are folded along the body which allows for embryonic growth of the traits (Laforsch and Tollrian, 2004). When we expose *D. lumholtzi* mothers to kairomones and remove them prior to conception, this results in TGP as lag times are shortened and defenses are boosted in the 2nd juvenile instar. Therefore, TGP must be inducible before embryogenesis. It is well possible that the environmental information is imprinted on the primordial germ cells that develop in the mother and thereby the offspring is prepared for future environmental conditions (Hemberger et al., 2009).

In comparison to offspring from mothers' that were exposed to kairomones until the 4th instar, permanently exposed *D. lumholtzi* have larger defenses. Similarly, the lag phase which is shortened by one instar in the offspring of not continuously exposed mothers, is shortened by two instars in the permanently exposed animals. Together with the fact, that the daphnia themselves are not sensitive to kairomones during embryogenesis this clearly shows that the shortening of the lag phase is a result of TGP. TGP initiates early defense expression so that WGP can start earlier and on a higher level. This TGP effect is intensified when the mothers are permanently exposed to kairomone, and could be an additive effect from the prolonged kairomone exposure.

Mechanistically TGP of inducible defenses is discussed to be associated with epigenetic inheritance where gene activity and/or accessibility is altered through differential methylation patterns, micro RNAs, or histone modifications (Vandegehuchte et al., 2010; Walsh et al., 2016; Fallet et al., 2020). These changes can be passed on to the next generation enhancing the offspring's performance (Feil, 2008; Sentis et al., 2018). Vandegehuchte et al. showed that *D. magna,* e.g., is able to pass on differential methylation patterns to the next two generations (Vandegehuchte et al., 2010). Furthermore, it was shown that methylation patterns from kairomone exposed *Daphnia ambigua* were passed on to following generations (Schield et al., 2016). The mechanisms that underly the formation of TGP in *D. lumholtzi* will be of interest in future experiments. Likewise, it remains to be investigated how long TGP persists in *D. lumholtzi* when predator cues are absent. In *D. cucullata* the TGP effect disappeared, after the disappearance of the predator cue (Agrawal et al., 1999). This scenario is well-imaginable for *D. lumholtzi*; when the predator disappears, the defenses will most likely stop growing and the animals will save the costs associated with defense expression. The following generation will most likely be undefended upon birth. It will be interesting to test if the offspring of predator exposed grandmothers and unexposed mothers, will react more sensitive to predator cues in comparison to offspring from naïve grandparents.

Taken together, *D. lumholtzi* shows a distinctive form of transgenerational phenotypic plasticity, where the defenses are developed faster and larger which enhances the defensive effect.

DATA AVAILABILITY STATEMENT

All data is provided in the **Supplementary Material** provided alongside of the manuscript.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ruhr University Bochum Animal Welfare Office.

AUTHOR CONTRIBUTIONS

LW and RT designed the study. AG, MJ, and MV performed the experiments. AG analyzed the data. AG, RT, and LW wrote the manuscript. All authors contributed to the final version 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.2021.637421/full#supplementary-material

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Predator-Induced Plasticity on Warning Signal and Larval Life-History Traits of the Aposematic Wood Tiger Moth, *Arctia plantaginis*

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Predator-induced plasticity in life-history and antipredator traits during the larval period has been extensively studied in organisms with complex life-histories. However, it is unclear whether different levels of predation could induce warning signals in aposematic organisms. Here, we investigated whether predator-simulated handling affects warning coloration and life-history traits in the aposematic wood tiger moth larva, *Arctia plantaginis*. As juveniles, a larger orange patch on an otherwise black body signifies a more efficient warning signal against predators but this comes at the costs of conspicuousness and thermoregulation. Given this, one would expect that an increase in predation risk would induce flexible expression of the orange patch. Prior research in this system points to plastic effects being important as a response to environmental changes for life history traits, but we had yet to assess whether this was the case for predation risk, a key driver of this species evolution. Using a full-sib rearing design, in which individuals were reared in the presence and absence of a non-lethal simulated bird attack, we evaluated flexible responses of warning signal size (number of orange segments), growth, molting events, and development time in wood tiger moths. All measured traits except development time showed a significant response to predation. Larvae from the predation treatment developed a more melanized warning signal (smaller orange patch), reached a smaller body size, and molted more often. Our results suggest plasticity is indeed important in aposematic organisms, but in this case may be complicated by the trade-off between costly pigmentation and other life-history traits.

Keywords: predation, Plastic response, Aposematism, Life-history, antipredator, Larva, Costs, maladaptation

INTRODUCTION

Organisms live in a constantly changing environment, and this variation may have important effects on an individual's fitness. Evolution (via genetic change) and plasticity (the flexibility of a genotype to change its phenotype in response to environmental cues) are key mechanisms upon which species adapt to environmental variation (Gotthard and Nylin, 1995; Ghalambor et al., 2007). Plasticity can be adaptive, when its response is in the same direction favored by selection (Ghalambor et al., 2007) and can allow a particular genotype to rapidly endure changes in the environment. On the other hand, a non-adaptive phenotype can be far from the adaptive peak and have a reduced fitness under environmental change (Ghalambor et al., 2007).

The role of plasticity in evolution is not straightforward. It is often argued that if plasticity is adaptive, it should deter evolution since it can hide genetic variation on which selection would act, and thus weaken selection (Ghalambor et al., 2007; Gordon et al., 2017). Yet, plasticity is also crucial for population persistence in a changing environment because it can buffer novel variants against purifying selection and may thus, facilitate long-term adaptation by maintaining higher genetic variation (Corl et al., 2018; Perry et al., 2018). Furthermore, induced-responses often involve costs, hindering the adaptive plastic responses by another environmental cue or stress response, thus reducing fitness and generating the pattern of maladaptive response (Morris and Rogers, 2013). Trade-offs in stress responses may limit plastic responses but may also constrain evolution and future adaptation (Koch and Guillaume, 2020).

Predation is one of the most important selective pressures in nature. Hence, predator-induced plasticity is perhaps one of the most relevant plastic responses, and has been shown to stimulate changes in prey morphology, behavior and life history for a variety of taxa (Tollrian, 1995; Tollrian and Dodson, 1999; Roff, 2002; Benard, 2004; Hammill et al., 2008). For example, studies using a variety of predator-simulated attack mechanisms have led to inducible variation in prey responses such as immunosuppression or variation in chemical defenses concentration (toxins) (Watson et al., 2004; Adamo et al., 2017; Bucciarelli et al., 2017; Cinel et al., 2020).

Predator-induced plasticity is common in organisms with complex life cycles, because each developmental phase exposes them to different predators, parasites, and environmental conditions (Benard, 2004; Friman et al., 2009; Mutamiswa et al., 2019). Prey life-history traits that are known to be plastic are growth rate, body size, and development time such as timing to metamorphosis or hatching (Stearns and Koella, 1986; Stearns, 1989; Nylin and Gotthard, 1998; Tollrian and Dodson, 1999). However, the direction and magnitude of plasticity in life-history traits vary widely (reviewed in Benard, 2004) and much is left to still discover and understand. In particular, it is still unknown how short-term selection pressures are linked to long-term fitness (Koch and Guillaume, 2020).

Plastic responses in life history may improve prey success under fluctuating predation risks, but may also involve trade-offs because individual survival might come at a cost to certain traits like reproduction (Tollrian, 1995; Tollrian and Dodson, 1999; Roff, 2002; Benard, 2004; Hammill et al., 2008). For instance, inducible morphological defenses, like neck spines and variation in body morphology in *Daphnia pulex* increase survival against predation from *Chaoborus* spp., compared to undefended morphs, but at the cost of a reduction in reproductive success (Hammill et al., 2008). The predatory rotifer *Asplanchna brightwellii* also induces plastic changes in the ultrastructure of lorica (shell), lorica thickness, lateral spines, and body size of *Brachionus* herbivores (*Brachionus calyciflorus* and *Brachionus angularis*). However, their development is also associated with decreased reproduction or reduced sex investment in herbivores (Yin et al., 2017).

Coloration is a common morphological defense that is often related to avoiding predator detection and attack (Endler, 1986, 1991a; Caro, 2005). Camouflage is the most common color strategy to avoid predation; it has been studied across a range of animal taxa including lepidopterans, crustaceans, cephalopods, reptiles, amphibians, and fish (Stevens, 2016), and has been shown to also be plastic in some cases. Some examples of background matching plasticity in response to different environmental cues are seen in pygmy grasshoppers, *Tetrix subulata* and *Tetrix ceperoi*. Their basic color is altered by the background substrate color, so when in a dark substrate, both species tend to change to a black and dark olive color, whereas in light substrates, the gray color morph dominates. The plasticity in body coloration increases camouflage and is likely to be an adaptation to reduce predation risk (Hochkirch et al., 2008). For organisms that live in complex environments, gaining optimal camouflage is tricky (Merilaita et al., 2017), in particular for mobile animals. Plasticity responses can solve this conundrum. A recent study by Corl et al. (2018) in side-blotched lizards (*Uta stansburiana*) established the crucial role of plasticity in background matching during the colonization of a new environment. Ancestral plasticity in coloration and divergence in two genes that increase pigmentation (regulators of melanin production), facilitate survival and persistence in novel darker habitats and allows time for subsequent genetic adaptation to fine-tune the plastic response to the new environment.

Aposematism is a complex defense strategy that is based on organisms displaying warning signals (typically conspicuous coloration) to advertise unpalatability or noxiousness (Poulton, 1890; Cott, 1940; Endler, 1991b; Inbar and Lev-Yadun, 2005; Speed and Ruxton, 2005). Although avoidance of conspicuous colors can be innate or inherited (Schuler and Roper, 1992; Mastrota and Mench, 1995; Lindström et al., 1999a), learning typically facilitates the negative association of a warning signal and unprofitability (Mappes et al., 2005). Predators learn to avoid unprofitable prey with conspicuous coloration more quickly compared to cryptic prey (Gittleman and Harvey, 1980; Lindström et al., 1999b). According to classic theory, predators acquire avoidance more quickly when warning signals are invariable (Müller, 1879). Thus, selection by predators should therefore favor the most common morph in the population, leading to positive frequency-dependent selection and monomorphism in aposematic species (Endler and Greenwood, 1988; Joron and Mallet, 1998; Endler and Mappes, 2004; Rowland et al., 2007; Ihalainen et al., 2008; Gordon et al., 2015). Why then, is variation in aposematic systems widespread (Briolat et al., 2019)?

Phenotypic plasticity could potentially provide a mechanism that allows for both monomorphism and variation in warning signals, through physiological plastic changes in color (e.g., changes in melanin or flavonoids production/synthesis triggered by temperature or other environmental stimuli) (Galarza et al., 2019). The desert locust, *Schistocerca emarginata*, is one of the most famous, but rare, examples of plasticity in aposematic species. Locusts display a shift in coloration as a response to local population density: at low-densities, individuals are solitary and cryptic (green), whereas at high-densities individuals shift to a more gregarious morph with aposematic coloration in the longer wings (yellow and black form) (Sword, 1999). This plasticity is suggested to reduce the cost of conspicuousness when locusts are rare and facilitate predator avoidance learning at high densities. However, this study involves a discrete trait, and there are currently very few studies investigating continuous plastic variation in warning signals.

The larvae of the wood tiger moth *Arctia plantaginis* [Arctiidae, formerly *Parasemia plantaginis* (Rönkä et al., 2016)] provide an excellent system to study predator-induced phenotypic plasticity in an aposematic species. Larvae are unpalatable and have a hairy black body with a dorsal continuous orange patch. The length of this patch varies significantly; from an almost entirely black phenotype to almost entirely orange (Ojala et al., 2007; Lindstedt et al., 2009, 2010, 2016; Galarza et al., 2019) and across and within populations (Ojala et al., 2007; Lindstedt et al., 2009, 2016). Previous studies in the species showed that the length of the orange patch is strongly heritable (Lindstedt et al., 2016) with temperature-induced (adaptive) plasticity (Lindstedt et al., 2009; Galarza et al., 2019). However, no study to date has evaluated predation-induced plasticity in the warning signal. Phenotypic plasticity may explain some variation observed in this system, because different signal sizes are beneficial under different conditions. A small signal (smaller orange patch) decreases the risk of predator detection (Lindstedt et al., 2008) but is associated with thermoregulation benefits (Lindstedt et al., 2009). A large orange patch is better at facilitating predator avoidance learning but less efficient at thermoregulation and immune response (Lindstedt, 2008; Lindstedt et al., 2008; Friman et al., 2009; Nielsen and Mappes, 2020). Having a plastic signal may therefore be the most efficient strategy for larvae in variable environments.

In this study we investigate to what extent predator-induced plasticity explains the continuous variation in larval warning coloration, and how predation risk influences larval life-history traits. To do so, we use a split full-sib rearing design, in which individuals are reared in the presence or absence of a non-lethal simulated bird attack. Typical bird attacks toward our hairy caterpillar include multiple pecking, handling, and dropping, which we stimulate. We then examine any changes in the length of the orange patch, as well as larval growth and development in the presence versus absence of this predation risk. If predator-induced plasticity plays a role in the size of the warning signal, we should expect differences in the length of the orange patch between the two predation treatments; this could be represented as a warning signal increase, as a more salient signal facilitates learning avoidance of avian predators, or as a warning signal decreases, as more melanic signals benefits from concealment when at high risk of detection or attack by naïve predators. We predict predation to have a negative effect on larval body size and developmental time (shorter developmental time with fewer instars) as risky environments seem to promote negative life-history shifts in many insects (e.g., Duong and McCauley, 2016).

MATERIALS AND METHODS

Study System

This experiment uses a laboratory population of wood tiger moth that had been reared and maintained in a greenhouse at the University of Jyväskylä in Central Finland since 2012 from wild Finnish stock. The caterpillars were fed with a mix of lettuce (*Lactuca sativa* var. crispa) and dandelion leaves (*Taraxacum* spp.) and kept under greenhouse temperatures that followed the outdoor temperature, between 20 and 30°C during the day (∼20 h) and 15–20°C during the night (∼4 h). In Finland, this species usually has one generation per year and *A. plantaginis* typically overwinter as a 3rd or 4th instar larva (Lindstedt et al., 2019).

Both adults and larvae are aposematic. Larvae are hairy and unprofitable (Lindstedt et al., 2008). Within the first two instars they are cryptically colored, but around their third instar larvae develop a warning signal that grows with age (Ojala et al., 2007) that can cover from 3 to 4 segments (30% of the body length) up to 7 segments (equivalent to 80% of the total body; **Figure 1**;

FIGURE 1 | Variation in larval warning signal. Large orange patch **(left)** and small orange patch **(right)**.

TABLE 1 | Full summary statistics for the influence of fixed effect, predation, random effect [family (all traits) and its interaction with predation (family-by-treatment)] effects on the warning signal and larval-history traits of *A. plantaginis*.

Trait	Statistics				
Orange signal size n = 448 families = 20	**Random effects**				
	Source of variation	Name	σ2	SD	
	Family	Intercept	0.398	0.631	
	Residual		2.246	1.498	
	Fixed effects				
	Source of variation	Estimate	s.e.	t	P
	Intercept	5.139	0.174	29.51	< 0.001
	Treatment	−0.305	0.141	2.153	0.031
Molts n = 331 families = 20	**Random effects**				
	Source of variation	Name	σ2	SD	
	Family	Intercept	0.184	0.43	
	Residual		0.853	0.923	
	Fixed effects				
	Source of variation	Estimate	s.e.	t	P
	Intercept	3.214	0.120	26.59	< 0.001
	Treatment	0.397	0.102	3.895	< 0.001
Growth rate n = 449 families = 20	**Random effects**				
	Source of variation	Name	σ2	SD	
	Family	Intercept	0.011	0.108	
	Residual		0.039	0.199	
	Fixed effects				
	Source of variation	Estimate	s.e.	t	P
	Intercept	0.4192	0.027	15.167	< 0.001
	Treatment	−0.083	0.018	−4.444	< 0.001
Development time n = 331 families = 20	**Random effects**				
	Source of variation	Name	σ2	SD	
	Family	Intercept	17.36	4.167	
	Residual		70.62	8.403	
	Fixed effects				
	Source of variation	Estimate	s.e.	t	P
	Intercept	47.666	1.138	41.87	< 0.001
	Treatment	1.418	0.93	1.525	0.128
Pupal weight n = 332 families = 20	**Random effects**				
	Source of variation	Name	σ2	SD	
	Family	Intercept	116.6	10.8	
	Residual		1296.8	36.01	
	Fixed effects				
	Source of variation	Estimate	s.e.	t	P
	Intercept	201.635	3.671	54.930	< 0.001
	Treatment	−35.224	3.974	−8.864	< 0.001

Lindstedt et al., 2008). This species is known to be a capital feeder, so emergence mass is set by resource allocation in the immature stages (Ojala et al., 2007). Individuals remain at the larval stage for most of their life cycle, on average 60 days. During this stage, larvae undergo four to seven instars. Following the larval stage, they pupate and emerge as adults 7–10 days later (Ojala et al., 2007).

Experimental Design

We evaluated predator-induced plasticity in a full-sib rearing design, in which we selected larvae from 20 families. From each family, we randomly selected 24 individuals of the same age (same hatching day), and these were evenly split and reared in two environments: presence (treatment; *N* = 240) and absence of a simulated bird predator attack (control; *N* = 240).

We started the experiment when the larvae were 10 days old (around instar 2), and reared them individually in petri dishes. At this point, all larvae still had cryptic coloration. We applied a non-lethal simulated bird attack stimulus in the predator treatment. We used a simulated predator handling to assume a predator induce response, as it has been shown in other studies that a physical non-lethal simulated predator attack induce prey responses to the perceived risk (Watson et al., 2004; Adamo et al., 2017; Bucciarelli et al., 2017; Cinel et al., 2020). Typical bird attack toward a hairy caterpillar include multiple pecking, dropping, and billing, which we tried to stimulate by taking the larva and gently handling it (squeezing) with soft tweezers (to avoid damaging the skin) 20 times. Most larvae returned to regular behavior not long after receiving the stimulus and no regurgitation and defecation were observed during simulated attack (Authors Pers. Obs.) Although the stimulated predator attack cannot exactly imitate a true bird attack with actual mortality, based on observing both real and simulated attacks we are rather convinced this manipulation was very close to a non-lethal predator attack. The treatment was applied every second day and continued until the larvae reached pupation, excluding the days when they were obviously molting. In the control environment, we reared the larvae under normal conditions without applying the bird attack stimulus. Furthermore, all individuals, from both treatments, were checked and fed daily, the petri dishes were clean (old food was removed and feces), and kept under greenhouse conditions. To ensure that the plastic responses were not a product of the handling stress, but an actual predator-induced response, the simulated predation handling was applied during the cleaning and feeding time so that both treatment groups were disturbed around similar times. We recorded the dates, when the stimulus was applied and the molting events, as well as the signal size after every molt in each petri dish. The experiment was conducted during the summer season (May to July 2015) at the University of Jyväskylä.

Data Collection

Traits were measured as follows: (A) warning signal size by counting the number of orange segments every time the larva molted (Lindstedt et al., 2008). Since the segments are distinguishable it was not necessary to disturb the larva to take the measurement. (B) molts by recording the dates and times the larva molted. This was done by checking daily the presence of dried skin from a molted larva. (C) growth rate: the difference between the final body size and the initial body size (mm) over the total days until the last measurement (before pupation or death). Body length in millimeters was measured using a digital caliper (Mitutoyo 500-181-21). All larvae were measured at the beginning of the experiment (when the treatment had not yet been implemented), and then approximately every sixth day until they reached pupation or died. Larvae were not manipulated during the measurements. If they were curled, they were left undisturbed until they had returned to seemingly normal behavior and were thus possible to measure. (D) development time by recording the days from hatching until pupation and (E) pupal weight was recorded in milligrams using a Mettler Xs204 digital scale.

Statistical Analysis

We used linear mixed-effects models to estimate the effects of the non-lethal predator stimulus on each of the following response variables separately: warning signal size, molting events, growth rate, developmental time, and pupal weight. The predator treatment was included as the only fixed factor for all the models. In the case of the orange signal size and molting, for which the data distribution were not clear, we selected the best model fit by comparing the AIC values of models with different distributions (**Supplementary Table 1**). To estimate the variation of the response due to the family, and to estimate the presence of family-by-treatment interaction, we compared models with the following random effect structures: (a) family and (b) family-by-treatment. The family-by-treatment interaction was retained in analyses if it improved the fit of the model, as judged by Likelihood Ratio tests (model comparison estimates reported in **Supplementary Table 2** and full models with random effect structures in **Table 1**). The analyses were done with all individuals except in the molting events and developmental time analyses, when only considered those that reached pupation.

We ran a survival analysis, to examine whether our light non-lethal predation stimulus induced high mortality between our treatments. Results importantly showed no significant differences between the control and the treatment [Cox proportional hazards model ($P = 0.15$), **Supplementary Table 3**].

The level of significance in all analyses was set at $p < 0.05$. The Satterthwaite approximation for degrees of freedom was applied in the linear mixed-effects models. All the statistical analyses were performed in R (v4.0.2; R Core Team 2020).

RESULTS

Larvae reared in the simulated predator handling environment displayed an orange band significantly smaller than individuals reared in the control environment ($F_{1,427.87} = 4.634$, $p = 0.031$; **Figure 2**). The phenotypic variation due to the family effect was about 15% (**Table 1**). In the predator treatment group, the orange band was 0.3 segments smaller than in the control (average signal 5 orange segments). Predation had a significant effect on most of the larval history traits. The larvae molted in the control group around three times after the experiment started, and 0.4 significantly more times in the predator environment ($F_{1,312.34} = 14.776$, $p \leq 0.001$; **Figure 3**), they grew 0.08 mm less per day ($F_{1,427.3} = 19.753$, $p \leq 0.001$) compare to the control ones (average 0.4 mm per day; **Figure 4**), the treatment did not, however, significantly affect larval developmental time ($F_{1,311.57} = 2.33$, $p = 0.127$; **Figure 5**) with 48 days in average until reach pupation. Finally, the average pupal weight in normal conditions was 201.635 mg, while in individuals from the predation treatment weighted 35.22 mg less ($F_{1,313.39} = 78.573$, $p \leq 0.001$; **Figure 6** and **Table 1**).

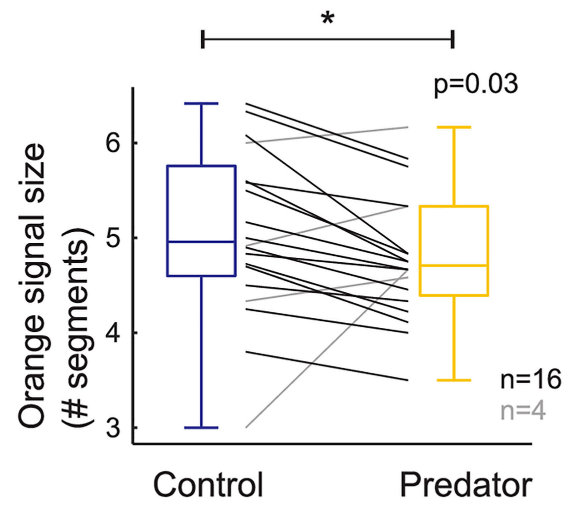

FIGURE 2 | Larval orange signal size based on raw data (i.e., each black and gray line represents a family). Boxplots show the mean values on control and predator treatment. * means significance.

DISCUSSION

Organisms with complex life cycles that express plasticity in their defense and life-history traits may have a selective advantage

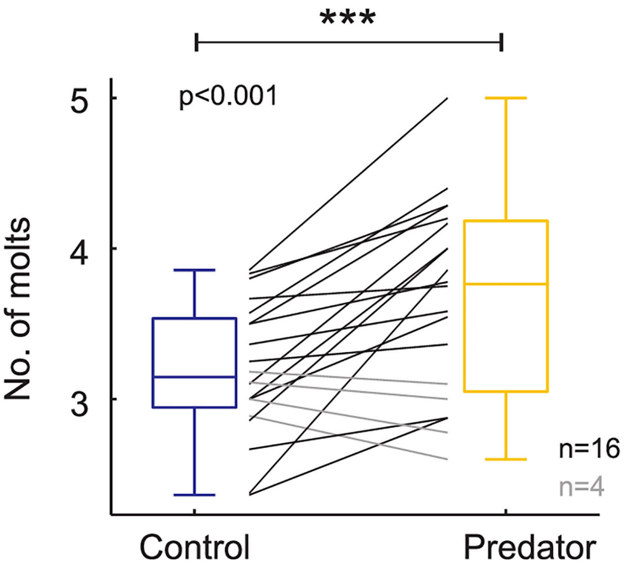

FIGURE 3 | Number of molting events based on raw data (i.e., each black and gray line represents a family). Boxplots show the mean values on control and predator treatment. *** means high significance.

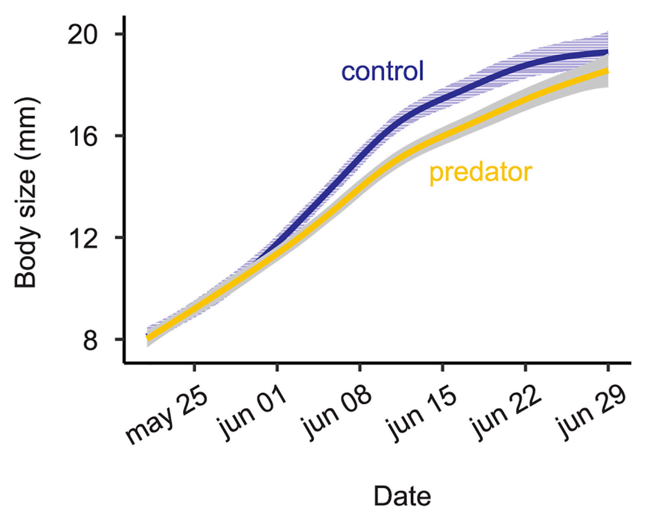

FIGURE 4 | Larval growth rate based on raw data.

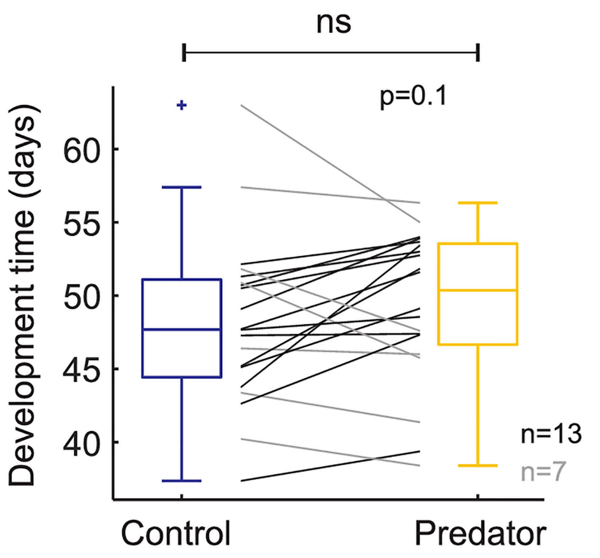

FIGURE 5 | Larval development time based on raw data (i.e., each black and gray line represents a family). Boxplots show the mean values on control and predator treatment. + means outlier.

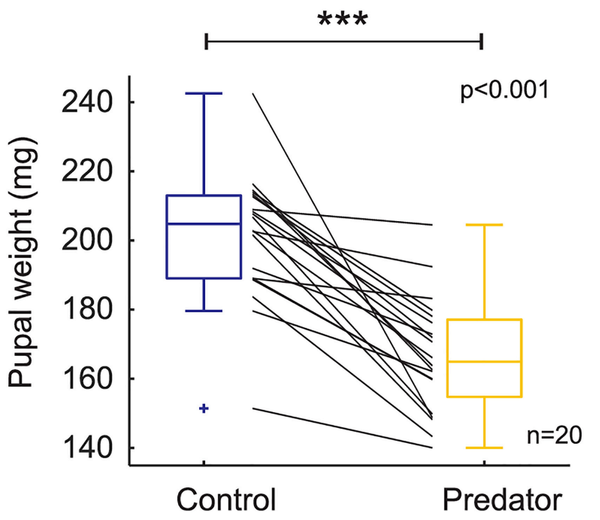

FIGURE 6 | Pupal weight based on raw data (i.e., each black and gray line represents a family). Boxplots show the mean values on control and predator treatment. *** means high significance; + means outlier.

because flexibility in strategies may allow a relatively rapid response to a range of threats and thus, increased survival. However, environmental change is often multivariate, and organisms are exposed simultaneously to multiple stressors that may have opposing effects. Furthermore, organisms can rarely can optimally respond to different stimulus since such responses may be costly and trade-off with other important traits leading to less optimal or even maladaptive responses.

We examined plastic responses to predation in the aposematic wood tiger moth larvae in warning signal efficacy and life-history traits. Aposematism is an interesting strategy to investigate potential plasticity for warning signal, since its efficacy is strongly dependent on both environments (light, background) and receivers' vision and cognition. Thus, it is unlikely that there is only one optimal strategy for warning signal conspicuousness and potential for the plastic responses could greatly improve prey survival. Interestingly, the only example of adaptive plasticity of warning signal we are aware of is the famous desert locust, *Schistocerca emarginata* (Sword, 1999) that change its coloration in response to population density. Our study species, wood tiger moth larvae show continuous variation in the size of their orange warning signal, which suppresses predators willingness to attack (Lindstedt et al., 2008; Nielsen and Mappes, 2020) but at the same time, when large, increases the prey conspicuousness which in turn increases the attack risk by naïve predators (Mappes et al., 2014). We investigated whether larvae could perceive predation risk and respond on it by rearing larvae in two different environments: with and without non-lethal predator stimulus. As we expected, we found that overall predation induced plasticity in defense and life-history traits. The predator stimulus reduced orange signal size, as predicted, increased molting events, decreased growth rate, body size, and pupal mass. In contrast, the treatment did not affect developmental time. We also found a clear family effect showing that there is additive genetic variation for all measured life history traits, but no interactions between the predator treatment. This means that families overall responded to the treatment similarly although they might have expressed differences in the mean plastic responses.

We expected predation to induce a positive (increase of orange patch length) or negative (decrease of orange patch length) plastic response in larval warning signals, because larger orange patches in *A. plantaginis* have been proven to enhance avoidance learning of avian predators (Lindstedt et al., 2008). On the other hand, a small signal decreases the risk of predator detection (Lindstedt et al., 2008) and it is also associated with thermoregulation benefits (Lindstedt et al., 2009).

Our results show that larvae from the predator environment expressed a smaller final orange signal, and thus a more melanized body. First, this might be explained by varying costs and benefits of warning signals under different predation environments. For example, a large signal can enhance predator avoidance learning (Lindstedt et al., 2008), but it may be costly against naïve predators because it increases conspicuousness and the risk of detection (Lindstedt et al., 2008). Smaller warning signals might therefore be favored by selection when the risk of detection by naïve predators is high (Endler and Mappes, 2004; Lindstedt et al., 2008).

Second, the contrast between the two colors (orange and black) might be more important in conspicuousness indicating prey unprofitability (Aronsson and Gamberale-Stille, 2012) and its detectability. Indeed, studies done by Tullberg et al. (2005); Bohlin et al. (2008), Caro et al. (2013); Barnett and Cuthill (2014), Barnett et al. (2016); Nielsen and Mappes (2020) suggest that contrasting color patterns are more efficient warning signals against predators than a homogeneous warningly colored signal. Furthermore, they proposed that contrasting color patterns might hold a double purpose: low detectability (camouflage) at a distance, but more conspicuous when close. Suggesting that intermedium levels of internal contrast seem to be a more efficient warning signal, because it keeps the balance between cryptic and aposematic function. In our case, reduction of the orange warning signal itself may have been a response of better highlighting this color contrast and an adaptive response to decrease conspicuousness.

Third, the plasticity in the signal might also indicate a non-adaptive response to the stress suggesting that our high predation risk simulation was perhaps too strong (squeezing the larvae 20 times every time stimulus was applied) or lasted too long (every second day until pupation or death). Under novel conditions, it is expected that many of the initial plastic responses are non-adaptive, because selection has not had the opportunity to shape the plastic response (Ghalambor et al., 2007, 2015). However, it should be noted that we did not find significant mortality differences between the treatments and all larvae returned to normal behavior soon after the stimulus was applied.

Fourth, increased larva cuticular melanin deposition is a common stress-response in insects to conditions such as exposure to UV radiation; probably due to the photoprotective properties (Debecker et al., 2015). It can also be a stress response to low temperatures (Goulson, 1994; Galarza et al., 2019), potentially a thermoregulation benefit to adjust to new conditions (Galarza et al., 2019), and predator risk. Under predation risk increased melanin can be an anticipatory response to the potential risk of cuticular wounding (Duong and McCauley, 2016), because of the important role of melanin in immune response and wound healing (Barnes and Siva-Jothy, 2000; Elliot et al., 2003; Friman et al., 2009; Beckage, 2011; Parle et al., 2016). Our stimulus, however, non-lethal caused larvae to suffer some hair loss during the attack simulation (Abondano Pers Obs). This loss could have stimulated an anticipatory risk of cuticular wounding in our larvae (Duong and McCauley, 2016) thus activating the melanization cascade, a physiological mechanism involved in immune responses and cuticle plugging and healing (De Gregorio et al., 2002; Beckage, 2011).

The plasticity responses observed in life-history traits under the treatment can be also considered non-adaptive response, according to life-history theory which should predict faster life histories under predation risk (Stearns and Koella, 1986; Abrams and Rowe, 1996; Arendt, 1997; Nylin and Gotthard, 1998; Roff, 2002; Benard, 2004). Instead, larvae in our predation treatment showed a lower growth rate, a smaller final body size, and a lighter pupal weight but similar developmental times compared to the control. A slow growth rate could be compensated with longer development times, but at the cost of increased risk of predation. On the other hand, a fast growth could mean avoiding predation risks but at the cost of reaching metamorphosis at an optimal size.

The lower growth rate and smaller final body size could be potentially explained by an indirect effect of the increase in melanization through the costly melanin hypothesis. Some studies suggest darker-induced phenotypes might be costly (as resources have to be allocated for melanin synthesis) in terms of lower growth rates and smaller body sizes (Goulson, 1994; Debecker et al., 2015; Galarza et al., 2019; Lindstedt et al., 2019). The results could also be explained by environmental stress during development. Here, constant manipulation or stress could alter overall physiological functioning, challenging the organism to keep metabolic functions normal (Chevin and Hoffmann, 2017; Taborsky et al., 2020). As a result of the cumulative stress, larvae in the predator treatment could have been limited to reach a minimal viable weight (MVW), defined by Davidowitz et al. (2003) as the minimal amount of resources necessary for a developing larva to successfully pupate. Lastly, our results could reflect altered foraging activity. Larvae could reduce their feeding activity as a behavioral defense to reduce predation risk, which results in less energy allocated in growth (Lima and Dill, 1990; Stamp and Bowers, 1993; Cressler et al., 2010). Because *A. plantaginis* is a capital breeder (Ojala et al., 2007), a lower growth rate, together with a limited developmental time, could constrain the resources allocated for pupation, leading to a lighter pupal weight. This could be considered as a stress-response based on the finding by Galarza et al. (2019), in which larvae of *A. plantaginis* reared in low temperatures, showed a lower growth rate, but a longer developmental time leading to similar pupal weights compared to larvae reared in high temperatures.

Larvae in the predator treatment underwent a higher number of molting events than the control ones. Individuals may increase their molting under conditions that constrain body size to compensate for this reduced growth rate, because body size increases at each larval molt. This stress response to unfavorable conditions is commonly observed in most insect species (Esperk et al., 2007), and may work as a compensatory mechanism for larvae to reach the threshold size and survive in adverse conditions (Nijhout, 1998). It could also help larvae reach pupation at the optimal size for hibernation under stressful conditions (Esperk et al., 2007; Barraclough et al., 2014). However, larvae did not differ in the age at pupation and the final body size was smaller in the predation treatment, suggesting the increase in molts did not work as a compensatory mechanism to increase body size or pupal mass. Perhaps the increment in molts could be explained as a response to restore the hair lost during the attack simulation in the predation treatment. Although hairiness does not decrease predator attacks or enhance avoidance learning of avian predators (Lindstedt et al., 2008), it can play an important role in protecting prey against another type of predators [e.g., insects; (Dyer, 1995)]. A higher number of molting events (increase in hairiness), together with increased melanization (decrease in orange patch size) may therefore be an investment in antipredator defenses, and a trade-off with investment in growth (larval growth rate and pupal weight).

Understanding how organisms and populations can adapt to changing environmental conditions is a crucial topic in evolutionary biology, considering the rapid environmental changes caused by human activity. Here, we have demonstrated that phenotypic plasticity is a potential mechanism for aposematic species to respond to novel stressful environments. We found that high predation risk, a novel condition for the larvae, induced plasticity in the warning signal by increasing melanin deposition. Furthermore, the predation treatment induced stress responses in life-history traits, indicating a potential trade-off in resource allocation for signal and life-history traits. However, to what extent phenotypic plasticity is beneficial, creating novel opportunities for selection, or harmful, constraining adaptive potential in a challenging environment, is yet unclear (Sgrò et al., 2016; Oostra et al., 2018). Future empirical studies are needed to investigate long-term fitness consequences of phenotypic plasticity, and whether plastic responses in warning signals are linked to changes in antipredator behavior. For example, larvae with a more conspicuous signal could be expected to spend more time in exposed locations compared to larvae with a more cryptic signal (Nielsen and Mappes, 2020). Finally, our study investigated phenotypic plasticity in laboratory-reared individuals with a homogenous population history. However, the evolutionary history of the population might have important effects on its plasticity, and future studies should therefore investigate phenotypic plasticity in wild populations with different evolutionary histories to better understand whether plastic responses are adaptive and how insect populations will perform under different scenarios.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

SG designed the study. DA conducted the experiments and led the writing of the manuscript. DA and SG analyzed the data. All authors contributed critically to the drafts, edited the manuscript, and approved the final version.

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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.2021.658177/full#supplementary-material

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Corrigendum: Predator-Induced Plasticity on Warning Signal and Larval Life-History Traits of the Aposematic Wood Tiger Moth, *Arctia plantaginis*

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In the published article, there was an error regarding the affiliation for Diana Abondano Almeida. As well as having affiliation 2, they should also have Department of Wildlife-/Zoo-Animal-Biology and Systematics, Faculty of Biological Sciences, Goethe Universität, Frankfurt, Germany.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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Parental Investment Under Predation Threat in Incubating Common Eiders (*Somateria mollissima*): A Hormonal Perspective

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Predation risk affects the costs and benefits of prey life-history decisions. Predation threat is often higher during reproduction, especially in conspicuous colonial breeders. Therefore, predation risk may increase the survival cost of breeding, and reduce parental investment. The impact of predation risk on avian parental investment decisions may be hormonally mediated by prolactin and corticosterone, making them ideal tools for studying the trade-offs involved. Prolactin is thought to promote parental care and commitment in birds. Corticosterone is involved in allostasis and may either mediate reduced parental investment (corticosterone-fitness hypothesis), or promote parental investment through a reallocation of resources (corticosterone-adaptation hypothesis). Here, we used these hormonal proxies of incubation commitment to examine the impact of predation risk on reproduction in common eiders (*Somateria mollissima*) breeding in the Baltic Sea. This eider population is subject to high but spatially and temporally variable predation pressure on adults (mainly by the white-tailed eagle *Haliaeetus albicilla* and introduced mammalian predators) and nests (by the adult predators and exclusive egg predators such as hooded crows *Corvus cornix*). We investigated baseline hormonal levels and hatching success as a function of individual quality attributes (breeding experience, female and duckling body condition), reproductive investment (clutch weight), and predation risk. We expected individuals nesting in riskier environments (i.e., on islands where predation on adults or nests is higher, or in less concealed nests) to reduce their parental investment in incubation, reflected in lower baseline prolactin levels and either higher (corticosterone-fitness hypothesis) or lower (corticosterone-adaptation hypothesis) baseline corticosterone levels. Contrary to our predictions, prolactin levels showed a positive correlation with nest predation risk. The unexpected positive relationship could result from the selective disappearance of low-quality females (presumably having low prolactin levels) from risky sites. Supporting this notion, female body condition and hatching success were positively correlated with predation risk on females, and baseline prolactin concentrations were positively correlated with duckling body condition, a proxy of maternal quality. In line with the corticosterone-adaptation hypothesis, baseline corticosterone levels increased with reproductive investment, and were negatively associated with nest predation risk. Hatching success was lower on islands where nest predation risk was higher, consistent with the idea of reduced reproductive investment under increased threat. Long-term individual-based studies are now needed to distinguish selection processes occurring at the population scale from individually plastic parental investment in relation to individual quality and variable predation risk.

Keywords: corticosterone, prolactin, individual quality, parental care, predation risk, reproductive success, common eider (*Somateria mollissima*)

INTRODUCTION

Predation exerts both direct and indirect effects on prey, and these effects show considerable spatiotemporal variation (Lima, 1998; Laundré et al., 2010). Whereas predators affect prey populations by direct killing, their mere presence also induces behavioral and physiological changes in prey (Lima, 1998). Such changes include increased aggregation tendency (Lehtonen and Jaatinen, 2016), altered habitat use (Turner, 1996; Creel et al., 2005), increased sensitivity to disturbance (Gómez-Serrano, 2020), and altered activity budgets (Pöysä, 1987; Blumstein and Daniel, 2005; Öst and Tierala, 2011). During the breeding season, the presence of predators affects the costs and benefits of parental investment, often leading to an exacerbated "cost of reproduction" (Slagsvold, 1984; Magnhagen, 1991). Indeed, reproducing prey are particularly exposed to predation by the risky behaviors they adopt to find a partner, mate, and successfully raise their offspring (Thomas, 1988; Magnhagen, 1991; Arnold et al., 2012). Consequently, decreased investment in current reproduction is expected under elevated predation risk, a prediction verified in birds laying smaller clutches when exposed to high risk (Doligez and Clobert, 2003; Eggers et al., 2006; Zanette et al., 2011; Noreikiene et al., 2021).

Reproduction is a costly life-history event not only because of its effect on survival (Magnhagen, 1991; Schwarzkopf and Shine, 1992; Arnold et al., 2012), but also because of elevated energy expenditure (Krapu, 1981). Life-history theory thus predicts a trade-off between individual investment in reproduction and survival (Williams, 1966). The cost of reproduction varies depending on individual state and quality (Feifarek et al., 1983; Reznick, 1985; Hamel et al., 2009), but also prevailing environmental conditions (De Steven, 1980; Reznick, 1985). In long-lived species, current parental investment is generally expected to be higher under favorable environmental conditions and individual states, i.e., when resources are plentiful (Whittingham and Robertson, 1994; Storey et al., 2017), weather conditions are benign (Thierry et al., 2013), predation risk is low (Ghalambor and Martin, 2000), and when individuals are in good condition (Chastel et al., 1995; Tveraa et al., 1998). On top of these factors, long-lived species should invest more into current reproduction as the prospects of future reproduction diminish with advancing age (the restraint hypothesis, Curio, 1983; Heidinger et al., 2006), even if environmental conditions are far from being optimal. This prediction has been well supported empirically (Forslund and Pärt, 1995; Préault et al., 2005; Angelier et al., 2007a,b). As a consequence of this survival-reproduction trade-off, individuals are predicted to modulate their allocation to current reproduction (Reznick, 1985), and may even completely refrain from current reproduction by skipping breeding under prohibitively high costs of reproduction (Chastel et al., 1995; Erikstad et al., 1998; Bruinzeel, 2007; Öst et al., 2018).

Hormonal mechanisms are involved in the mediation of life-history decisions when coping with a range of stressors including predation risk (Ricklefs and Wikelski, 2002). In birds, prolactin and corticosterone are especially relevant to study parental investment. Prolactin is the main hormone involved in the expression of parental behavior, and prolactin levels can be a reliable proxy of parental investment and reproductive involvement (Angelier and Chastel, 2009; Angelier et al., 2016; Smiley, 2019). Consequently, circulating blood prolactin concentrations are elevated during reproduction (Criscuolo et al., 2002; Vleck and Vleck, 2011). Furthermore, individuals with higher prolactin levels may provide better parental care (Angelier and Chastel, 2009; Smiley and Adkins-Regan, 2016, 2018), such as higher brood provisioning (Miller et al., 2009; Smiley and Adkins-Regan, 2016, 2018) and incubation commitment (Angelier et al., 2015; Wang et al., 2020), or even shorter latency to return to the nest after a disturbance (Angelier et al., 2009; Hope et al., 2020). Importantly, circulating prolactin levels have been shown to decrease in response to stressor, poor body condition, or low food availability (Delehanty et al., 1997; Groscolas et al., 2008; Angelier and Chastel, 2009; Riechert et al., 2014a). This is associated with a concomitant reduction of parental care, suggesting that prolactin may mediate parental responses to environmental conditions (Angelier et al., 2016).

Corticosterone, on the other hand, is involved in the maintenance of homeostasis (McEwen and Wingfield, 2003; Romero et al., 2009) and in the response to environmental challenges (Angelier and Wingfield, 2013; Wingfield, 2013). The corticosterone-fitness hypothesis states that circulating corticosterone levels are negatively associated with performance and fitness, because elevated baseline corticosterone levels may reflect higher energetic demand induced by deteriorating environmental conditions (Wingfield et al., 1983; Kitaysky et al., 2007), and may be associated with reduced survival and breeding success (Silverin, 1986; Angelier et al., 2010; Goutte et al., 2010; Vitousek et al., 2018; Breuner and Berk, 2019). For example, high circulating corticosterone levels have been shown to be involved in a reduction of parental care (Silverin, 1986; Angelier et al., 2009; Ouyang et al., 2011; Lynn, 2016; Schoenle et al., 2017), or even induce nest abandonment (Wingfield et al., 1983; Robin et al., 1998; Groscolas and Robin, 2001; Spée et al., 2011). In contrast, circulating corticosterone levels may also be positively associated with fitness, as stated by the corticosterone-adaptation hypothesis (Bonier et al., 2009). Indeed, elevated baseline corticosterone levels may enhance energy mobilization (Romero, 2002; Breuner, 2011) and therefore promote reproductive effort (Riechert et al., 2014a). For example, elevated baseline corticosterone levels have been associated with higher incubation effort (Ouyang et al., 2013), higher brood provisioning (Miller et al., 2009; Bonier et al., 2011; Ouyang et al., 2013), or higher offspring production (Bonier et al., 2011; Love et al., 2014). Importantly, the association between fitness and corticosterone may be dose-dependent (Bonier et al., 2009). In line with hormetic mechanisms (Costantini et al., 2010; Costantini, 2014), the corticosterone-fitness hypothesis may be validated under high levels of baseline corticosterone, while the corticosterone-adaptation hypothesis may be validated under lower baseline corticosterone levels.

Here, we investigate to what extent prolactin and corticosterone—two endocrine mediators of parental investment—are affected by predation risk in a long-lived ground-nesting bird, the common eider (*Somateria mollissima*), hereafter eider. Specifically, baseline prolactin and corticosterone levels have been shown to be closely related to incubation commitment in several species (Ouyang et al., 2013; Schoenle et al., 2017; Bowers et al., 2019; Hope et al., 2020), including the eider (Criscuolo et al., 2002, 2005). We therefore expect female eiders, as a long-lived species, to display lower baseline prolactin levels and either higher (corticosterone-fitness hypothesis) or lower (corticosterone-adaptation hypothesis) baseline corticosterone levels at the end of incubation if they reduce their investment in incubation as a response to increased predation threat. In our study area in SW Finland, northern Baltic Sea, female eiders nest on islands showing substantial spatial and temporal variation in predation pressure on adults and their nests (Öst et al., 2018) as well as in nest concealment (Öst and Steele, 2010), allowing us to test the impact of predation risk on variation in these endocrine traits and hatching success. We analyzed baseline hormone levels (prolactin and corticosterone) as a function of direct or indirect proxies of female quality (i.e., breeding experience, female body condition, offspring body condition), energetic investment in current reproduction (clutch weight), and predation risk on incubating female eiders and nests. In addition, we examined the endocrine correlates of reproductive success (i.e., hatching success). We expect (1) higher baseline prolactin and either lower (corticosterone-fitness hypothesis) or higher (corticosterone-adaptation hypothesis) baseline corticosterone levels in high-quality breeders and/or birds producing heavier clutches, as experience and condition are known to influence hormone levels (Angelier et al., 2007b; Bókony et al., 2009; Breuner, 2011). We also expect (2) birds nesting in riskier environments (i.e., on islands where predation on adults or nests is higher, or in less concealed nests) to have lower prolactin levels and either higher (corticosterone-fitness hypothesis) or lower (corticosterone-adaptation hypothesis) corticosterone levels, reflecting lower investment in incubation under increased threat. Finally, due to the link between parental investment and both prolactin and corticosterone levels (Angelier and Chastel, 2009; Bonier et al., 2009), we expect (3) lower baseline prolactin levels and either higher (corticosterone-fitness hypothesis) or lower (corticosterone-adaptation hypothesis) baseline corticosterone levels to be associated with poorer reproductive performance (i.e., reduced hatching success).

MATERIALS AND METHODS

Study Area and Species

Study Area and Female Monitoring

Fieldwork was conducted in the archipelago surrounding Tvärminne Zoological Station (59°50′N, 23°15′E), in the western Gulf of Finland, in 2013. In this area, female eiders nest on islands of various sizes (N= 17 islands, mean area ± SD = 1.78 ± 2.72 ha, min = 0.13 ha, max = 10.22 ha). Vegetation cover varies from bare rocks and sparse patches of herbs or stands of juniper (*Juniperus communis*) to pine (*Pinus sylvestris*) dominated forest. The study islands are relatively close to each other, typically only hundreds of meters apart, in a total study area of ca 15 km^2. Nevertheless, islands vary considerably in predation risk on both incubating females and their nests (Öst et al., 2011), due to variable forest cover (Ekroos et al., 2012; Öst et al., 2018) and range of available nest micro-habitats in terms of concealment (Seltmann et al., 2014). Importantly, the breeding period represents a period of elevated predator-induced mortality in this ground-nesting bird, and thus the survival of adult females is lower than that of males in eider populations subject to significant predation risk (Ramula et al., 2018).

Eiders are long-lived ground-nesting birds, with a mean life expectancy of ca. 21 years (Coulson, 1984). The eider breeding population at Tvärminne has been monitored every year since 1990. Incubating female eiders were trapped on their nest with hand nets between May and early June to measure hormone levels (N = 148 out of 355 breeding females in 2013). Females incubate for about 26 days (Korschgen, 1977), and incubating birds were captured predominantly during the later phase of incubation to minimize nest abandonment (mean ± SD = 17.6 ± 4.6 days after the start of incubation, range = 6.5–25.5 days). Incubation stage was estimated using an egg floatation test (Kilpi and Lindström, 1997). Each trapped female was ringed with a standard metal ring and a unique color-ring combination, weighed to the nearest 5 g with a Pesola spring balance, and its radius-ulna length was measured to the nearest 1 mm with a wing ruler as a proxy of body size.

Breeding Experience, Body Condition, and Clutch Weight

We characterized female breeding experience using a qualitative indicator (Jaatinen and Öst, 2011). We categorized females as either experienced (already ringed when captured) or inexperienced breeders (unringed at capture; only adult breeders are ringed in the study area). As eiders are philopatric (Swennen, 1990, 1991; Coulson, 2016) and show high nest-site fidelity (Öst et al., 2011; Coulson, 2016), and as the majority of breeding females are captured annually (trapping success in 2013 = 65.1%), with a constant trapping effort since 1996 (Jaatinen and Öst, 2011), this indicator of breeding experience can be considered reliable.

For each trapped female, we calculated two indices of body condition: one measured when the bird was trapped and sampled for blood (physiological parameters are expected to vary according to the instantaneous condition at the time of sampling, Kitaysky et al., 1999; Riechert et al., 2014b), and the other estimated at hatching (to allow direct comparison between females by taking into account weight loss during incubation, see below). To avoid any weight bias due to females potentially still laying eggs, only females that had incubated for more than 5 days were kept for further analyses. Body condition at trapping was calculated as the standardized residuals of a linear regression of log-transformed weight on log-transformed radius-ulna length. As eiders fast and lose weight during incubation (Criscuolo et al., 2000), it is essential to take into account the incubation stage to compare individual differences in energy reserves, in addition to structural size. Hence, we estimated body condition at hatching. To do so, we used the standardized residuals of a linear regression of log-transformed estimated weight at hatching on log-transformed radius-ulna length (Öst et al., 2008). Weight at hatching was estimated as the weight of the female at trapping subtracted by the estimated weight that the female was expected to lose during the remaining incubation time (estimated log-transformed daily weight loss: E ± SE = -0.221 ± 0.004, $t = -51.64$, $P < 0.001$). This estimate of mean weight loss rate during incubation was derived as the slope of the linear regression of log-transformed body mass on log-transformed incubation time and projected hatching date (see Öst and Steele, 2010). In addition, for each trapped female, total clutch weight was measured to the nearest 1 g in order to control for a potential influence of clutch weight on endocrine traits. Because eggs lose mass during development, we corrected clutch mass for incubation stage, evaluated by egg floatation, by extracting the residuals obtained from the linear regression of clutch weight on incubation stage.

Endocrine Correlates of Parental Investment

In 2013, a total of 148 breeding female eiders were trapped on the nest and sampled for blood (∼1.5 mL) from the ulnar vein within 3 min following capture (mean ± SD = 2.42 ± 0.35 min, range = 1.67–3.22 min) in order to obtain baseline hormonal levels (Romero and Reed, 2005). Baseline corticosterone levels were not related to sampling time (linear regression: $t = 1.23$, $P = 0.22$, $r^2 = 0.004$); hence the two blood samples taken after 3 min (3.12 and 3.22 min) were kept for further analyses. Blood samples were centrifuged and plasma was stored at −20°C until assay. Plasma concentrations of corticosterone and prolactin were determined by radioimmunoassay at the Centre d'Etudes Biologiques de Chizé (CEBC), as previously described (corticosterone: Lormée et al., 2003; Öst et al., 2020); prolactin: Criscuolo et al., 2002) (corticosterone, 5 assays, inter-assay CV: 14.31%, intra-assay CV: 10.97%; prolactin, 2 assays, inter-assay CV: 12.05%, intra-assay CV: 11.32%).

Hatching Success and Offspring Condition

For each nest, hatching success (0: failure, 1: success) was determined by returning to the nest at the end of incubation and monitoring the presence of ducklings. The eider is a precocial species and ducklings usually leave the nest within 24 h of hatching (Öst and Bäck, 2003). If no ducklings were found in the nest, they had either already hatched and left the nest, or the nest had been depredated. Consequently, egg shells were examined to determine if the breeding attempt had been successful or not. Hatched eggs were recognizable by their intact leathery membrane while depredated nests had either no eggs or eggs broken into pieces, usually with a bloody membrane still attached to the shells (Bolduc et al., 2005; Öst and Steele, 2010). A nest was considered as hatched if at least one duckling or one hatched egg membrane was found.

If ducklings ($N = 209$) were found in the nest ($N = 66$ out of 93 successful nests), their tarsus length was measured to the nearest 0.1 mm with a Vernier caliper and they were weighed to the nearest 1 g. Duckling body condition was estimated as a proxy of offspring quality by extracting the standardized residuals of the linear regression of log-transformed body weight against log-transformed tarsus length (Öst et al., 2020).

Assessment of Predation Risk

Nest-Site Micro-Habitat

Females breed on the ground under variable cover and mainly rely on crypsis to avoid being detected by predators. Concealment mainly offers protection from avian predators, and to a lesser extent from mammalian predators. To quantify nest concealment, we measured the amount of nest cover (including all layers of vegetation from ground level to canopy, including rocks) above each nest based on hemispherical photographs. To do so, we used a 42-mm fisheye lens mounted on an Olympus C-740 digital camera placed in each nest cup and aimed vertically (Öst and Steele, 2010; Jaatinen et al., 2014). Each picture was converted to black and white and the proportion of black pixels (corresponding to vegetation or other obstacles, e.g., rocks) was measured using Image Tool (version 3.00; University of Texas Health Science Center, San Antonio).

Island-Specific Female and Nest Predation Risk

Breeding females and their eggs are subject to predation from native and non-native species. The main predators of incubating females are the native white-tailed eagle (*Haliaeetus albicilla*), the population of which has increased rapidly since the 1990s (Stjernberg et al., 2005; Öst et al., 2018), the eagle owl (*Bubo bubo*) as well as two introduced mammals: the American mink (*Neovison vison*) and the raccoon dog (*Nyctereutes procyonoides*) (Jaatinen and Öst, 2013; Öst et al., 2018). In addition, eggs are depredated either by primary predators of female eiders, or by nest predators such as hooded crows (*Corvus cornix*) or gulls (*Larus* spp.) (Jaatinen et al., 2014). The latter consume eggs either when the female is absent from the nest or act as secondary predators after the female has been attacked on the nest.

In order to distinguish between these different types of predation, we determined for each island an index of adult female and nest predation risk, respectively. These two indices are based on confirmed cases of depredated females or nests rather than unsuccessful predation attempts, as the latter are impossible to document in the field without camera surveillance. However, one should expect a close correlation between the incidence of successful and unsuccessful predation attempts. Female predation risk was calculated as the island-specific proportion of killed nesting females (number of killed females divided by the number of breeding attempts). Island-specific nest predation risk was calculated based on hatching success information (see above). The number of successful (i.e., hatched) nests at the final nest fate census was divided by the number of nests on the island for whose fate (success or failure) was known (326 known nest fates out of 355 monitored nests; 91.8% of known nest fates; mean number of nests per island ± SD = 31 ± 17 nests). The main reason for nest failure was depredation (136 out of the 155 failed nests; 87.7%) and the low number of non-depredated but failed nests could be explained by females incubating infertile or unviable eggs. Thus, island-specific nest predation was calculated as: 1-island-specific proportion of successful nests. Both island-specific female and nest predation risk varied considerably between islands (female predation risk: mean ± SD = 0.03 ± 0.04, min = 0.00, max = 0.10; nest predation risk: mean ± SD = 0.42 ± 0.20, min = 0.00, max = 0.86, $N = 17$ islands).

Statistical Analyses

All statistical analyses were conducted in R 3.6.1. (R Core Team, 2018). We used linear mixed models (LMMs) (R function "lme," nlme package; Pinheiro et al., 2019) and generalized linear mixed models (GLMMs) (R function "glmer," lme4 package; Bates et al., 2015) to investigate variation in baseline prolactin and corticosterone levels, reproductive success and duckling body condition, in relation to the aforementioned individual and environmental explanatory variables. We checked that all models met the assumptions of normality and homoscedasticity of residuals. Consequently, baseline corticosterone levels were log-transformed as $\ln(x + 1)$ to meet model assumptions. Model selection was performed using a stepwise backward elimination method in which non-significant terms ($P > 0.05$) were eliminated at each step. We preferred a stepwise model selection approach due to its ease of interpretation and presentation, but acknowledge its potential limitations (Smith, 2018). To verify the robustness of our model selection procedure, we therefore also performed conditional model averaging across best-ranked models (ΔAICc < 2) for the dependent variables, using the function "dredge" in the R-package MuMIn (Barton, 2020). This robustness check confirmed that we had correctly identified the final models and significance of each included explanatory variable for nearly all analyses in this paper. The only exception was the analysis of female body condition at hatching; here, the association with adult predation risk was only a trend when performing model averaging (see Results below). For each model, we relied on the variance inflation factor (VIF) to look for multicollinearity among candidate variables. We did not detect any multicollinearity among the candidate variables of selected models (all VIF < 2.5; Allison, 2012).

Physiological Responses

To study the variation in female physiology in relation to proxies of individual quality, maternal investment and predation risk, we constructed two sets of LMMs, where baseline prolactin and corticosterone levels were included as the dependent variable, respectively. The explanatory individual state variables were body condition at trapping, breeding experience, incubation stage, radius-ulna length, and incubation-stage corrected clutch weight. Baseline corticosterone was also included in the models investigating baseline prolactin variation to assess the potential effect of corticosterone on prolactin levels (Angelier and Chastel, 2009; Angelier et al., 2009; Vleck and Vleck, 2011). Furthermore, the following predation risk related explanatory variables were included in the models: nest cover, nest predation risk and female predation risk. One extremely high baseline prolactin value (value = 343.82 ng/mL, mean ± SD = 149.56 ± 43.81 ng/mL, min = 57.56 ng/mL, max = 343.82 ng/mL) was removed from statistical analyses. To account for the potential non-independence of physiological responses of individuals breeding on the same island, island identity was added to all models as a random effect.

Hatching Success and Duckling Quality

As reproductive success followed a binomial distribution (0: failure, 1: success), we built GLMMs with a binomial error distribution, with reproductive success as the dependent variable and baseline prolactin and corticosterone levels, individual characteristics (body condition at hatching, breeding experience, radius-ulna length, and clutch weight) and environmental factors (nest cover, island-specific nest and female predation risk) as explanatory variables. As the probability of a nest being depredated is likely to decrease as it gets closer to hatching (due to a shorter time left for the predator to detect the nest), we also included incubation stage (i.e., the number of days since egg laying) as an explanatory variable. Island identity was included as a random effect to account for the potential pseudoreplication arising from repeated observations from each island.

We used LMMs to study the association between female baseline prolactin and corticosterone levels (explanatory variables) and duckling body condition (dependent variable), a proxy of offspring quality. As ducklings belonging to the same clutch are not independent from each other, and as we expect non-independence between the body conditions of ducklings hatching on the same islands, clutch and island identity were added as random effects in these models.

Body Condition at Hatching

Covariation between individual quality and reproductive success may influence the observed relationship between maternal hormone profiles and parental investment, e.g., if early breeding failure disproportionately affects poor-quality individuals. To assess whether such selective disappearance may occur, we analyzed variation in female body condition, a proxy of female quality, in relation to female attributes and predation risk.

In these LMMs, female body condition at hatching was the dependent variable and breeding experience, nest predation risk and female predation risk were explanatory variables. Island identity was included as a random effect to account for the non-independence of body conditions of females nesting on the same island.

RESULTS

Baseline Prolactin in Relation to Individual and Environmental Characteristics

The final model explaining variation in baseline prolactin levels only included nest predation risk (**Table 1**). Thus, baseline prolactin levels showed a significant positive association with increasing nest predation risk (nest predation risk effect, parameter estimate ± standard error: E ± SE = 45.41 ± 21.24; **Table 1** and **Figure 1A**), but showed no significant association with female predation risk. Likewise, the final model explaining variation in baseline prolactin did not show a significant association between baseline prolactin levels and breeding experience, structural size, condition at trapping, clutch weight, or nest cover.

Baseline Corticosterone in Relation to Individual and Environmental Characteristics

The final model explaining variation in baseline corticosterone levels included breeding experience, clutch weight and nest predation risk (**Table 1**). Baseline corticosterone levels were significantly higher in experienced birds compared to first-time breeders (baseline corticosterone concentration: inexperienced females: mean ± SE = 9.87 ± 0.71 ng/mL; experienced females: mean ± SE = 17.06 ± 1.25; **Table 1** and **Figure 2**). Baseline corticosterone also showed a significantly positive relationship with clutch weight (E ± SE = 0.0013 ± 0.0005; **Table 1** and **Figure 2**). In contrast, female structural size, body condition or nest cover were not related to baseline corticosterone concentrations. With respect to our predation indices, increasing island-specific nest predation risk had a significant negative association with corticosterone levels (E ± SE = −0.97 ± 0.39; **Table 1** and **Figure 1B**), whereas female predation risk did not explain variation in baseline corticosterone levels.

TABLE 1 | Final LMMs and GLMM explaining baseline prolactin variation, baseline corticosterone variation, hatching success or body condition of incubating female eiders in relation to individual and environmental explanatory variables after stepwise backward elimination.

Dependent variable	Explanatory variable	χ^2	*P*-value
Baseline prolactin concentration	Nest predation risk	4.570	0.033
Baseline corticosterone concentration	Breeding experience	14.359	< 0.001
	Clutch weight	7.094	0.008
	Nest predation risk	6.153	0.013
Hatching success	Baseline prolactin	5.333	0.021
	Radius-ulna length	3.101	0.078
	Female predation risk	4.278	0.039
	Nest predation risk	12.984	< 0.001
Female body condition at hatching	Breeding experience	8.738	0.003
	Female predation risk	4.506	0.034

Island identity was included as a random effect.

Hatching Success in Relation to Physiology, Individual and Environmental Characteristics

The final model predicting hatching success included baseline prolactin, radius-ulna length, and female and nest predation risk, as explanatory variables (**Table 1**). Hatching success showed a significant negative relationship with increasing baseline prolactin levels (E ± SE = −0.012 ± 0.005; **Table 1** and **Figure 3A**), while it did not significantly vary with baseline corticosterone levels (**Figure 3B**). Hatching success tended to increase with female structural size, although this effect was not significant (**Table 1**). Other individual explanatory variables (incubation stage, body condition at hatching and breeding experience) were not selected in the final model. We also did not find any significant association between nest cover and hatching success. However, we also found that hatching success was significantly negatively related to nest predation risk (E ± SE = −8.85 ± 2.46; **Table 1** and **Figure 3C**) and significantly positively associated with female predation risk (E ± SE = 14.18 ± 6.86; **Table 1** and **Figure 3D**).

Female Body Condition in Relation to Individual Attributes and Predation

Female body condition at hatching was related to individual and environmental factors (**Table 1**). Body condition at hatching was significantly higher in more experienced birds (body condition at hatching: inexperienced females: mean ± SE = 0.16 ± 0.08; experienced females: mean ± SE = 0.62 ± 0.08; **Table 1** and **Figure 4**). Body condition at hatching also showed a significant positive correlation with female predation risk (E ± SE = 4.03 ± 1.90; **Table 1** and **Figure 4**), although this association was only marginally significant (E ± SE = 0.15 ± 0.08; $Z = 1.756$, $P = 0.079$) when performing conditional model averaging. In addition, female body condition at hatching was not significantly related to nest predation risk.

Offspring Body Condition and Maternal Hormone Profiles

Duckling body condition was significantly positively associated with maternal baseline prolactin concentrations (E ± SE = 0.006 ± 0.003; $\chi^2 = 4.481$, $P = 0.034$; **Figure 5A**), but showed no association with maternal baseline corticosterone levels ($\chi^2 = 4.481$, $P = 0.907$; **Figure 5B**).

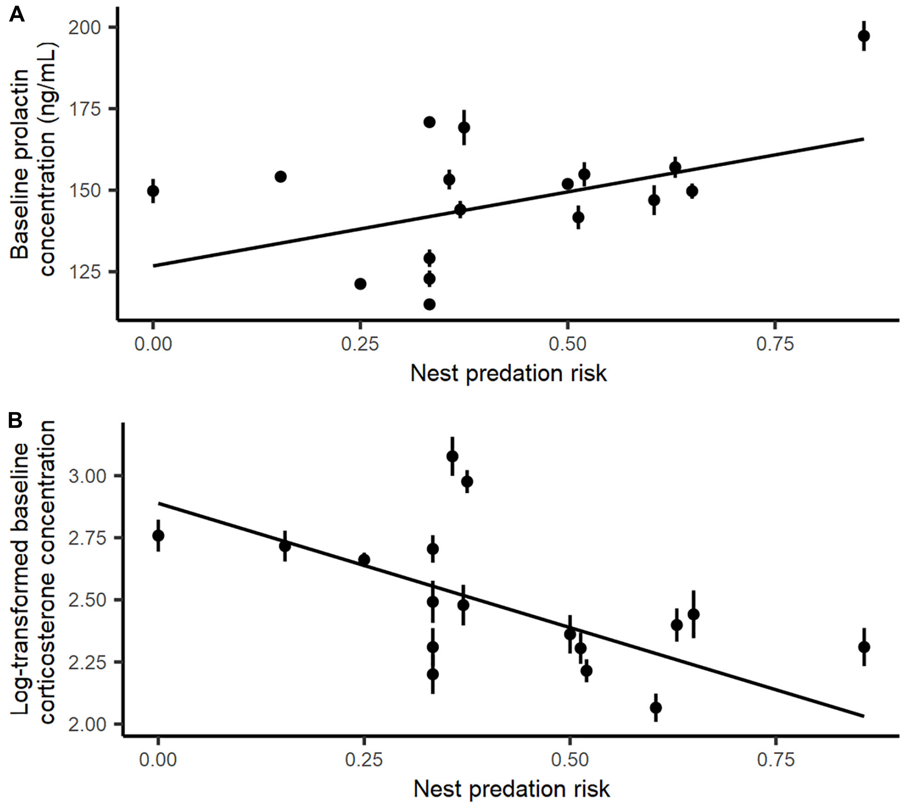

FIGURE 1 | Relationship between baseline **(A)** prolactin and **(B)** log-transformed corticosterone levels of incubating female eiders and island-specific nest predation risk. Black dots correspond to island-specific mean hormone levels and bars account for the standard error.

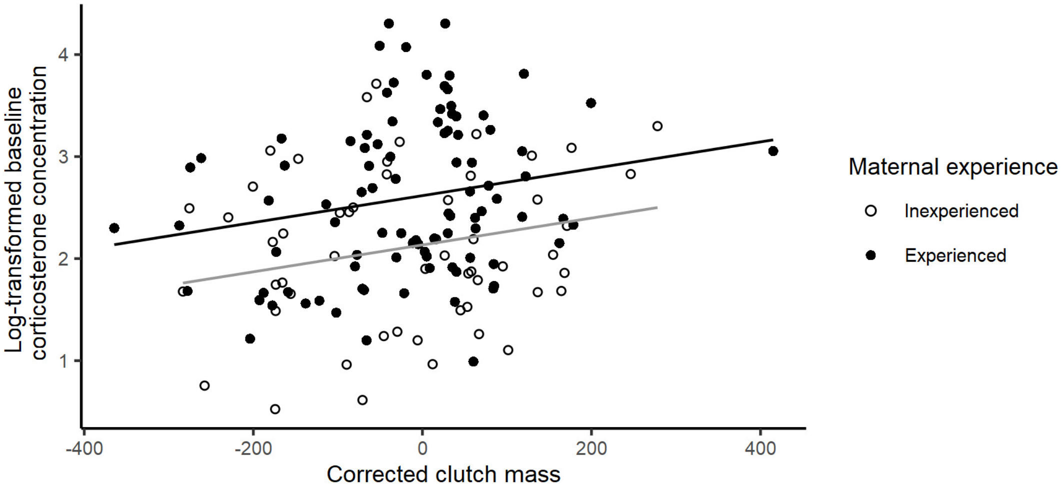

FIGURE 2 | Relationship between baseline log-transformed baseline corticosterone levels and incubation-stage corrected clutch weight of incubating female eiders for experienced (in black) and inexperienced (in gray) breeders.

DISCUSSION

In this study, we investigated the role of baseline prolactin and corticosterone levels, two hormonal correlates of parental investment (e.g., Criscuolo et al., 2002, 2005; Ouyang et al., 2013, in modulating incubation commitment of female eiders in response to variable predation risk on themselves and their offspring. Baseline prolactin levels were unrelated to direct proxies of female quality (body condition and breeding experience) or reproductive investment (clutch weight).

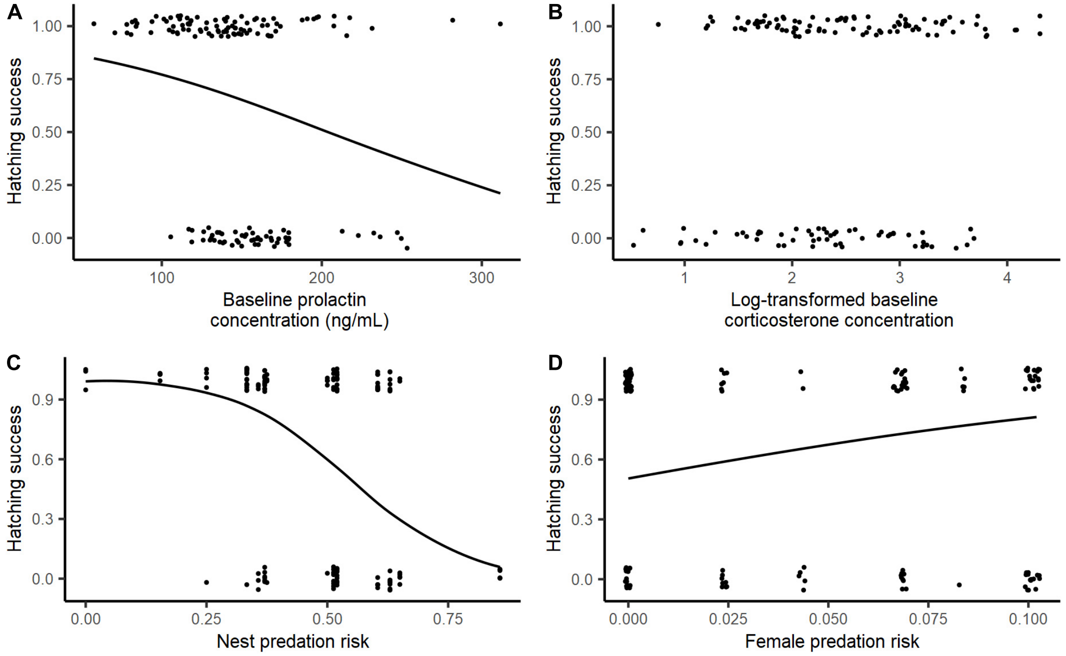

FIGURE 3 | Relationship between hatching success of female eiders and **(A)** baseline prolactin levels, **(B)** baseline log-transformed corticosterone levels, **(C)** island-specific nest predation risk, and **(D)** island-specific female predation risk.

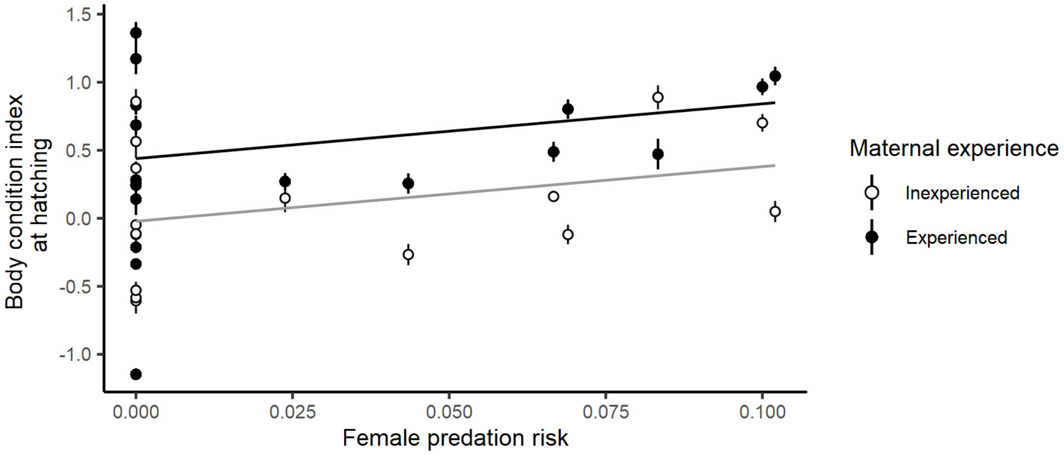

FIGURE 4 | Relationship between the body condition index at hatching of female eiders and island-specific female predation risk for experienced (in black) and inexperienced (in gray) breeders. Dots correspond to island-specific mean body condition indices at hatching for experienced (in black) and inexperienced (in white) breeders, respectively, and bars account for the standard error.

However, elevated maternal prolactin levels were associated with better duckling body condition (**Figure 5A**), suggesting a positive association between female baseline prolactin levels and female parental commitment. Surprisingly, and contrary to our expectations, birds breeding on riskier islands (characterized by higher nest predation risk) displayed higher baseline prolactin levels. This unexpected finding may reflect an absence of low-quality individuals, characterized here by low prolactin levels, from risky nesting sites. In favor of this hypothesis, incubating females exposed to a higher threat of predation on themselves were, on average, in better body condition (i.e., presumably of higher quality) than those nesting in less dangerous sites (**Figure 4**), even though we did not detect any direct association between female body condition and baseline prolactin levels.

In addition, we found that baseline corticosterone levels increased along with clutch weight, our proxy of energetic investment in reproduction, and was higher in experienced females than in inexperienced breeders. These two findings are in favor of the corticosterone-adaptation hypothesis and support the idea that elevated baseline corticosterone levels may

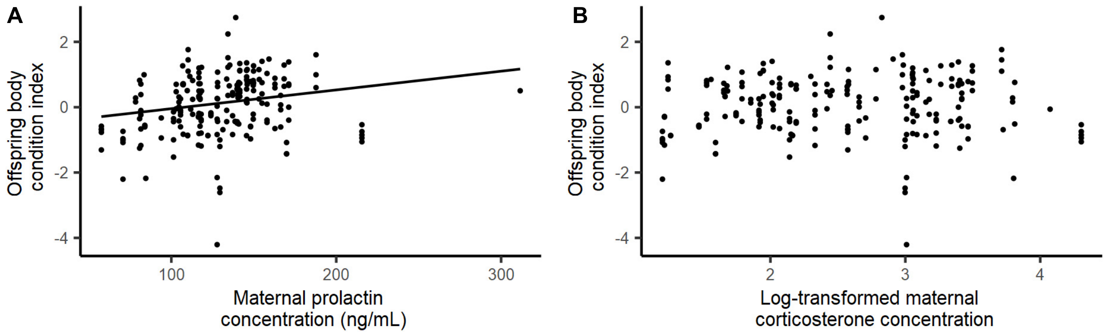

FIGURE 5 | Relationship between the duckling body condition index and maternal baseline concentrations of **(A)** prolactin and **(B)** corticosterone (log-transformed) in eiders.

promote parental investment (Bonier et al., 2009). Accordingly, we found that birds breeding on islands with a high risk of nest predation displayed lower circulating corticosterone levels. Consequently, high nest predation risk and low prospects of successful hatching may lead females nesting in these risky areas to reduce their incubation commitment in the current clutch, through a lower allocation of resources to reproduction, mediated by lower baseline corticosterone levels.

Prolactin, Individual Quality, and Predation Risk

Contrary to our expectations, we found no influence of body condition at trapping, a proxy of individual quality, on baseline prolactin levels. This result suggests that, in incubating eiders, baseline prolactin levels are not directly affected by individual body condition. Although this result is not in line with the results from other studies (Criscuolo et al., 2002, 2003, 2006; Angelier and Chastel, 2009; Schmid et al., 2011), it could be explained by the non-linear link between prolactin and body condition in capital breeders (Drent and Daan, 1980). In such species, prolactin levels only decrease when individuals reach a very low threshold of body mass (Cherel et al., 1988). In our study, all individuals were above this threshold, which is around 1.10 kg for eiders (Korschgen, 1977). Accordingly, this lack of relationship between baseline prolactin levels and body condition has been highlighted in other capital-breeding birds with large amount of body reserves (Angelier et al., 2006; Verreault et al., 2008; Angelier and Chastel, 2009).

Likewise, and contrary to previous studies (Angelier et al., 2006, 2007b; Christensen and Vleck, 2008; Angelier and Chastel, 2009; Riechert et al., 2012; Smiley and Adkins-Regan, 2016), we did not find any significant increase in baseline prolactin levels with breeding experience, a proxy of individual quality in female eiders (Jaatinen and Öst, 2011). This discrepancy may result from the time of sampling: we caught all eiders toward the end of the incubation period, when a substantial fraction of the inexperienced breeders with low prolactin levels may already have failed. Overall, we did not find any direct evidence of a significant positive association between baseline prolactin levels and proxies of female quality (body condition and experience). Higher prolactin levels were also not related to clutch weight (a proxy of energetic investment in reproduction), but they were positively associated with duckling body condition at hatching. This finding is noteworthy because the survival and recruitment of female eiders is related to their relative body condition as ducklings (Christensen, 1999), and thus the production of heavy offspring may have positive effects on maternal fitness. Altogether, these results suggest that maternal baseline prolactin levels may be positively associated with some but not all aspects of parental quality.

Baseline prolactin levels were positively associated with nest predation risk, but unrelated to adult predation risk and nest cover. This result is not in line with most studies evidencing lower prolactin levels in individuals subject to nest predation risk (Angelier et al., 2016). However, we showed that baseline prolactin levels were positively associated with duckling body condition, an indirect proxy of female quality, and this finding could indicate that only high-quality females were found on islands where nest predation risk was high. In line with this idea, we found that female body condition, a proxy of individual quality (Lehikoinen et al., 2010), increased along with adult predation risk. Two mutually non-exclusive explanations are possible. First, only high-quality females with high prolactin levels may opt to breed at risky sites in this species frequently exhibiting intermittent breeding (the reproductive suppression model; Wasser and Barash, 1983). Supporting this idea, a long-term analysis of female body condition at Tvärminne shows that body condition has been increasing over the past 15 years, concomitantly with a steeply increasing trend in white-tailed eagle numbers and in nest predation risk (Öst et al., 2018).

Second, higher baseline prolactin levels on risky islands could be explained by the early breeding failure of low-quality females on these sites. In our study, females were trapped toward the end of the incubation period and high prolactin levels and incubation commitment may be required to reach this stage of breeding on risky sites, whereas less committed females may reach this stage on safer islands. Indeed, prolactin is known to play a role in nest attendance and high prolactin levels are associated with lower tendency to abandon the nest (Spée et al., 2010; Angelier et al., 2016; Smiley and Adkins-Regan, 2018) and a shorter latency to return to the nest after a disturbance (Angelier et al., 2009; Hope et al., 2020).

In addition, and contrary to both our *a priori* expectations and the previous literature (Angelier et al., 2007b, 2009, 2016; Ouyang et al., 2011; Riechert et al., 2014a), females displaying higher levels of baseline prolactin were less rather than more successful than those with lower levels. However, this result is consistent with the observed positive relationship between female prolactin levels and nest predation risk, likely arising from the selective disappearance of low-quality females with low baseline prolactin levels from the breeding pool on high-risk islands. Consequently, a putative link between high prolactin levels and high nest predation risk may lead to an indirect negative relationship between prolactin levels and reproductive success. In further support of this conclusion, there was a positive correlation between reproductive success and predation risk on adult females. This finding is consistent with the view that only high-quality females with high baseline prolactin levels opt to breed, or, alternatively, have nests that survive until the late incubation stage under high threat of predation. A positive correlation between female body condition at hatching and female predation risk (**Figure 3D**) is also consistent with this explanation. Summing up this evidence, we conclude that high-quality breeders characterized by high prolactin levels are likely to be overrepresented on risky sites at the end of the incubation period.

Corticosterone, Reproductive Investment, and Predation Risk

As for prolactin, we did not find any significant relationship between baseline corticosterone levels and female body condition. Such a lack of association between baseline corticosterone and body condition or body mass has also been shown in other eider populations (Bourgeon and Raclot, 2006; Criscuolo et al., 2006; D'Alba et al., 2011), and, more generally, in other capital breeders (Angelier et al., 2006; Groscolas et al., 2008). As for prolactin, this lack of a relationship could result from the fact that incubating female eiders have not reached a low threshold in body mass (Cherel et al., 1988) at the time of capture. However, we found that experienced breeders displayed higher baseline corticosterone levels than inexperienced breeders, a result in favor of the corticosterone-adaptation hypothesis (Bonier et al., 2009). Accordingly, some studies have found that circulating corticosterone levels increase with age and breeding experience in capital breeders with extended fasting periods (Angelier et al., 2006). In such species, elevated baseline corticosterone levels may be associated with an increased investment in reproduction, especially when individuals have not reached a lower threshold in body condition (Cherel et al., 1988), as is the case in our study.

In further support of the corticosterone-adaptation hypothesis, we found that baseline corticosterone levels were positively associated with clutch weight. Despite the possible occurrence of partial clutch predation (Erikstad et al., 1993; Öst et al., 2008) and conspecific nest parasitism, the frequency of which is low in our population (ca 6% of eggs; Waldeck et al., 2004), clutch weight or size can be used as a reasonable proxy for female initial energetic investment in reproduction in eiders (Hanssen et al., 2003; Jaatinen et al., 2013). Overall, corticosterone may promote energy mobilization (Romero, 2002; Breuner, 2011) and incubation effort (Breuner, 2011; DuRant et al., 2013), and moderately elevated baseline corticosterone levels could be a proxy for increased incubation commitment in our study system. Accordingly, other studies have shown that increased baseline corticosterone levels do not trigger nest abandonment in eiders (Criscuolo et al., 2005), nor do they induce nest failure unless corticosterone levels reach very high values (D'Alba et al., 2011), a result consistent with the hormetic dose response framework (Costantini et al., 2010; Costantini, 2014). The positive association between baseline corticosterone levels and both clutch weight and maternal experience may thus indicate that incubating females increase their investment in a current reproductive event when its value is elevated (i.e., heavy clutch), and when the prospect of future reproduction diminish (the restraint hypothesis, Curio, 1983; Heidinger et al., 2006).

In addition, we showed that baseline corticosterone levels were negatively related to nest predation risk, but unrelated to adult predation risk and nest cover. These results are consistent with the corticosterone-adaptation hypothesis, and lower baseline corticosterone levels in risky nesting sites may therefore suggest that female eiders reduce their investment in incubation in the face of increased nest predation risk. This interpretation is supported not only by the fact that baseline corticosterone levels in this study were unrelated to hatching success and offspring quality, but also by the positive effect of breeding experience on both parental investment (Öst and Steele, 2010) and corticosterone levels in eiders (this study). Interestingly, similar effects of increasing nest predation risk on parental investment have been demonstrated in other species (Doligez and Clobert, 2003; Eggers et al., 2006; Zanette et al., 2011).

While baseline corticosterone levels can be seen as a proxy of parental investment, they can also mirror the response to prolonged exposure to chronic environmental perturbations, such as predation risk (Scheuerlein et al., 2001; Clinchy et al., 2004; Travers et al., 2010; Angelier and Wingfield, 2013). In that context, high baseline corticosterone levels could also be related to high stress sensitivity. Under that scenario, lower baseline corticosterone levels on risky sites could result not only from individuals reducing their parental investment under high predation threat, but also from the selective disappearance of highly sensitive individuals from these risky sites. Highly sensitive female eiders could either have already abandoned their breeding attempts prior to our trapping efforts on these sites, or they could have failed their breeding attempt at the time of trapping, toward the end of incubation. Females exhibiting higher baseline corticosterone levels would thus be expected to skip breeding more often and/or to be prone to abandon their nest when nesting on sites with a high risk of nest predation (Vitousek et al., 2014). Supporting this possibility, it has been found in other species that incubating individuals displaying lower baseline corticosterone levels returned more quickly to the nest following a disturbance (Angelier et al., 2009; Edwards et al., 2013), or tended to abandon their nest less often than more sensitive individuals (Spée et al., 2010; Ouyang et al., 2012).

PERSPECTIVES

In this study, we investigated the role of baseline prolactin and corticosterone levels in modulating parental investment decisions under variable predation threat in incubating female eiders. We showed that birds breeding on islands subject to higher nest predation threat displayed higher baseline prolactin levels, and that this could reflect an absence of low-quality individuals with low prolactin levels at risky nesting sites. We also found that birds breeding on riskier islands displayed lower baseline corticosterone levels, and this may suggest that females reduce their incubation commitment on islands where predation risk on offspring is high (the corticosterone-adaptation hypothesis). Although our findings allow a better understanding of how predation risk may interact with hormonal regulation of parental investment, the correlative nature of the study does not allow us to draw causal relationships between hormone levels, parental investment and predation risk, highlighting the need for experimental studies to further clarify these relationships. In addition, our results do not allow us to distinguish effects of predation threat at the population level from effects of predation risk at the individual level. Longitudinal individual-based studies are now needed to disentangle within- and between-individual variation in hormonal levels in relation to individual quality and variable predation risk. This would allow us to distinguish natural selection processes from individual plasticity in responding to a changing environment. Such studies will eventually pave the way for a better understanding of the ability of prey to physiologically and behaviorally cope with environmental perturbations and how this impacts their reproductive life-history strategies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Handling of birds was approved by the National Animal Experiment Board (permit number ESAVI/1697/04.10.03/2012) and complied with the specific regulations of Tvärminne Zoological Station.

AUTHOR CONTRIBUTIONS

MÖ and KJ conceived the study and conducted fieldwork. CP and FA conducted laboratory analyses. BM analyzed the data. BM, MÖ, FA, and KJ interpreted the results. All authors contributed to writing the article and approved the submitted version.

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Multimodal Aposematic Defenses Through the Predation Sequence

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Aposematic organisms warn predators of their unprofitability using a combination of defenses, including visual warning signals, startling sounds, noxious odors, or aversive tastes. Using multiple lines of defense can help prey avoid predators by stimulating multiple senses and/or by acting at different stages of predation. We tested the efficacy of three lines of defense (color, smell, taste) during the predation sequence of aposematic wood tiger moths (*Arctia plantaginis*) using blue tit (*Cyanistes caeruleus*) predators. Moths with two hindwing phenotypes (genotypes: WW/Wy = white, yy = yellow) were manipulated to have defense fluid with aversive smell (methoxypyrazines), body tissues with aversive taste (pyrrolizidine alkaloids) or both. In early predation stages, moth color and smell had additive effects on bird approach latency and dropping the prey, with the strongest effect for moths of the white morph with defense fluids. Pyrrolizidine alkaloid sequestration was detrimental in early attack stages, suggesting a trade-off between pyrrolizidine alkaloid sequestration and investment in other defenses. In addition, pyrrolizidine alkaloid taste alone did not deter bird predators. Birds could only effectively discriminate toxic moths from non-toxic moths when neck fluids containing methoxypyrazines were present, at which point they abandoned attack at the consumption stage. As a result, moths of the white morph with an aversive methoxypyrazine smell and moths in the treatment with both chemical defenses had the greatest chance of survival. We suggest that methoxypyrazines act as context setting signals for warning colors and as attention alerting or "go-slow" signals for distasteful toxins, thereby mediating the relationship between warning signal and toxicity. Furthermore, we found that moths that were heterozygous for hindwing coloration had more effective defense fluids compared to other genotypes in terms of delaying approach and reducing the latency to drop the moth, suggesting a genetic link between coloration and defense that could help to explain the color polymorphism. Conclusively, these results indicate that color, smell, and taste constitute a multimodal warning signal that impedes predator attack and improves prey survival. This work highlights the importance of understanding the separate roles of color, smell and taste through the predation sequence and also within-species variation in chemical defenses.

Keywords: aposematism, *Arctia plantaginis*, *Cyanistes caeruleus*, defense mechanisms, multimodal signaling, predator-prey interactions, chemical defense, warning signals

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INTRODUCTION

Predation is one of the main threats to an organism's survival. As a result, there are many different traits that have evolved to help organisms avoid predation and most organisms use more than one line of defense. In some cases, these multiple defenses can act simultaneously (Ruxton et al., 2018). For example, prey may evolve behaviors such as background choice (Sargent, 1966; Kang et al., 2012; Kjernsmo and Merilaita, 2012; Green et al., 2019) or body orientation (Kang et al., 2012; Rowland et al., 2020) and also to have a color pattern that is camouflaged against their surroundings, all of which help avoid detection by predators (Stevens and Ruxton, 2018). However, many animals have defense mechanisms that act sequentially by impeding different stages of attack (Endler, 1991; Caro, 2005; Ruxton et al., 2018). Primary defenses act to prevent physical contact between predator and prey (i.e., at the encounter, detection, identification and approach stages of an attack), whereas secondary defenses deter attack after or just before the predator has made physical contact with the prey (i.e., at the subjugation and consumption stages of attack) (Ruxton et al., 2018). Whether selection favors investment in primary and/or secondary defenses depends on the relative properties of those defenses such as their energetic cost and efficacy against predators (Broom et al., 2010).

Aposematism is a defense strategy that relies on communication to signal unprofitability to predators (Poulton, 1887, Poulton, 1890; Cott, 1940; Stevens, 2013). Aposematic prey (the signaler) use a warning signal to inform predators (the receiver) of unpleasant or harmful defenses to reduce the likelihood or extent of attack by the predator and to promote, enhance, or maintain learned avoidance of that prey type in future encounters (Poulton, 1887, Poulton, 1890; Cott, 1940). Such warning signals may act as a primary defense if the predator has an innate color bias (Smith, 1975; Roper, 1990; Schuler and Roper, 1992; Mastrota and Mench, 1995; Lindström et al., 1999) or has learned to avoid the warning signal through prior experience (Gittleman and Harvey, 1980; Roper and Wistow, 1986; Alatalo and Mappes, 1996; Ham et al., 2006; Green et al., 2018). Conversely, warning signals may act as a secondary defense if increased predator wariness improves the chance that prey will escape or reduces harm to prey after subjugation (Halpin et al., 2008; Ruxton et al., 2018) or if the warning signal is "switchable" and only becomes apparent after the predator has engaged with the prey (Blest, 1964; Sivinski, 1981; Grober, 1988; Broom et al., 2010; Umbers and Mappes, 2015; Kang et al., 2016; Umbers et al., 2017; Song and Jablonski, 2020). Often, visual or auditory warning signals are combined with chemical defenses, which deter predators through some combination of taste (Marples et al., 1994; Skelhorn and Rowe, 2006, 2010), smell (Rowe and Guilford, 1996, 1999; Lindström et al., 2001; Jetz et al., 2001; Kelly and Marples, 2004; Rojas et al., 2019), or toxicity (Cortesi and Cheney, 2010; Arenas et al., 2015). These chemical defenses are typically considered secondary defenses, which act to prevent consumption after subjugation has occurred or to dissuade predators from attacking such prey in the future (Ruxton et al., 2018). However, chemical defenses may also be detected before subjugation and influence the predator's likelihood or latency to approach or attack the prey (Guilford et al., 1987; Rowe and Halpin, 2013; Rojas et al., 2017, 2019). Therefore, the dichotomy between primary and secondary defenses is not perfect, and it is possible for a single defense mechanism to protect prey across multiple stages of a predator's attack.

Aposematism is formed by multimodal signaling (Rowe and Halpin, 2013). That is, it involves the use of signal components that are received through two or more sensory modalities by a single receiver (Stevens, 2013). Warning signals are usually conspicuous *visual* or *auditory* signals that are combined with some form of chemical defense (either sequentially or simultaneously), which predators perceive through certain *smell* or *taste* receptors. However, smell and taste reception are thought to have evolved largely to help animals avoid the inadvertent consumption of harmful, toxic food (Shi et al., 2003; Fischer et al., 2005; Chandrashekar et al., 2006; Reed and Knaapila, 2010). In this way, smell and taste can also be considered signals that warn predators of toxicity (Eisner and Grant, 1981; Weldon, 2013). However, as with Batesian mimics, which imitate visual aposematic signals, the information content of such chemical signals may not always be truthful, as not all chemicals that are perceived to have an unpleasant smell or taste are toxic (Ruxton and Kennedy, 2006; Nissim et al., 2017; Winters et al., 2018; Lawrence et al., 2019). In addition, there is evidence that defensive smell and bitter taste alone are not necessarily sufficient to prevent successful attack from predators (Eisner and Grant, 1981; Guilford et al., 1987; Moore et al., 1990; Rowe and Guilford, 1996; Kelly and Marples, 2004; Siddall and Marples, 2011), therefore the function of defense chemicals as honest signals of toxicity within aposematic systems requires further study (Holen, 2013).

Many chemically defended species use complex chemical mixtures that contain different types of chemicals, and utilizing multiple defensive compounds can be an adaptive strategy in a number of different ways. Chemical diversity may help prey defend themselves against multiple enemies, whereby different compound types are used to target different predators. For example, in *A. plantaginis* neck fluids defend against bird predators (but not invertebrates) and abdominal fluids defend against invertebrates (but not birds) (Rojas et al., 2017). It may also be more difficult for predators to evolve immunity to a suite of toxins compared to just one (Zhao et al., 2003). In addition, multiple defense compounds may be used as a multimodal signal if a single predator uses both smell (of volatile compounds) and taste (of non-volatile, bitter compounds) to assess the toxicity of chemically defended prey (Marples et al., 1994). Smell and taste may also act at different stages of attack. Smell can be used to detect volatile odorants from a distance, potentially allowing predators to perceive chemical defenses before prey capture (Rowe and Halpin, 2013; Rojas et al., 2017, 2019). Whereas non-volatile compounds require predators to first capture prey before the chemical defense can be perceived via taste receptors. Despite this, the effect of smell and taste is rarely differentiated in studies of multimodal aposematic displays, but see Marples et al. (1994), and therefore it remains unclear how interactions between smell and taste fit within the theoretical framework of multimodal aposematic signals (Rowe and Halpin, 2013). Thus, measuring the individual and combined effects of smell, taste, and warning coloration is essential for understanding the evolution and maintenance of both chemical diversity and multimodal warning signals.

Here, we investigate a multi-modal aposematic defense (visual warning signal, smell, taste) in the polymorphic wood tiger moth *Arctia plantaginis* to blue tit (*Cyanistes caeruleus*) predators. In a recent study, blue tits used the potent smell of methoxypyrazines as context-setting signals for the aposematic colors of *A. plantaginis* (Rojas et al., 2019). Birds only delayed attack in response to the white model color when the methoxypyrazine smell was present. This finding differed from previous studies that found the yellow morph to be better protected when live moths were offered to blue tits in the lab (Nokelainen et al., 2012), when dead moths were placed in the field (Nokelainen et al., 2012) and when moth models (i.e., dummies) were placed in the field (Nokelainen et al., 2014). One explanation for the difference between the response of blue tits to yellow and white morphs under laboratory conditions could be the use of models (Rojas et al., 2019) rather than live prey (Nokelainen et al., 2012). To address this issue, we use live moths in this study. In addition, Rojas et al. (2019) found that chemically treated models differed to controls in terms of proportion of moth's body eaten and beak wiping behavior (a common disgust response), suggesting the presence of both an aversive smell and taste in this species and highlighting the need to disentangle these two modalities from the defense fluid (Rojas et al., 2019). *A. plantaginis* also have the ability to sequester pyrrolizidine alkaloids, which they distribute to all tissues including neck fluids (Anne Winters unpublished data), which might explain the aversive taste. Pyrrolizidine alkaloids are well documented to defend against invertebrate predators (Brown, 1984; Dussourd et al., 1988; Masters, 1990; Eisner and Eisner, 1991; Conner et al., 2000; Eisner et al., 2000). However, evidence for their defense against vertebrates is less robust (Ritland, 1991; Rowell-Rahier et al., 1995; Yosef et al., 1996; Cardoso, 1997). Therefore, while lepidopterans that sequester pyrrolizidine alkaloids widely exhibit conspicuous coloration (Nishida, 2002), further evidence is needed to support the role of pyrrolizidine alkaloids in aposematic defenses against vertebrates (Nishida, 2002). In the present study, live moths of each color morph that were manipulated to have only the smell (methoxypyrazines), only the bitter taste (pyrrolizine alkaloids) or with both present, were offered to birds to test whether color, smell and taste constitute a multimodal warning signal in *A. plantaginis*. We test whether combined modalities improve discrimination of toxic prey by predators and enhance aversion learning. We also investigate at which stage of attack (approach, attack, subjugation, consumption) each defense modality is effective at influencing predator behavior and whether multimodality improves prey survival.

MATERIALS AND METHODS

Study Species *A. plantaginis*

The aposematic wood tiger moth (*Arctia plantaginis*, formerly *Parasemia plantaginis*) is a member of the Erebidae family (Rönkä et al., 2016) and widely distributed across the Northern hemisphere (Hegna et al., 2015). There is geographical variation in warning coloration (Hegna et al., 2015). In Europe, male hindwings are either yellow or white and female hindwings vary continuously from yellow to red (Lindstedt et al., 2011; Nokelainen et al., 2012; Hegna et al., 2015). Hindwings are often exposed at rest in this species, particularly if moths are alerted, preparing to fly, or if the weather is cool. The discrete variation in male hindwing coloration follows a one-locus two allele model, where the yellow allele (y) is recessive to the white (W), resulting in three genotypes (WW, Wy, yy). Both homozygous white (WW) and heterozygous (Wy) genotypes have white hindwing coloration, while the homozygous recessive genotype (yy) has yellow (Suomalainen, 1938; Nokelainen et al., in prep a). Therea are also differences between genotypes in the white hue of the forewings, which is perceptible to birds (Nokelainen et al., in prep a). The color polymorphism is under selection by bird predators in the wild (Rönkä et al., 2020). In predation experiments, birds respond differently toward the hindwing morphs, avoiding either yellow (Nokelainen et al., 2012, 2014) or white (Rojas et al., 2019), but see Rönkä et al. (2018). Rojas et al. (2019) speculate that the variable response by predators could be due to differences in cues between the moths and their model stimuli, differences in light environment between experiments (Nokelainen in prep b), or the presence or absence of methoxypyrazine odor.

Arctia plantaginis is chemically defended, with two uniqe defense secretions that target different predators (Rojas et al., 2017). One secretion is released between the head and thorax when the thorax is grabbed or pinched and deters birds (neck fluid), and a second secretion is released from the abdomen when the moth is disturbed and deters ants (abdominal fluid) (Rojas et al., 2017). Two main methoxypyrazine compounds are released from the neck fluids: 2-sec-butyl-3-methoxypyrazine (SBMP) and 2-isobutyl-3-methoxypyrazine (IBMP) (Burdfield-Steel et al., 2018). These are produced *de novo* by the moth when raised on an artificial diet (Burdfield-Steel et al., 2018). These methoxypyrazines emit a potent odor that is aversive to blue tit predators, causing delayed attack, increasing disgust behaviors such as beak wiping, and reducing the amount or likelihood of consumption (Rojas et al., 2017, 2019). In addition, *A. plantaginis* is efficient at sequestering pyrrolizidine alkaloids from their diet (**Table 1**). These alkaloids are present in wild-caught moths, and distributed to all body parts of the moths including both neck and abdominal defense fluids of moths (Anne Winters, unpublished data). The efficacy of pyrrolizidine alkaloids sequestered by *A. plantaginis* in defense against predation has not yet been tested.

Manipulation of Color, Smell, and Taste of *A. plantaginis*

The wood tiger moth (*Arctia plantaginis*) is well suited to examine the role of color, smell and taste in the multimodal

TABLE 1 | Quantification of pyrrolizidine alkaloids (PA) seneciphylline and senecionine in the larvae, food, and feces of *A. plantaginis* raised on an artificial diet with 10% freeze-dried *Senecio vulgaris*.

PA	Sample	μg/mg	s.e	n
Seneciphylline	Larvae	2.42	0.48	6
	Food	0.24	0.02	2
	Feces	0.01	0	2
Senecionine	Larvae	0.14	0.03	6
	Food	0.05	0.04	2
	Feces	0	0	2

This may differ from the final concentration at adulthood.

aposematic display of a live insect because each of these three components can be independently manipulated (**Figure 1**). Importantly, the use of live prey accounts for the actual nutritional value of prey (Halpin et al., 2014) and natural delivery mechanism(s) of the chemical defense secretions (Eisner and Meinwald, 1966), both of which improve the ecological significance of results compared experiments using models as stimuli (Rowe and Halpin, 2013).

Color Manipulation

To control the color morph of male *A. plantaginis* used in this experiment, moth families were purpose bred from 3rd generation 2019 lab stock of known (color morph) genotype at the University of Jyväskylä. Moths were paired to produce offspring of WW, Wy, and yy genotypes and mate pairings were staggered so that adults would continuously emerge from November-March, providing a sufficient time period to conduct the behavioral experiment.

Taste Manipulation

To control the pyrrolizidine alkaloid "taste" of the moth, each clutch was then split between two artificial diet treatments: a control diet with no dietary source of pyrrolizidine alkaloids (4.6 agar, 8.58 g yeast, 32.1 g semolina, 8.3 g wheat germ, 150 ml boiling water, 1.76 g Vanderzant vitamin mix, 1800 μl nipagen and 180 μl acetic acid) and an artificial diet with 10% freeze-dried *Senecio vulgaris*, as a dietary source of pyrrolizidine alkaloids (4.6 g agar, 8.15 g yeast, 30.5 g semolina, 7.89 g wheat germ, 150 ml boiling water, 2.5 g freeze-dried *Senecio vulgaris*, 1.76 g Vanderzant vitamin mix, 1800 μl nipagen and 180 μl acetic acid). Larvae of each family/diet treatment were housed together in plastic containers until pupation and fed daily with fresh food spooned onto small squares of baking paper. To confirm the sequestration of pyrrolizidine alkaloids from the second diet, six larvae were selected and subject to chemical analysis along with 2 samples of their diet treatment, food and feces. Briefly, the samples were first freeze-dried and then weighed to the nearest 0.1 mg. Samples were then homogenized, extracted and processed through LC-MS/MS following the protocol outlined in Reinwaldt et al. (2017). Seneciphylline and Senecionine were identified as major compounds and quantified using a stock solution of standards (2 mg each of Monocrotaline, Monocrotaline N-oxide, Jacobine, Jacobine N-oxide, Intermedine, Intermedine N-oxide, Retrorsine, Seneciphylline, Seneciphylline N-oxide, Senecionine, Senecionine N-oxide, and Senkirkine, in 20 ml of 5% methanol solution). *A. plantaginis* efficiently sequestered pyrrolizidine alkaloids. Both major compounds identified were accumulated, rather than excreted by the larvae, resulting in a greater concentration of pyrrolizidine alkaloid in the moth compared to their dietary source. Seneciphylline was 10× as concentrated in the larvae compared to their food, while only trace amounts were excreted in the feces. Senecionine was 3× as concentrated in the larvae compared to their food, while only trace amounts were excreted in the feces (**Table 1**).

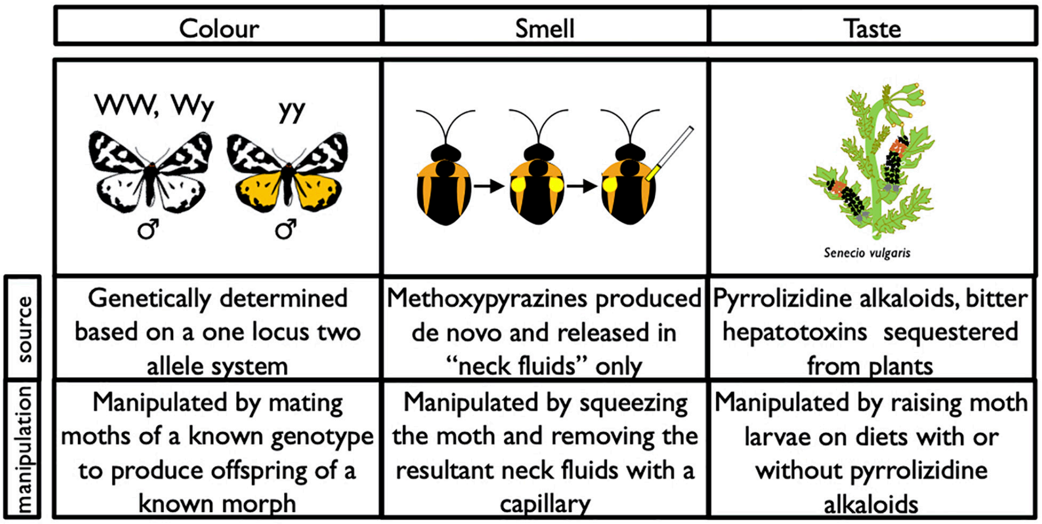
Smell Manipulation

After pupation, individuals were placed singly in vials with a sponge cap, which was sprayed daily with water to prevent desiccation until they eclosed. After the moth eclosed, it was stored in a refrigerator at ∼4°C until use in the experiment (12 days ± 0.5 SE). To manipulate the methoxypyrazine "smell" of the moth, neck fluids were removed from a subset of the emerging adults by squeezing the thorax between the fingers and collecting the resultant fluid using a microcapillary. Moths were squeezed the day before they were used in the experiment so that the majority of the methoxypyrazine smell could be released and dissipated and then again 15 min before the experiment on the day of the trial (see below for further details), to remove any remaining methoxypyrazines. The moth was removed from the refrigerator 30 min before each sampling and the sponge cap was sprayed with water to allow the moth to warm up and hydrate for 30 min. Moths that retained their neck fluids for the experiment underwent the same protocol except the neck fluids were not collected. Neck fluids were sampled in a separate room with closed doors so that the odor was not pervasive in the bird housing or experimental enclosures.

From these manipulations, 251 adult male moths were spread between 12 treatment groups with at least $n = 9$ moths per treatment (**Table 2**). Treatments with moths of the yellow morph have lower sample size for two reasons: (1) Moths of the yellow morph have poor fecundity compared to other genotypes (Nokelainen et al., 2012; Gordon et al., 2018); poor mating success and small clutch sizes resulted in fewer offspring with yellow hindwings. (2) The experiment was ended prematurely due to COVID-19, and the recently eclosed and yet to eclose yellow males that could have increased the sample size had to be discarded.

C. caeruleus Capture and Husbandry

In total, 84 wild blue tits (*C. caeruleus*) were trapped from a feeding station at Konnevesi Research Station in Central Finland between November 2019 and March 2020. Birds were weighed on the day of capture and then individually housed in plywood enclosures (65 cm × 50 cm × 80 cm) on a 11 h : 13 h (light : dark) cycle for at least one day (8 days ± 0.5 SE) before the experiment started so that they acclimatized to captive conditions. During this time, birds had *ad libitum* access to sunflower seeds, peanuts, a vitamin enriched food supplement and water. After the experiment, birds were ringed for identification purposes, aged and sexed according to established methods published in: "Svensson (1992) Identification Guide to European Passerines



	Colour	Smell	Taste
source	Genetically determined based on a one locus two allele system	Methoxypyrazines produced de novo and released in "neck fluids" only	Pyrrolizidine alkaloids, bitter hepatotoxins sequestered from plants
manipulation	Manipulated by mating moths of a known genotype to produce offspring of a known morph	Manipulated by squeezing the moth and removing the resultant neck fluids with a capillary	Manipulated by raising moth larvae on diets with or without pyrrolizidine alkaloids

FIGURE 1 | Illustration of the source for each component (color, smell, and taste) in the multimodal warning signal of *A. plantaginis* and brief description of the method used to manipulate that component in live moths for this experiment.

TABLE 2 | Number of birds (and moths) in each of treatment group including those of each hindwing phenotype: white and yellow, genotype: WW, Wy, or yy, those with (+) and without (–) methoxypyrazine smell (MP), and those with (+) and without (–) pyrrolizidine alkaloid taste (PA).

Hindwing	Genotype	Chemical Treatment			
Phenotype		– MP smell		+ MP smell	
		– PA taste	+ PA taste	– PA taste	+ PA taste
White	WW	12 (36)	7 (21)	11 (33)	8 (23)
White	Wy	6 (18)	6 (18)	5 (15)	7 (21)
Yellow	yy	7 (21)	3 (9)	6 (18)	6 (18)

ISBN: 9789163011184 Publisher: British Trust for Ornithology," and then released at their site of capture. Birds were captured and housed with permission of Central Finland Centre for Economic Development, Transport and Environment (VARELY/294/2015) and a license from the National Animal Experiment Board (ESAVI/9114/04.10.07/2014).

Behavioral Experiment

Birds were transported to a separate experimental room and placed inside masonite enclosures (50 cm × 50 cm × 70 cm), which were equipped with a perch and water bowl and lit with an Exo Terra Repti Glo 25 W 5.0 UVB compact light bulb (see Waldron et al., 2017 **Supplementary Material** for irradiance measurements). Spectral reflectance measurements of the masonite background along with the forewings and hindwings for each genotype are included in the **Supplementary Material** (**Supplementary Figure 1**). Birds were observed through a mesh-covered opening at the front of the cage and by a video camera (Sony DSC-HX1) at the top of the cage (**Figure 2**). During an acclimation/training period, birds were offered two sunflower seeds through a hatch behind a visual barrier, which was used to accurately measure when the moth was seen, approached, and attacked. The first sunflower seed was offered immediately after the bird was placed in the cage. After 1 h, if the bird ate the first sunflower seed, it was then offered a second sunflower seed and monitored every 15 min until the second seed was eaten. If after 1 h the bird had not eaten the first seed, it was monitored every 15 min and offered the second seed only after the first was eaten. To ensure the birds were sufficiently hungry, the behavioral experiment was initiated 1 h after the bird ate the second sunflower seed. To measure predator avoidance learning across trials, each bird was presented with 3 moths (one moth per day for three consecutive days) from one of the treatment groups (**Table 2**).

During the experiment, the observing room was kept dark and silent to reduce the effect of the researcher on bird behavior. Moths were held with forceps by the forewing and then placed into the experimental enclosure using the same hatch used for the sunflower seeds (**Figure 2**). We observed moths to hold their wings slightly open, partially exposing their polymorphic hindwings, which is a common natural resting position for the moth. After the bird saw the moth, it had 15 min to attack, if it did not attack, the experiment ended. After the bird attacked the moth, the experiment ended when the bird showed no further interest in any part of the moth for one full minute. During the assay, birds and moths were observed by two authors (JL

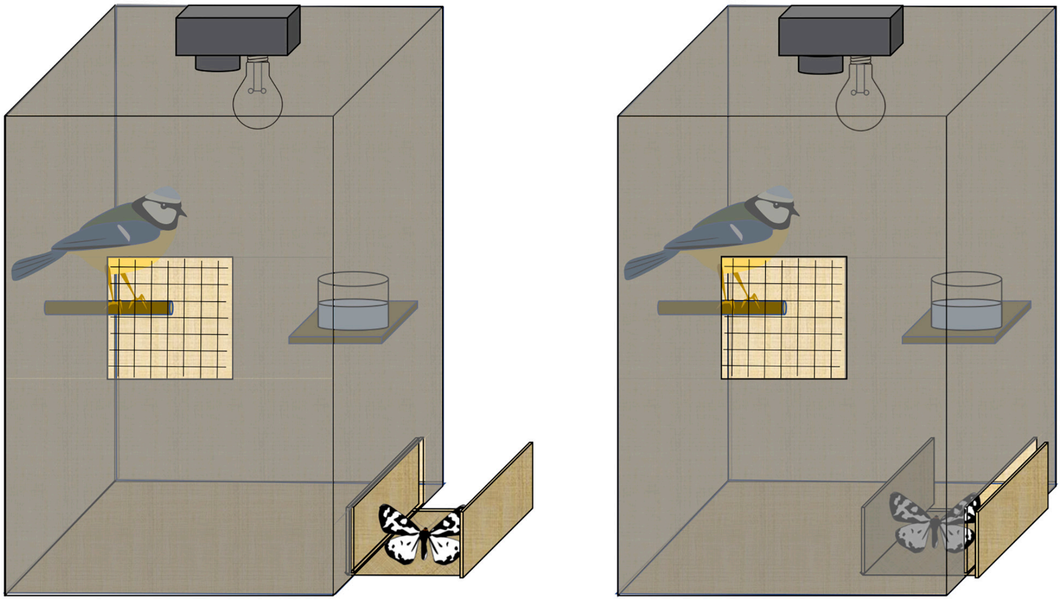


FIGURE 2 | Experimental setup of the behavioral experiment demonstrating the placement of perch, water, camera, light source, mesh opening for observation, and hatch for inserting moths into the enclosure.

and AEW) and data was recorded to measure the following 16 variables (**Supplementary Table 1**): (1) *approach probability* measured whether or not the bird approached the moth (yes/no, for all moths) (2) *approach latency* was measured as the time (in seconds) from seeing the moth (tilts head to look down at it) to approaching it (landing beside the moth, usually on the movable platform the moth was placed unless the moth moved) (3) *attack probability* measured whether or not the bird attacked the moth (yes/no, for moths that were approached) (4) *attack latency* measured the time (in seconds) from approaching the moth to grabbing it (5) *drop probability* measured whether or not the bird dropped the moth at least once before eating it (yes/no, for moths that were attacked) (6) *prey drop latency* measured the time (in seconds) from grabbing the moth to dropping it for the first time for birds that ate <50% of the moth (7) *prey dropping* counted the number of times the bird dropped the moth before beginning to eat it (8) *handing duration* was a sum of the time (in seconds) the bird spent holding the moth (grabbing the moth until dropping the moth). Includes each occurrence the moth was held and includes *eating duration* (9) *eating probability* measured whether or not the bird ate at least part of the moth (yes/no, for moths that were attacked) (10) *eating duration* was a sum of the time (in seconds) the bird spent eating the moth (started eating the moth until stopped eating the moth). Includes each occurrence the moth was eaten. (11) *proportion eaten* was calculated by adding together the proportion of each of six body parts eaten (antennae, head, thorax, abdomen, legs, wings), as estimated by eye, and dividing by six to calculate the total proportion of the moth that was eaten by the bird (12) *kill latency* was measured as the time (in seconds) from seeing the moth to killing it (usually by eating or removing the head) (13) *beak wiping*, which is a common disgust behavior (Evans and Waldbauer, 1982; Skelhorn and Rowe, 2009; Rowland et al., 2015; Rojas et al., 2017, 2019) was measured as the number of bouts of beak wiping the bird performed after grabbing the moth until the end of the trial, (14) *water drinking*, which may increase after the bird has consumed something distasteful (Burdfield-Steel et al., 2019), was measured as the number of "sips" taken from the water bowl after grabbing the moth until the end of the trial. In addition, (15) moth activity, sum of the time (in seconds) the moth spent crawling, flying, or flexing which includes each occurrence the moth was active, and (16) moth survival (yes/no, for all moths) were also recorded. These behaviors were first recorded on datasheets during the experiment using a stopwatch (to nearest second) and then confirmed by JL watching the video afterward. If there was a discrepancy between the video and the original observation in terms of the timing or counts of a behavior, the video observation was used because these behaviors could be measured more accurately using the video. However, *kill latency* was always measured using the original observation because it is difficult to ascertain the time of death from the video. Birds remained under observation for 30 min following the experiment to monitor for ill effects from moth consumption, but none were observed. After the observation period, birds were offered 8 g of meal worms. The weight of mealworms eaten within 10 min was used as a measure of the bird's *hunger level* (Stevens et al., 2010). If the bird did not eat the moth or the mealworms (2 individuals), it was excluded from the experiment.

Statistical Analysis

All analyses were conducted using R version 4.0.3 (R Core Team, 2011). All models include the fixed effects of moth morph (white, yellow), methoxypyrazine smell (present, absent), pyrrolizidine alkaloid taste (present, absent), and trial number (1, 2, 3). We then used a forward stepwise selection process to include interactions and co-variates based on AICc and a threshold of Δ 2. As there are a large number of interactions in our multimodality study to consider that may have potential to be biologically meaningful, we used the dredge function in the MuMin Package (Barton and Barton, 2015) for this step. In all cases the simplest model within Δ 2 of the top model was selected. Then, we compared models using genotype (WW, Wy, yy) or color morph (w, y). If genotype improved the AICc score of the model by greater than Δ 2, moth genotype was used instead. Finally, after selecting interactions and morph or genotype, additional relevant co-variates were selected to be included in the model if they improved the AICc score of the model by greater than Δ 2. These co-variates that have potential to influence the predation sequence include: *moth activity* which can influence the bird's required effort, *hunger level* which can influence the bird's motivation, *bird age* which may relate to experience, *bird sex* where physiological differences may influence behavior and motivation, and *bird weight* which may relate to body condition and motivation. In all models, except for moth survival probability, bird ID was included as a random factor to account for multiple trials per bird. Model assumptions were checked and distributions were chosen accordingly. Follow-up analyses were conducted to determine which treatments differed from the control. Tables detailing model selection (**Supplementary Table 2**) and model summaries (**Supplementary Table 3**) are provided in the **Supplementary Material**.

First, we tested whether the probability that blue tits would progress through the predation sequence (binomial response variables = *approach probability*, *attack probability, drop probability*, *eating probability,* or *moth survival*) differed among treatments. To do this, we used generalized linear mixed-effects models (GLMM) with binomial distributions using the package lme4 (Bates et al., 2015). *Bird weight* improved the AICc score for the models of *drop probability, eating probability,* and *moth survival* by more than Δ 2, so it was included as a co-variate in those models (**Supplementary Table 2**).

Next, we tested whether timed bird behaviors (approach and attack latencies, eating and handling durations, *drop latency*, and *kill latency*) differ among treatments to using cox proportional hazards models (Therneau and Therneau, 2015). *Moth genotype* improved the AICc score for the models of *approach latency*, *attack latency*, *drop latency*, and *eating duration* by more than Δ 2 AICc, so *moth genotype* was used instead of *moth morph* for these models (**Supplementary Table 2**). For the model of *attack latency*, the interaction between *moth genotype* and *pyrrolizidine alkaloid taste* improved the AICc score by more than Δ 2, and for *kill latency* the interaction between *methoxypyrazine smell* and *pyrrolizidine alkaloid taste* improved the AICc score by more than Δ 2, so these interactions were included in the models (**Supplementary Table 2**). Based on AICc comparison, *bird age* was selected to be included as a co-variate in the model of *attack latency*, and *bird weight* was included in the models of *drop latency, handling duration*, and *eating duration*, while bird *hunger level* was included in the model of *kill latency* (**Supplementary Table 2**).

Then, we tested whether counts of bird disgust behaviors after attacking the moth (*prey dropping*, *beak wiping*, *water drinking*) differed among our treatments using GLMM with poisson distributions (except for *beak wiping*). The sum of squared Pearson residuals indicated that the model for *beak wiping* behavior was overdispersed, so a negative binomial distribution was used instead. For the model of *water drinking*, the interaction between methoxypyrazine smell and trial number improved the AICc score by more than Δ 2, so it was included in the model (**Supplementary Table 2**). Bird *weight* improved the AICc score for the model of *prey dropping* by more than Δ 2, so it was included as a co-variate in that model (**Supplementary Table 2**).

Finally, we tested whether the *proportion eaten* differed among treatments. Model residuals were normally distributed, therefore we used a linear mixed effects model with a Gaussian distribution. *Bird weight* improved the AICc score by more than Δ 2, so it was included as a co-variate in the model (**Supplementary Table 2**).

RESULTS

Approach

Birds approached the moths in each of the 251 trials (except for one case, trial 2, Wy, white morph, both chemical defenses). Independent of neck fluids, *approach latency* was longer for moths of the WW genotype, but not the Wy genotype, compared to moths of the yy genotype (estimate ± SE = −0.6958 ± 0.2143, $z = -3.25$, $p = 0.001$; **Figure 3A** and **Supplementary Table 3**). However, when neck fluids with methoxypyrazine smell were present, birds approached both white morph genotypes more slowly compared to yy moths (WW estimate ± SE = −0.8214 ± 0.2998, $z = -2.4$, $p = 0.006$; Wy estimate ± SE = −0.6445 ± 0.3324, $z = -1.94$, $p = 0.052$, **Figure 3A** and **Supplementary Table 3**) suggesting that the methoxypyrazine smell of heterozygote moths is especially important at this stage of attack. *Approach latency* was longer for juvenile birds compared to adults (estimate ± SE = −0.4321 ± 0.2054, $z = -2.10$, $p = 0.035$; **Supplementary Figure 2A** and **Supplementary Table 3**). Overall, *approach latency* decreased as the trials progressed (estimate ± SE = 0.2899 ± 0.0873, $z = 3.32$, $p = 0.001$; **Supplementary Figure 3A** and **Supplementary Table 3**). *Approach latency* was not affected by the presence of neck fluids or pyrrolizidine alkaloids alone (**Supplementary Table 3**).

Attack

Birds attacked the moths in 94% of the trials (236 out of 251), with a trend for *attack probability* to increase with trial number (estimate ± SE = 0.8207 ± 0.4833, $z = 1.698$, $p = 0.089$, **Supplementary Figure 3B** and **Supplementary Table 3**). There was no effect of moth morph, methoxypyrazine smell, or pyrrolizidine alkaloid taste on *attack probability* (**Supplementary Table 3**).

The interaction between moth genotype and pyrrolizidine alkaloid taste influenced bird *attack latency* (estimate ± SE = −1.1902 ± 0.3563, $z = -3.34$, $p = 0.001$, **Figure 3B** and **Supplementary Table 3**). The pyrrolizidine

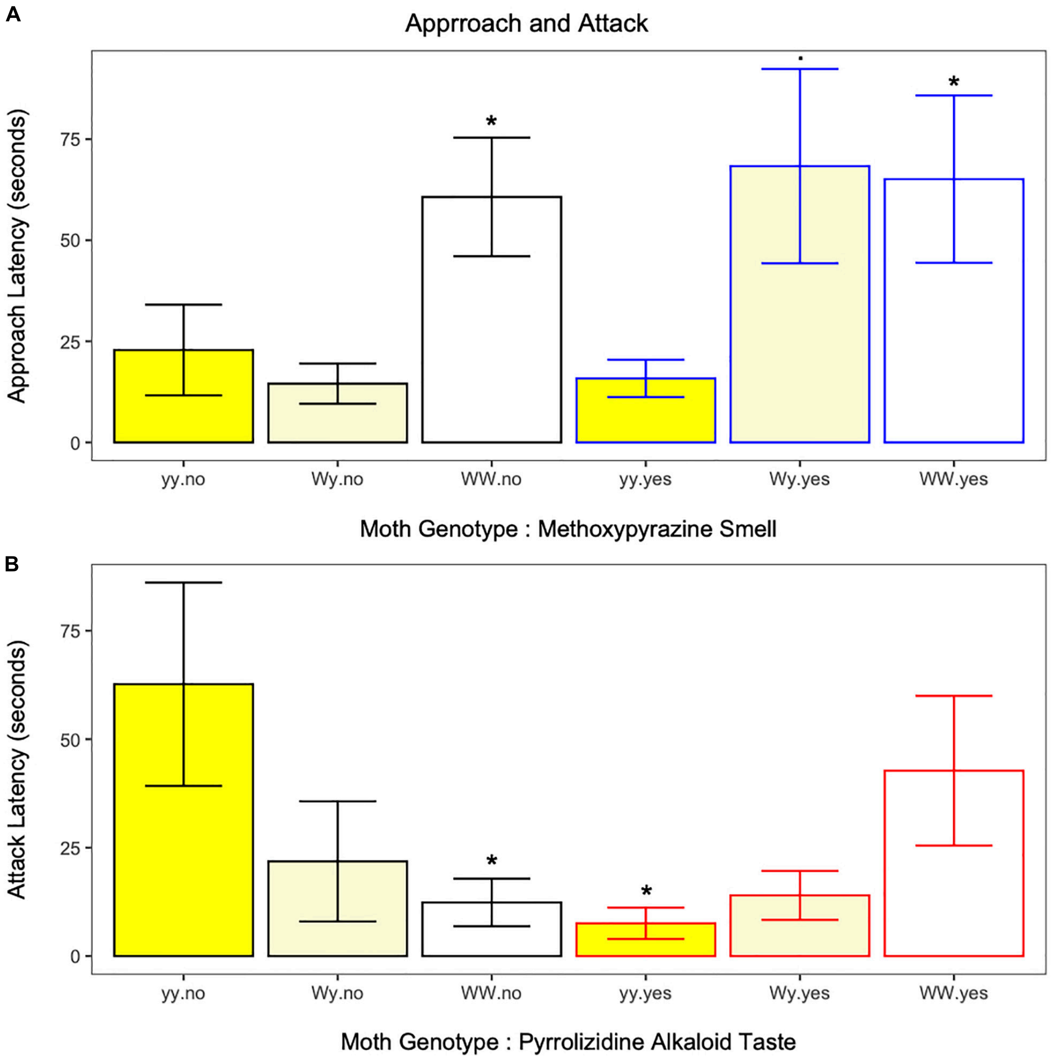


FIGURE 3 | (A) Approach latency in response to each moth genotype with (blue outline) and without (black outline) methoxypyrazine smell. Latency to approach moths of the Wy genotype (white hindwings) morph was dependent on the presence of methoxypyrazine smell. Whereas, birds hesitated longer to approach moths of the WW genotype (white hindwings) compared to moths of the yy genotype (yellow hindwings) irrespective of methoxypyrazine smell. *Indicates significant differences (. = trend) from yy moths without methoxypyrazine smell. **(B)** Attack latency in response to each moth genotype with (red outline) and without (black outline) pyrrolizidine alkaloid taste. Latency to attack moths of the yy genotype (yellow hindwings) was lower for moths on the pyrrolizidine alkaloid diet, while latency to attack moths of the WW genotype (white hindwings) was higher for moths on the pyrrolizidine alkaloid diet. Pyrrolizidine alkaloid diet did not impact attack latency for moths of the Wy genotype (white hindwings) *Indicates significant differences from yy moths without pyrrolizidine alkaloid taste.

alkaloid diet increased bird *attack latency* for moths of the WW genotype, but decreased bird *attack latency* for moths of the yy genotype (**Figure 3B**), suggesting the diet treatments affect the primary anti-predator defenses of genotypes in different ways. Bird *attack latency* decreased as the trials progressed (estimate ± SE = 0.3244986 ± 0.08131366, $z = 3.99$, $p < 0.001$, **Supplementary Figure 3C** and **Supplementary Table 3**). *Attack latency* was not affected by methoxypyrazine smell.

Subjugation

Following attack, birds dropped the moth at least once in 28% of the trials (65 out of 236), and independent of moth morph, bird *drop probability* was higher for moths that had neck fluids than those that did not (estimate ± SE = 1.2688 ± 0.5532, $z = 2.294$, $p = 0.0218$; **Figure 4A** and **Supplementary Table 3**). However, when investigated separately, it was only white moths with methoxypyrazine smell that significantly differed from yellow moths with none (estimate ± SE = 2.4375 ± 1.0445, $z = 2.334$, $p = 0.0196$; **Figure 4A** and **Supplementary Table 3**). *Drop probability* was positively associated with bird weight, heavier birds were more likely to drop the moth (estimate ± SE = 1.4527 ± 0.4463, $z = 3.255$, $p = 0.0011$, **Supplementary Figure 2B** and **Supplementary Table 3**). Independently, there was no effect of moth morph, pyrrolizidine alkaloid taste, or trial number on *drop probability* (**Supplementary Table 3**).

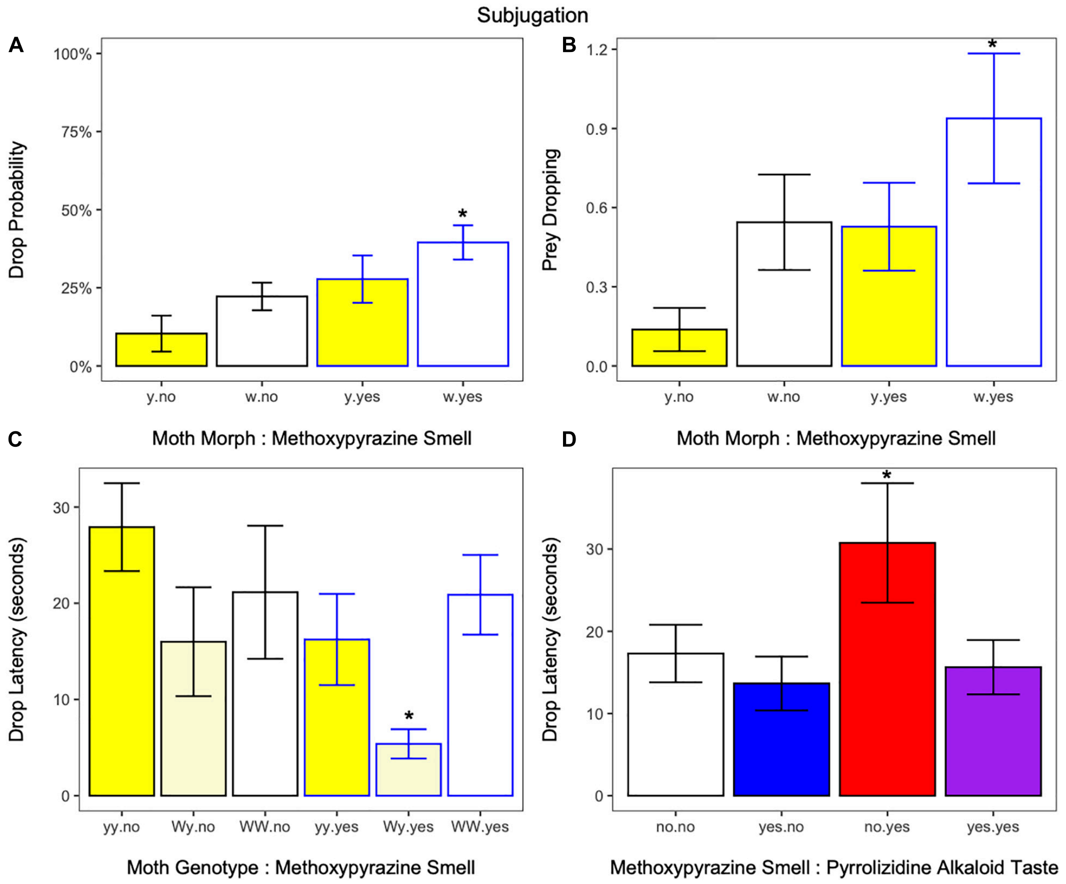


FIGURE 4 | (A) % of moths that were dropped at least once after being grabbed in response to moth morph (yellow or white) and methoxypyrazine smell. Moths with white hindwings and methoxypyrazine smell were more likely to be dropped and with (blue outline) or without (black outline) methoxypyrazine smell. Panel **(B)** were also dropped a greater number of times compared to yellow moths without methoxypyrazine smell. *Indicates significant differences from yellow moths without methoxypyrazine smell. **(C)** Drop latency (time between grabbing and dropping the moth for the first time for birds that ate<% 50 of the moth) was quicker for moths of the Wy genotype with methoxypyrazine smell compared to yellow moths without (*indicates significant differences from moths of the yy genotype without methoxypyrazine smell), suggesting a more potent defense upon contact and **(D)** was slower for moths that were raised on a pyrrolizidine alkaloid diet compared to moths with no chemical defenses, suggesting toxin sequestration is costly to other (primary) defenses (*indicates significant difference from moths without methoxypyrazine smell or pyrrolizidine alkaloid taste). Bar graphs show mean ± SE [or percent (Dropped = yes)].

In addition, *prey dropping* behavior (number of times the bird dropped the moth before eating it) increased if the moth had methoxypyrazine smell (estimate ± SE = 0.9679, ± 0.4286, $z = 2.258$, $p = 0.0239$, **Figure 4B** and **Supplementary Table 3**) and heavier birds exhibited more *prey dropping* behavior (estimate ± SE = 0.8179 ± 0.3261, $z = 2.508$, $p = 0.0121$, **Supplementary Figure 2D**). However, again it seems likely this effect is driven by moths of the white morph, as this was the only treatment to independently differ from yellow moths without methoxypyrazine smell (estimate ± SE = 1.9527 ± 0.8343, $z = 2.341$, $p = 0.0193$, **Figure 4B** and **Supplementary Table 3**) in a separate analysis. Independently, there was no effect of moth morph, pyrrolizidine alkaloid taste, or trial number on *prey dropping* behavior.

Of the birds that ate less than half of the moth, birds had a shorter *drop latency* for moths with methoxypyrazine smell compared those without (estimate ± SE = 0.6173 ± 0.2360, $z = 2.62$, $p = 0.0089$, **Figure 4C** and **Supplementary Table 3**), and moths of the Wy genotype compared to those of the WW (estimate ± SE = 0.9142 ± 0.2886, $z = 3.17$, $p = 0.0015$) or yy (estimate ± SE = 0.7022 ± 0.3040, $z = 2.31$, $p = 0.0210$, **Supplementary Table 3**) genotypes, with a shorter period of time between grabbing and abandoning Wy moths (**Figure 4C**). This effect is likely being driven by the *drop latency* for Wy moths with methoxypyrazine smell, which was the only treatment to significantly differ from yellow moths without methoxypyrazine smell (estimate ± SE = 1.5433, 0.4460, $z = 3.46$, $p = 0.0005$, **Figure 4C** and **Supplementary Table 3**). Surprisingly, *drop latency* was quicker for moths raised on the control diet compared to those raised on a diet with pyrrolizidine alkaloids (estimate ± SE = −0.5087 ± 0.2288, $z = -2.2$, $p = 0.0260$, **Figure 4D** and **Supplementary Table 3**), which is likely being driven by *drop latency* for moths with pyrrolizidine alkaloids but without methoxypyrazine smell, which was the only treatment to significantly differ from moths with no chemical defenses (estimate ± SE = −0.8317 ± 0.3934, $z = -2.11$, $p = 0.0350$), suggesting a trade-off between pyrrolizidine alkaloid sequestration and synthesis of methoxypyrazine reserves. Heavier birds had quicker *drop latency* (estimate ± SE = 0.6337 ± 0.1748, $z = 3.63$, $p = 0.0003$, **Supplementary Figure 2C**). There was no effect of trial number on drop latency (**Supplementary Table 3**).

Handling duration decreased as the trials progressed (estimate ± SE = 0.6538 ± 0.0978, $z = 6.68$, $p < 0.001$; **Supplementary Figure 3D** and **Supplementary Table 3**). Heavier birds had a shorter *handling duration* (estimate ± SE = 0.62076875 ± 0.20082539, $z = 3.09$, $p = 0.002$, **Supplementary Figure 2E** and **Supplementary Table 3**). Moth morph, methoxypyrazine smell, and pyrrolizidine alkaloid taste did not influence *handling duration* (**Supplementary Table 3**).

Consumption

Birds ate at least part of the moth in 81% of the trials (48 out of 251), and were less likely to eat moths that had methoxypyrazine smell than those that did not (estimate ± SE = −2.2490 ± 0.9158, $z = -2.456$, $p = 0.0141$; **Figure 5A** and **Supplementary Table 3**). There was no effect of moth morph, pyrrolizidine alkaloid taste, or trial number on *eating probability* (**Supplementary Table 3**).

There was a significant effect of genotype on *eating duration*, where birds took longer to eat moths of the WW genotype compared to the yy genotype (estimate ± SE = −0.6592 ± 0.2532, $z = -2.60$, $p = 0.0092$, **Figure 5B** and **Supplementary Table 3**). *Eating duration* decreased with trial number (estimate ± SE = 0.44751035 ± 0.1026699, $z = 4.36$, $p < 0.001$, **Supplementary Figure 3E**), and heaver birds had a shorter *eating duration* (estimate ± SE = 0.46967725 ± 0.1540265, $z = 3.05$, $p = 0.0023$, **Supplementary Figure 2G**). Neither methoxypyrazine smell nor pyrrolizidine alkaloid taste affected *eating duration* (**Supplementary Table 3**).

In addition, the *proportion eaten* decreased if the moth had methoxypyrazine smell compared to those without [$t(79) = -2.405621$, $p = 0.0185$; **Figure 5C** and **Supplementary Table 3**]. However, it seems likely this effect is driven by the treatment where moths that have both methoxypyrazine smell and pyrrolizidine alkaloid taste in their neck fluids. When analyzed separately, the *proportion eaten* was smaller from moths with both defenses compared to moths with no defenses [$t(78) = -2.2654$, $p = 0.0263$; **Figure 5C** and **Supplementary Table 3**], but the *proportion eaten* did not differ between moths with only methoxypyrazine smell and those with no chemical defenses [$t(78) = -1.2821$, $p = 0.2036$; **Figure 5C** and **Supplementary Table 3**]. The *proportion eaten* increased with trial number [$t(151) = 2.0533$, $p = 0.0418$]; **Supplementary Figure 3F** and decreased with bird weight [$t(78) = -3.6513$, $p = 0.0005$; **Supplementary Figure 2H** and **Supplementary Table 3**]. There was no effect of pyrrolizidine alkaloid taste or moth morph on the *proportion eaten* (**Supplementary Table 3**).

Disgust

Beak wiping behavior decreased with trial number (estimate ± SE = −0.7963 ± 0.1089, $z = -7.311$, $p < 0.001$; **Supplementary Figure 3G** and **Supplementary Table 3**). There was no effect of moth morph, methoxypyrazine smell, or pyrrolizidine alkaloid taste on *beak wiping* (**Supplementary Table 3**).

The interaction between methoxypyrazine smell and trial number influenced bird *water drinking* behavior (estimate ± SE = 0.6299 ± 0.2814, $z = 2.238$, $p = 0.0252$, **Figure 5D** and **Supplementary Table 3**). *Water drinking* decreased with trial number if the moth did not have neck fluids (**Figure 5D**). Neither the moth morph nor pyrrolizidine alkaloid taste affected *water drinking* behavior in the birds (**Supplementary Table 3**).

Survival

The interaction between methoxypyrazine smell and pyrrolizidine alkaloid taste influenced bird *kill latency* (estimate ± SE = −1.1483162 ± 0.4830955, $z = -2.38$, $p = 0.017$, **Figure 6A** and **Supplementary Table 3**). *Kill latency* was quicker if the moth did not have a methoxypyrazine smell and was raised on a diet with pyrrolizidine alkaloids, suggesting a trade-off between toxin sequestration and methoxypyrazine synthesis. *Kill latency* decreased with trial number (estimate ± SE = 0.6360943 ± 0.1096281, $z = 5.80$, $p < 0.001$; **Supplementary Figure 3H** and **Supplementary Table 3**), and with hunger level (estimate ± SE = 1.0167007 ± 0.2755151, $z = 3.69$, $p < 0.001$; **Supplementary Figure 2J** and **Supplementary Table 3**). There was no effect of moth morph on *kill latency* (**Supplementary Table 3**).

Moths survived in only 43 (17%) trials. *Moth survival* increased if they had methoxypyrazine smell (estimate ± SE = 1.25092 ± 0.40567, $z = 3.084$, $p = 0.0021$; **Figure 6B** and **Supplementary Table 3**), and *moth survival* increased with bird weight (estimate ± SE = 1.48241 ± 0.33329, $z = 4.448$, $p < 0.001$, **Supplementary Figure 2I** and **Supplementary Table 3**). When analyzed separately, white moths with methoxypyrazine smell were the only moths with higher survival compared to yellow moths without (estimate ± SE = 1.6612 ± 0.7887, $z = 2.106$, $p = 0.0352$, **Figure 6B** and **Supplementary Table 3**), and moths with both methoxypyrazine smell and pyrrolizidine alkaloid taste were the only moths with higher survival compared to moths with no chemical defenses (estimate ± SE = 1.1442 ± 0.4999, $z = 2.289$, $p = 0.0221$, **Figure 6C** and **Supplementary Table 3**), however, there was a trend for moths with only methoxypyrazine smell to also have higher survival compared to moths with no chemical defenses (estimate ± SE = 0.9899 ± 0.5201, $z = 1.903$, $p = 0.0570$). Neither pyrrolizidine alkaloid taste alone nor trial number affected *moth survival* (**Supplementary Table 3**).

DISCUSSION

Summary

This study investigated multimodal anti-predator defenses through the predation sequence (**Figure 7**), and how multimodality impacts predator avoidance learning and moth survival. Bird approach latency toward moths of the Wy (white hindwings) morph was dependent on the presence of

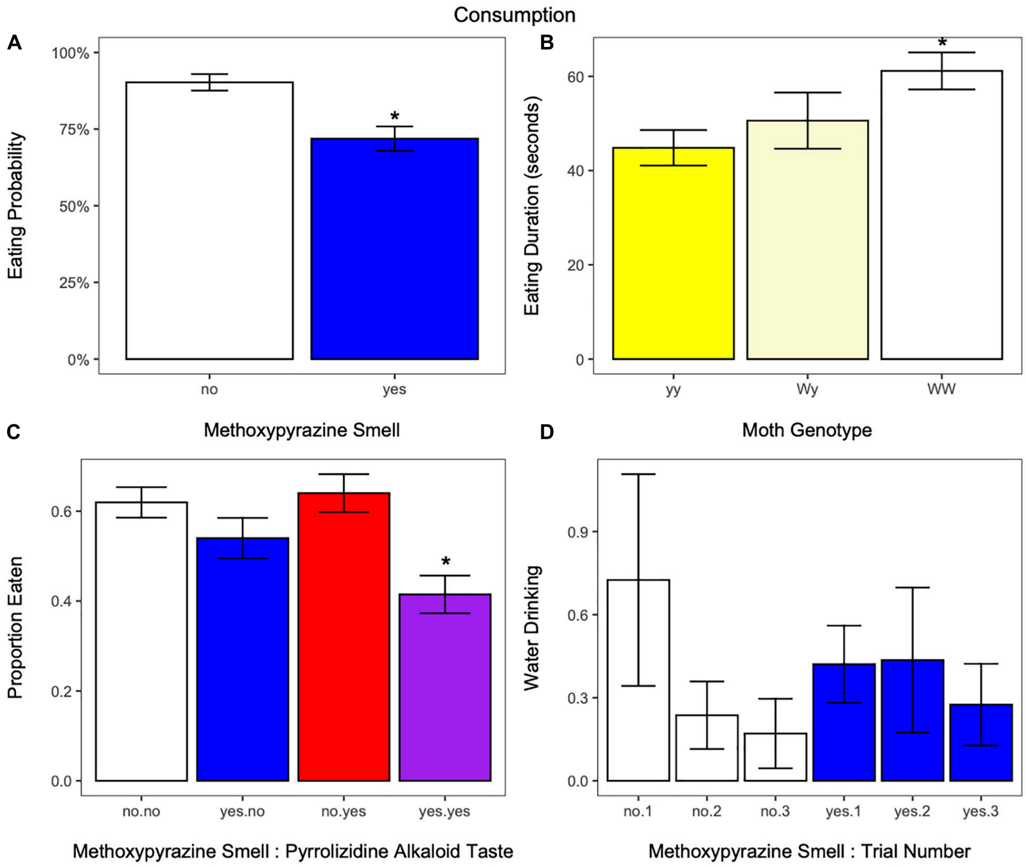


FIGURE 5 | (A) The probability for birds to eat at least part of the moth in response to the presence (blue) or absence (white) of methoxypyrazine smell. Birds were more likely to eat moths without methoxypyrazine smell. **(B)** The amount of time birds spent eating moths of each genotype. Birds ate moths of the WW (white hindwings) genotype more slowly compared to moths of the yy genotype (yellow hindwings). *Indicates significant differences from the yy genotype. **(C)** The proportion of the moth's body eaten in response to methoxypyrazine smell and pyrrolizidine alkaloid taste. The proportion eaten was smaller if the moth had both types of chemical defense. *Indicates significant difference from moths without methoxypyrazine smell or pyrrolizidine alkaloid taste. **(D)** The number of times birds took a drink of water in response to trial number and methoxypyrazine smell. Birds reduced their water drinking as trials progressed to a greater degree for moths that did not have methoxypyrazine smell compared to those that did, suggesting that moths with methoxypyrazine smell maintain aversion across trials. Bar graphs show mean ± SE [or percent (Eaten = yes)].

neck fluids with methoxypyrazine smell, but approach latency was longer for moths of the WW (white hindwings) compared to moths of the yy genotype (yellow hindwings) irrespective of methoxypyrazine smell. Color and smell had additive effects on dropping behavior, where moths of the white morph with neck fluids were more likely to be dropped and were also dropped a greater number of times. Drop latency (time between grabbing and dropping the moth for the first time) was quickest for moths of the Wy genotype that had neck fluids, suggesting a more potent defense upon contact for this genotype. Furthermore, taste alone did not deter bird predators. Birds were less likely to eat moths with neck fluids compared to those without, but only responded to the presence of pyrrolizidine alkaloids (taste and toxicity) after they had started to eat the moth and when the methoxypyrazine smell was also present, causing them to eat a smaller proportion of the moth's body with both chemical defenses. Surprisingly, the pyrrolizidine alkaloid diet had a detrimental effect on predator deterrence in the early stages of attack including *attack latency*, *drop latency*, and *kill latency* (**Figure 7**), suggesting a possible trade-off between secondary (toxin sequestration) and primary (methoxypyrazine synthesis and/or wing pigmentation) defenses. Overall, moths of the white morph with methoxypyrazine smell had the greatest chance of survival. However, even though pyrrolizidine alkaloids had a negative impact on attack stage progression, toxin sequestration did not negatively impact survival. Indeed, moths with both methoxypyrazine smell and pyrrolizidine alkaloid taste had the highest survival overall. We did not find support for predator aversion learning, although birds adjusted their water drinking behavior across trials in response to methoxypyrazine smell. We suggest that methoxypyrazines act as context setting signals for warning colors and as attention alerting or "go-slow" signals for distasteful toxins, thereby mediating the relationship between warning signal and toxicity. The effect of each modality on each stage of attack (**Figure 7**) is detailed below.

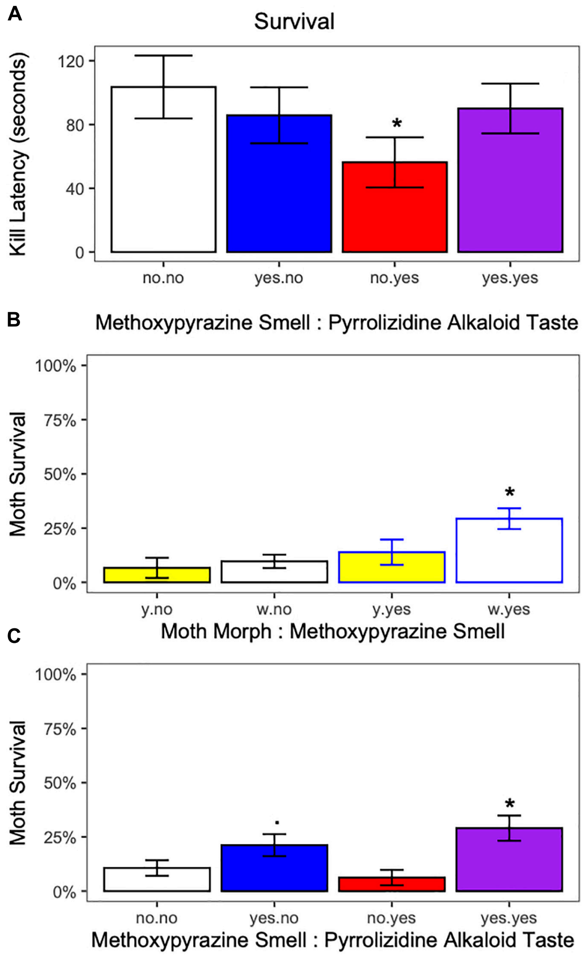


FIGURE 6 | (A) Bird latency to kill the moth in response to methoxypyrazine smell and pyrrolizidine alkaloid taste. Moths raised on a pyrrolizidine alkaloid diet, but without methoxypyrazine smell were killed more quickly than moths without either chemical defense. *Indicates significant difference from moths without either chemical defense. **(B)** Moth survival in response to moth morph (yellow or white) and methoxypyrazine smell. Moths of the white morph with = blue methoxypyrazine smell had higher survival compared to moths of the yellow morph without = black methoxypyrazine smell. *Indicates significant difference from yellow moths without methoxypyrazine smell. **(C)** Moth survival in response to methoxypyrazine smell and pyrrolizidine alkaloid taste. Moths with both methoxypyrazine smell and pyrrolizidine alkaloid taste had higher survival than moths without either chemical defense. *Indicates significant differences (. = trend) from moths without either chemical defense. Bar graph shows percent (Survived = yes)±.

Approach

At the approach stage (**Figure 7**), birds hesitated longer to approach moths of the WW genotype compared to the yy genotype but only hesitated to approach moths of the Wy genotype if they had a methoxypyrazine smell. In addition, adult birds approached moths more quickly than juveniles, which suggests juveniles were more cautious with their prey. In a previous study, Rojas et al. (2019) found that blue tits took longer to approach models with white wings when neck fluids were present, regardless of whether they were coated with fluids from yellow (Y) or white (W) males, suggesting that methoxypyrazine smell did not differ between the morphs. While, neck fluids from Wy and WW males were not differentiated in Rojas et al. (2019), examination of **Figure 3** suggests that variation in approach latency is largest for white morphs, which is consistent with the idea that fluid properties may differ between WW and Wy genotypes. Further chemical analysis is necessary to determine genotype differences in the type or quantity of *de novo* synthesized methoxypyrazines.

The difference in approach latency between WW and Wy moths without neck fluids, both of which have white hindwings, suggests that there is a perceptible visual difference between these two genotypes to blue tits. Ultraviolet (UV) components of the color pattern differ between these two genotypes especially in the forewings (Nokelainen in prep a), and therefore it is possible that birds are responding to UV-reflectance by delaying their attack of prey. Indeed, UV reflective white color is used as a warning signal in other lepidopteran species (Corral-Lopez et al., 2020), although in some earlier experiments UV-reflectance was found to invite rather than deter attacks by birds (Lyytinen et al., 2001, 2004).

Our results support the findings of Rojas et al. (2019) that, in the presence of neck fluid odor, birds take longer to approach *A. plantaginis* with white hindwings compared to those with yellow hindwings. Rojas et al. (2019) presented moths on a green background, while we presented moths against brown masonite (**Supplementary Figure 1**), which suggests that the white morph elicits longer approach hesitation even when presented against different colored backgrounds. These findings are at odds with previous studies where the yellow morph was found to be better protected (Nokelainen et al., 2012, 2014), but confirms that this discrepancy is not simply a difference in cues between model stimuli and natural prey. As suggested by Rojas et al. (2019) and experimentally tested by O. Nokelainen et al. (in prep b) there is an interaction between color pattern and light environment on predator response to *A. plantaginis* hindwing coloration. This could explain differences between experiments. Furthermore, natural prey that are not alive, such as some of those used in the Nokelainen et al. (2012) field experiment, may lack chemical delivery mechanisms to effectively release methoxypyrazine odors, and these volatile compounds may not have been present when the moths were presented to birds. These results highlight the importance of considering the interplay between multiple modalities, but also variation in natural environmental conditions, such as light environment, that can influence predator responses to defended prey.

Attack

Surprisingly, bird attack latency (time from approaching to attacking the moth) depended on the interaction between moth genotype and the pyrrolizidine alkaloid diet treatment (**Figure 7**), with birds hesitating longest to attack homozygous yellow moths from the control diet and homozygous white moths if they were raised on the pyrrolizidine alkaloid diet. Birds could be more motivated to attack larger prey, however, diet did not impact pupal weight for the WW and yy genotypes and attack latency did not differ between diet treatments for heterozygous moths, which were heavier when raised on the pyrrolizidine alkaloid diet (**Supplementary Figure 4**). It is puzzling that birds could perceive these non-volatile toxins before they are tasted and that the presence of toxins could cause birds accelerate their attack (for homozygous yellow moths). A more likely explanation is that sequestering toxins from the diet can be costly, limiting investment in other defense mechanisms such as methoxypyrazine synthesis or wing pigmentation, and that this cost differs between the genotypes. For example, it is possible that small reserves of methoxypyrazines are present even in moths that had their neck fluids removed, that the potency of these reserves is impacted by diet, and that the detection of these reserves requires close range (between approach and attack). Similarly, Lindstedt et al. (2010) found that a diet with iridoid glycoside toxins was costly to primary defenses of female *A. plantaginis*, resulting in hindwings with a lighter hue. The cost of pyrrolizidine alkaloid sequestration, and particularly its impact on other anti-predator defenses in *A. plantaginis* warrants further study.

Subjugation

After attack, bird likelihood to drop the moth and the number of times they dropped the moth differed in response to the additive effects of methoxypyrazine smell and color morph. Birds increased dropping behavior when moths had methoxypyrazine smell, and even more so when the moth had white hindwings. It is possible that these behaviors vary in response to the combination of visual and chemical signals. Such a relationship between methoxypyrazines and warning coloration is common in the literature, where it is suggested that methoxypyrazines act as context-setting signals (Marples and Roper, 1996; Rowe and Guilford, 1996, 1999; Lindström et al., 2001; Jetz et al., 2001; Kelly and Marples, 2004; Rowe and Halpin, 2013; Vickers and Taylor, 2018, 2020). However, it is also possible that white morph moths, and in particular those that are heterozygous for hindwing coloration, have more potent chemical defense, which causes differences in predator response between the genotypes. These effects are not mutually exclusive. Indeed, Rojas et al. (2019) found that moths of the white morph may have a more aversive taste.

Bird drop latency (the time between grabbing and dropping the moth) was quicker for heterozygous moths and moths with methoxypyrazine smell, but slower for moths from the pyrrolizidine alkaloid diet, and particularly for moths with pyrrolizidine alkaloids but no methoxypyrazines (**Figure 7**). Again, one explanation for the apparent eagerness for birds to pursue moths that have pyrrolizidine alkaloids is that toxin sequestration may be costly and reduce investment in other defenses, such as methoxypyrazines, which in turn may reduce defense potency in the earlier stages of attack before the bird has encountered the taste of pyrrolizidine alkaloids.

Consumption

Birds were less likely to eat moths with a methoxypyrazine smell than those without. In addition, birds took longer to eat moths of the WW genotype compared to moths of the yy genotype (**Figure 7**). Bird water drinking, which was correlated with eating duration (**Supplementary Figure 5**), decreased across trials, but only if the moths did not have neck fluids. However, despite being the only component that is intrinsically linked to the concentration of hepatotoxic pyrrolizidine alkaloids, taste alone did not deter bird predators. Birds only reduced the proportion eaten of the moth's body with pyrrolizidine alkaloids compared to other treatments when neck fluids containing methoxypyrazines were also present. One possibility is that methoxypyrazines alert predators to the presence of bitter toxins. In the attention-altering hypothesis 'one signal can increase the degree to which a receiver focuses attention on another sensory field, and by doing so, improves discrimination within that field' (Hebets and Papaj, 2005). For instance, Guilford (1994) first suggested that visual warning signals might be 'go-slow' signals that alert predators to pay better attention in their assessment of prey palatability. Our findings suggest that smell may also provide a 'go-slow' signal for taste and toxicity.

Fluid secretion may be an important mechanism for the delivery of chemical defenses, discharging a distasteful chemical cocktail into the bird's mouth before the bird has had a chance to bite into and taste the more nutritious tissues of the moth (Eisner and Meinwald, 1966). Indeed, in a study of leaf beetles, birds were more likely to reject prey that had their defense secretion intact compared to those that only had pyrrolizidine alkaloids sequestered into their body tissues (Rowell-Rahier et al., 1995). Therefore, it is possible that we have underestimated the effect of pyrrolizidine alkaloid defense, and that pyrrolizidine alkaloids in the neck fluids (Anne Winters Unpublished data) might contribute to moth defense at earlier stages of the attack sequence.

The role of pyrrolizidine alkaloids in defense against invertebrates is well-documented (Brown, 1984; Dussourd et al., 1988; Masters, 1990; Eisner and Eisner, 1991; Hare and Eisner, 1993; Conner et al., 2000; Eisner et al., 2000). Rojas et al. (2017) found that *A. plantaginis* abdominal fluids were deterrent to ants, but not to birds, but the compounds in the abdominal fluid were not identified. Pyrrolizidine alkaloids are present in the abdominal fluids of *A. plantaginis* (Anne Winters unpublished data) and it is possible that these contribute toward defense against invertebrates. Birds did not find the abdominal fluids (which do not contain methoxypyrazines) aversive, and this is in line with our finding that birds only reduced consumption of pyrrolizidine alkaloids when methoxypyrazines are also present. Invertebrate predators may also respond to visual aposematic signals. Similar to findings with birds, jumping spiders alter their response to visual signals in response to odor (Vickers and Taylor, 2018, 2020). Therefore, multimodal displays of color, smell, and taste are likely under selection from multiple, taxonomically distinct, predators. Defenses may asymmetrically target these predators, providing marginal protection for some types of predators and strong protection against others. Thus, multiple predators may create different selection pressures that shape the evolution of multimodal aposematic signals.

Survival

Moths raised on the pyrrolizidine alkaloid diet that had their neck fluids (methoxypyrazine smell) removed were killed more quickly than moths with no defenses (**Figure 7**), which, as mentioned above, suggests a trade-off between toxin

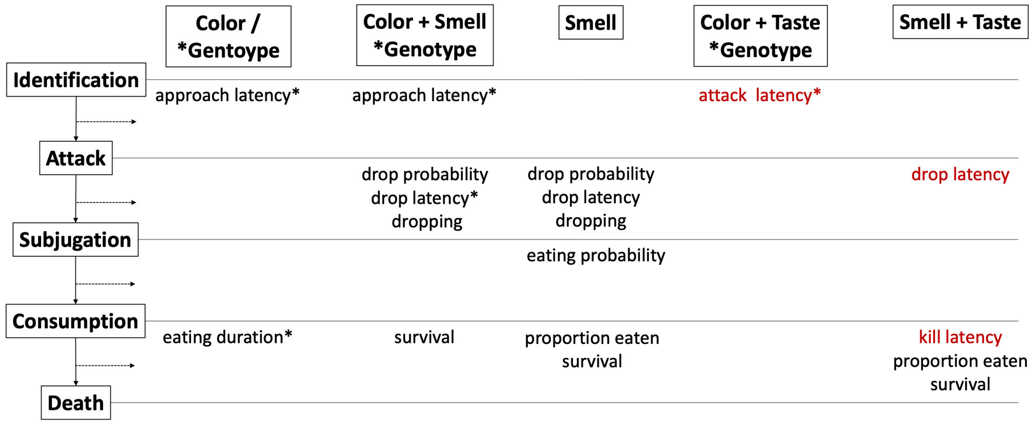


FIGURE 7 | Summary of the results indicating the bird behaviors (approach latency, drop probability, drop latency, prey dropping, eating probability, eating duration, proportion eaten, kill latency, moth survival) that were influenced by each defense modality (color, color + smell, smell, color + taste, smell + taste) and the stage of the attack sequence that was inhibited. *Indicates that genotype rather than moth color morph was included in the model based on AICc. Text in red indicates reduced defense efficacy for that defense treatment/bird behavior (cost of pyrrolizidine alkaloid toxin sequestration may trade-off with primary defenses), while black texts indicates improved efficacy.

sequestration and investment in other anti-predator defenses such as methoxypyrazine reserves and/or wing pigmentation. In addition, while moth activity was not selected to be included in the model based on AICc, moths on the pyrrolizidine alkaloid diet were less active compared to moths raised on the control diet (**Supplementary Figure 6**), which could reduce the amount of time needed for birds to capture and kill them. Overall, moth survival was highest for moths of the white morph with methoxypyrazine smell (**Figure 7**) and, despite the detrimental effects of pyrrolizidine alkaloid sequestration in terms of attack latency, drop latency, and kill latency, pyrrolizidine alkaloids did not negatively impact moth survival (**Figure 7**). Indeed, moths with both methoxypyrazine smell and pyrrolizidine alkaloid taste had the highest survival.

Predator Learning

We did not find evidence for aversion learning in this study. Instead, the time birds took to approach, attack, and handle the moths decreased with trials and birds were more likely to attack the moths as the trials progressed. Birds were quicker and more likely to attack moths in all treatments, including the treatment with no chemical defenses, suggesting a protective benefit of prey novelty that decreases with predator experience.

Birds that are no longer surprised by chemical defenses might still be expected to avoid them if those defenses are toxic or cause harm. However, birds in our experiment did not learn to avoid moths that contained toxic pyrrolizidine alkaloids. There are a number of reasons birds might decide to consume toxic prey, even after they have been warned about it (Barnett et al., 2007, 2012). For example, Hämäläinen et al. (2020) found that great tits differ in taste perception, but that their decision to eat toxic prey depended on the bird's body condition, and not taste perception. Similarly, we found that bird body weight (as a proxy for condition), impacted behaviors across the predation sequence including dropping, handling, killing and eating the moth. Likewise, decisions about eating chemically defended prey may also relate to the presence and nutritional value of alternative food sources (Brower et al., 1968; Turner and Speed, 1999; Kokko et al., 2003; Sherratt, 2003). It is possible that birds would have learned to avoid toxic moths if they were given the choice of a nutritious and non-toxic alternative. In addition, these experiments took place in the winter, when food, and especially live insects, are scarce and the ambient temperature is cooler compared to summer months, which could influence the choice to consume toxic prey. Indeed, Chatelain et al. (2013) found that starlings increased consumption of prey that they knew to contain toxins when the ambient temperature was cooler. Stevens et al. (2010) found that birds are more likely to eat unpalatable, aposematic prey when they are hungry, and similarly, we found that hungrier birds killed the moths more quickly.

Intra-Specific Differences in Chemical Defense

As described above, bird approach latency changed based on the presence of methoxypyrazines, but only for the Wy genotype. In addition, birds abandoned Wy moths with methoxypyrazine smell more quickly than other genotypes. And, contrary to our expectation, moth genotype also interacted with pyrrolizidine alkaloid taste at the attack latency stage (the time from approaching to attacking the moth). Together, these results suggest that the neck fluid defenses of moths that are heterozygous for hindwing coloration may be particularly potent, and that the potential cost of toxin sequestration is unequal across the genotypes. Using life history data of moths obtained from this experiment, male moths of the Wy genotype were the only moths that were differentially impacted by the diet manipulation in terms of pupal weight. The pupae of Wy males were heavier when raised on the diet containing pyrrolizidine alkaloids compared to the diet without (**Supplementary Figure 4**), suggesting the Wy genotype may perform better on this diet compared to the other genotypes. Further research is required to determine whether the Wy genotype differs in the quantity or ratio of *de novo* synthesized methoxypyrazines SBMP and IBMP and whether the Wy genotype more efficiently sequesters or differentially utilizes pyrrolizidine alkaloids from their diet. However, our findings suggest that heterozygotes may have an advantage when it comes to the dietary sequestration of chemical defenses and in defense against predation, which could help to explain the persistence of color polymorphism in this species (see also Gordon et al., 2018).

Conclusion

Altogether these results suggest that color, smell, and taste function as a multimodal warning signal, and that there may be trade-offs between defense modalities, which impact different stages of attack such that primary defenses may dishonestly signal pyrrolizidine alkaloid content. Color and smell provided protection from a distance and during the initial encounter, while during consumption, methoxypyrazine smell may alert predators to the presence of pyrrolizidine alkaloids, reducing the proportion eaten in the treatment with both chemical defenses compared to the control. Overall, moth survival was highest for moths of the white morph with methoxypyrazine smell and, despite the detrimental effects of pyrrolizidine alkaloid sequestration on defense in the early attack stages, toxin sequestration did not negatively impact moth survival. Indeed, of the chemical defense treatments, moths with both methoxypyrazine smell and pyrrolizidine alkaloid taste had the highest survival. The smell of methoxypyrazines seems to be an especially important signal, facilitating predator responses to both color and taste perception.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by The Central Finland Centre for Economic Development, Transport and Environment, a license from the National Animal Experiment Board (ESAVI/9114/04.10.07/2014) and the Central Finland Regional Environmental Centre (VARELY/294/2015).

AUTHOR CONTRIBUTIONS

JL participated in the design of the study, lab work, fieldwork, data analysis, and drafting the manuscript. JK participated in the lab work, fieldwork, and drafting the manuscript. ON participated in the design of the study and drafting the manuscript. JM participated in the conception and design of the study and drafting the manuscript. AW conceived, coordinated, and designed the study, participated in lab work, field work, data analysis, and drafting the manuscript. All authors approved the submitted version.

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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.2021.657740/full#supplementary-material

Supplementary Figure 1 | Spectral reflectance (% reflectance from 300 to 700 nm wavelengths) of the masonite enclosure (background) and for the forewings (FW) and hindwings (HW) and black portion of the wing (BK) of each genotype: WW, white hindwings; Wy, white hindwings; yy, yellow hindwings.

Supplementary Figure 2 | Effect of co-variates: **(A)** approach latency in response to bird age, **(B–I)**, drop probability, drop latency, prey dropping, handling duration, eating probability, eating duration, proportion eaten, and moth survival in response to bird weight, **(J)** kill latency in response to hunger level.

Supplementary Figure 3 | Approach latency **(A)**, attack probability **(B)**, attack latency **(C)**, handling duration **(D)**, eating duration **(E)**, proportion eaten **(F)**, beak wiping **(G)**, and kill latency **(H)** in response to trial number.

Supplementary Figure 4 | Pupal weight in response to diet for male *A. plantaginis* raised on a diet with (AG) and without (ART) the addition of pyrrolizidine alkaloids (10% freeze dried *Senecio vulgaris*). Moths of the Wy genotype raised on the pyrrolizidine alkaloid diet were heavier compared to Wy moths raised on the artificial diet.

Supplementary Figure 5 | Bird water drinking (number of sips) increased in response to eating duration.

Supplementary Figure 6 | Moth activity in response to pyrrolizidine alkaloid taste. "no," moths that were not raised on a pyrrolizidine alkaloid diet; "yes," moths that were raised on a pyrrolizidine alkaloid diet. Moths raised on a pyrrolizidine alkaloid diet were less active.

Supplementary Table 1 | General definition, operative definition and unit of measure for each type of variable.

Supplementary Table 2 | Model selection using AICc for each response variable. Interactions and co-variates were selected to be included in the model and genotype replaced color morph if it improved the AICc score by more than Δ 2.

Supplementary Table 3 | Model summaries and follow up analyses for each response variable.

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Risk-Induced Foraging Behavior in a Free-Living Small Mammal Depends on the Interactive Effects of Habitat, Refuge Availability, and Predator Type

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Predators have a major influence on prey populations and broader ecosystem dynamics through both their consumptive and non-consumptive effects. Prey employ risk-induced trait responses such as shifts in habitat use or changes in foraging behavior in response to the presence of predators. Risk-induced changes in foraging depend upon both the predator community and the environmental context; however, the influence of these factors have rarely been concurrently examined in free-living animals. We investigated the interactive effects of habitat, refuge availability, and predator type on the foraging behavior of free-living white-footed mice, accounting for the abiotic factor moonlight. We used auditory calls of a local terrestrial cursorial predator and a local avian ambush predator to simulate predation risk in both a forest edge and a forest interior habitat, and measured the foraging of mice under different experimental refuge types. We found that, while mice had reduced foraging when exposed to predation risk, the degree of this response depended on an interaction among habitat, refuge use, and type of predator. Prey had the greatest reduction in foraging and used refuges the most when exposed to cursorial-hunting foxes at the open forest edge. The risk-induced reduction in foraging and the use of refuges was much weaker in the forest interior, but even here foxes elicited a greater response as compared to owls. Generally, foraging tended to decrease with increasing moonlight, but this was not significant. We suggest that it is the temporal nature of cursorial vs. ambush predators in our system that drives such effects as opposed to their hunting mode, and that prey responses to temporal hotspots of risk need further examination. Generally, our results show that wild small-mammal prey species have variable responses to predation risk depending on the environmental context in which risk occurs.

Keywords: predation risk effects, risk-induced trait responses, non-consumptive effects (NCEs), giving-up-density (GUD), white-footed mice, *Peromyscus leucopus*

INTRODUCTION

Predators have a major influence on prey populations and broader ecosystem dynamics through both their consumptive (i.e., killing; Sih et al., 1985; Krebs et al., 2001) and non-consumptive effects (Lima and Dill, 1990; Werner and Peacor, 2003; Sheriff et al., 2020b). For example, the mere presence of predators can result in risk-induced trait responses in prey behavior, morphology, and physiology (Lima, 1998; Peacor et al., 2020). These responses can scale up to alter prey fitness (Sheriff et al., 2009; Zanette et al., 2011), with potential effects on prey population size and community dynamics (Sheriff et al., 2020b). These changes have been observed across a wide range of taxa, including mammals (e.g., Creel et al., 2007; Sheriff et al., 2011; Cherry et al., 2016), birds (e.g., Fontaine and Martin, 2006), fish (e.g., McGhee et al., 2020), and invertebrates (e.g., Hermann and Thaler, 2014). Importantly, these changes may depend upon the environment in which they are elicited. Thus, understanding risk-induced effects in free-living prey under different contexts is an important part of understanding risk effects in natural systems (Peacor et al., 2020; Sheriff et al., 2020b).

One of the most well-studied risk-induced trait response in prey is a change in behavior (Lima and Dill, 1990; Lima, 1998). Many studies show a reduction in activity and a shift in habitat use corresponding to reduced foraging and increased refuge use, respectively, in response to predation risk (e.g., Werner et al., 1983; Heithaus and Dill, 2002; Ferrari et al., 2009). Sih (1984) showed that greater risk induces greater refuge use by prey. Longland and Price (1991) showed that various rodent species alter habitat use in response to increased predation risk, preferentially foraging in habitats with greater refuge availability. Orrock and Fletcher (2014) found that deer mice reduced their foraging in response to increased predation risk by re-introduced foxes; however, the reduction in foraging was highly dependent on the availability of shelter and moonlight. On nights with low moon illumination, mice foraged less in patches that had no shelter and more in patches that had shelter as fox abundance increased, but the effect was opposite during nights of low moon illumination. This may be because shelter provided overhead cover, allowing mice to avoid being detected by avian predators on nights with lower moon illumination but perhaps providing less of a benefit on nights with higher moon illumination, when the mice were more vulnerable to fox attacks (Orrock and Fletcher, 2014). These results suggest that the effect of predation risk on refuge use is highly dependent on other contextual factors, including moonlight illumination.

Predator type (e.g., terrestrial vs. avian, which in some cases may influence hunting mode) has also been shown to affect the risk-induced behavioral responses of prey (Preisser et al., 2007; Schmitz, 2008). For example, Embar et al. (2014) found that gerbils reduced their foraging more in response to the presence of owls than they did in response to the presence of snakes. Predator type has also been shown to influence microhabitat and refuge use. Kotler et al. (1991) found that gerbils forage more under the cover of bushes than they do in the open when exposed to cues that signify the presence of owls. However, gerbils exposed to cues that signify the presence of snakes preferentially forage in open microhabitats and avoid foraging under bushes (Kotler et al., 1993). Although its impact is highly context-dependent, predator hunting mode has also been shown to affect the behavior of prey, with sit-and-wait ambush predators generally expected to elicit stronger prey responses when compared to actively hunting predators (Preisser et al., 2007). This is likely because the attacks of ambush predators are more predictable in space and time than those of cursorial predators, causing prey to exhibit anti-predator behavior on a more constant basis.

Thus, risk-induced changes in foraging depend upon refuge availability, the predator community, and the environment in which these interactions occur. Although many studies have examined the interactive effects of some of these factors (e.g., Preisser et al., 2007; Wilson and Cooper, 2007; Miller et al., 2013), the influence of all three of these factors have not been examined concurrently in free-living animals. In addition, the effects of refuge availability and microhabitat on foraging behavior can be influenced by environmental characteristics, such as moon phase. Generally, mammals tend to forage more actively when there is lower moonlight intensity, presumably because these conditions make it less likely that they will be spotted by a predator (Prugh and Golden, 2014; Loggins et al., 2019). For example, Kotler et al. (2010) showed that gerbils forage less and display more vigilant behavior during brighter moon phases. Also, white-footed mice have been shown to only forage under a certain threshold of ground-level moonlight intensity (Guiden and Orrock, 2019).

Here, we investigate the combined effects of refuge availability, predator type (which also influences predator hunting mode), and habitat on prey foraging behavior in free-living white-footed mice, *Peromyscus leucopus*, while accounting for the environmental covariate of moon illumination. White-footed mice are an ideal species to investigate the combined effects of contextual factors on risk-induced behavioral responses; they are preyed on by both avian and terrestrial predators, and studies have shown reduced foraging responses to both predator types (e.g., Orrock and Fletcher, 2014; Giordano et al., submitted[1]). They also occupy a wide range of habitats (which may be more or less risky depending on their predators; Witmer and Moulton, 2012). Using auditory playbacks of either avian (ambush sit-and-wait hunting) or mammalian (cursorial chase hunting) predators, we measured the foraging behavior of white-footed mice with access to various refuge types at the forest edge (open habitat, 10 m from a distinct forest edge) and forest interior (closed canopy with dense understory, 70 m from the same distinct forest edge). We tested the hypothesis that the risk-induce trait responses of prey can vary according to the risk perceived during a foraging attempt and the environment in which the attempt occurs. We expected that mice exposed to predation risk would forage less overall and would forage more under refuges compared to in patches without refuge. Furthermore, we expected that mice would forage more in the forest interior compared to at the forest edge. We predicted that:

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[1] Giordano, A., Hunninck, L., and Sheriff, M. (2021). *Prey Responses to Predation Risk Under Chronic Road Noise* (Manuscript submitted for publication).

1. Mice would forage the most under the refuge that provided the greatest protection from the specific predator's hunting mode. Specifically, we predicted that mice would forage most under the refuge that provided vertical cover (i.e., closed top but open sides) when exposed to owl playbacks, but forage most under the refuge that provided horizontal cover (i.e., closed sides but open top) when exposed to mammalian playbacks. In both cases, we predicted that mice would forage the least in habitats without refuges due to higher perceived predation risk in open habitats regardless of predator type.
2. Mice would have the lowest foraging effort in response to the predator that posed the most risk in a given habitat. As such, we predicted that mice at the forest edge would reduce foraging the most in response to owl playbacks (given the more open habitat), and that mice in the forest interior would reduce foraging the most in response to mammalian playbacks (given the denser understory protecting them from owls but not as much from foxes, who are shorter than the understory canopy).
3. Alternatively, prey may reduce foraging the most to owls regardless of habitat or refuge, given that they are sit-and-wait ambush predators, which are generally predicted to elicit a greater anti-predator response (Preisser et al., 2007; Schmitz, 2008).

MATERIALS AND METHODS

Study Location and Study Species

This study occurred within a forested tract of land 2 km long and 0.65 km wide surrounded by low density rural housing and farms in Westport, Massachusetts from August 8th to September 20th, 2019. The study area was dominated by oak trees (*Quercus* sp.) and holly trees (*Ilex* sp.) with a thick understory of *Vaccinum* sp. The edge habitat also included various willow species. Deer, coyotes, bobcats, foxes, fishers, rabbits and small mammals are readily seen or heard. Based on observations from trapping and camera footage, the small mammal community in the area is dominated by white-footed mice (*P. leucopus*) and chipmunks (*Tamias striatus*) and includes few other species. Barred owls (*Strix varia*) and red-tailed hawks (*Buteo jamaicensis*) are the prevalent avian predators in the area, with great horned owls (*Bubo virginianus*) and eastern screech owls (*Megascops asio*) also present at a lower density. The study design aimed to primarily target white-footed deer mice (*P. leucopus*), a nocturnal small mammal species. To attempt to exclude diurnal species such as chipmunks (*T. striatus*), data was only collected between the hours of 7 pm and 7 am. To exclude larger seed-eating species, mice could only gain access to seeds through small openings in the feeding trays. Although footprints characteristic of white-footed mice were observed in trays over the course of the experiment, there were no footprints that suggested the presence of other species.

Predation Risk Manipulation

To manipulate predation risk, we used auditory calls/playbacks of local predators and non-predators (Zanette et al., 2011; Suraci et al., 2016). A speaker system was positioned approximately 40 m into the forest, such that it was equidistant from foraging trays at the forest edge (10 m into the forest) and forest interior (70 m into the forest) habitats. The speaker system played calls for 40% of the time between 7 pm and 7 am at approximately 70 dB (at the source of sound), which attenuated to approximately 68 dB at the foraging trays. In addition, given that predators do not call while they are attacking, the speaker system included motion detecting infrared sensors, such that any motion within 1 m of the foraging trays triggered the speaker system to stop playbacks for 30 s. Playbacks included: a barred owl (*S. varia*) avian predator treatment; a red fox (*Vulpes vulpes*) mammalian predator treatment; and a spring peeper (*Pseudacris crucifer*) noise control treatment.

Giving-Up Density Measurements

We measured foraging behavior using the giving-up density (GUD) technique (Brown et al., 1988). The GUD technique involves setting out trays filled with a set amount of food homogenously distributed in an inedible substrate like sand or soil. The amount of food that remains after a forager gives up on feeding from the tray is measured as its GUD. This indicates the point at which the benefits of foraging are equal to or less than the costs of continuing to forage, which include metabolic costs, missed-opportunity costs, and the risk of predation (Brown, 1988; Brown et al., 1997).

We placed three foraging trays (32 cm × 11 cm × 17.5 cm) 1 m apart both 10 m (forest edge) and 70 m (forest interior) into the forest (i.e., from the forest edge). In each group of three trays, two were enclosed by either a table-like refuge (30 cm tall with a 60 × 60 cm covered top and open sides) or an open-topped box refuge (30 cm tall with 60 cm wide closed sides and an open top, walls sloped inward at 30 degrees; mice could easily access the foraging tray under the sloping walls which remained at least a few inches above the substrate). One foraging tray was left in a patch without a refuge. Refuges were constructed with plywood and painted brown with non-toxic, non-scented paint. Two infrared sensors per group of foraging trays were embedded in 1 m tall PVC pipe stands, which were also painted brown. These sensors detected small mammal movement within 1 m of any foraging tray and triggered the speaker system to stop playbacks for 30 s. Each foraging tray contained 0.5 L sand and 2.5 g seeds. All trays were placed in the forest at 7 pm and collected at 7 am. Once collected, seeds were sifted out of the sand. The seeds were then dried at 60°C for 2 h to remove moisture accumulated in them from ambient

TABLE 1 | Model output showing the effects of habitat, refuge, risk treatment, moon illumination, and day on the amount of food eaten by white-footed mice.

Predictor	SS	MSS	Num. Df	Den. Df	*F*-value	*p*-value
Habitat – Forest edge	34.37	34.37	1	87.45	140.28	<0.001
Treatment – Control	1.63	0.81	2	14.87	3.32	0.064
Refuge – Enclosed	2.10	2.10	1	86.43	8.59	0.004
Moon	0.67	0.67	1	14.19	2.73	0.121
Day – Day 1	0.03	0.03	1	14.40	0.14	0.715
Habitat × Treatment	2.74	1.37	2	87.39	5.59	0.005
Habitat × Refuge	0.13	0.13	1	86.43	0.52	0.474
Treatment × Refuge	1.07	0.54	2	86.43	2.19	0.119
Habitat × Treatment × Refuge	0.19	0.10	2	86.43	0.39	0.680

Results obtained through a Type III ANOVA with Satterthwaite's method. SS, Sum of Squares; MSS, Mean sum of squares; Num. Df, Numerator Degrees of Freedom; Den. Df, Denominator Degrees of Freedom.

humidity. Seeds were then weighed to estimate the GUD for each tray on each night.

Study Design

The speaker system described above was set up at the study site. Each auditory call treatment was played for 2 consecutive nights with 1–2 nights of silence (no playbacks) in between. Separate trays were deployed on each of these consecutive nights for the purpose of collecting GUDs. There were six trays deployed on each of the two playback nights: three at the forest interior and three at the forest edge. At each location, these trays were spaced 1 m apart so that each of them was under a different refuge condition (1 under a table-like refuge, 1 under a box-like refuge, and 1 in a non-refuge patch). This resulted in the generation of 6 GUDs per night, and 12 GUDs per treatment round. This procedure was repeated for 10 rounds, resulting in 120 GUDs total (3 of which were discarded because they were improperly dried before weighing). The position of the refuge types was randomized between rounds. We semi-randomized the order of the treatments to control for potential variation due to the timing of the experiment. The treatments were run in the order of: Control (C), Owl (O), Control (C), Fox (F), O, C, F, O, F, C. No experiments were conducted concurrent with rainfall.

Statistical Analysis

GUDs ($n = 117$) were analyzed using a linear mixed regression model examining the interactive effects of predator treatment (control/fox/owl), habitat (forest interior/forest edge), and refuge (enclosed/non-refuge patch) on mice foraging behavior, thereby controlling for the block design of our study. The degrees of freedom (here: denominator-degrees-of-freedom) were adjusted using the Satterthwaite method. We found no statistical difference between the two different types of enclosed refuges ($t = -0.179$, $p = 0.858$), so we grouped them. To simplify the presentation of the data, each GUD was converted into the amount of food eaten by subtracting the GUD from the initial weight of the seed (2.5 g seed – GUD). These values were then log-transformed. One observation was excluded to obtain normality of model residuals. We included moon illumination as a covariate in the regression model (R package *lunar* v.0.1-04). Moon illumination was calculated from moon phase, which was determined by the date of data collection. A full moon was recorded as 100% illumination, whereas a new moon was recorded as 0% illumination. Day was also included as a fixed effect to control for potential differences between consecutive experiment days within experiments. Day could not be included as a random effect, as this variable only has two levels and the recommended minimum levels of a factor to be included as a random effect is 5 (Zuur et al., 2009). Experiment (defined as each set of 2 consecutive days in one replicate; $N = 10$) was included as a random effect to control for environmental differences between replicates, such as ambient temperature or seasonal change. *Post hoc* tests were conducted with the *emmeans* package (v.1.5.2-1), using the Holm-Bonferroni *p*-value correction for multiple testing. Model residuals were visually checked to conform to the assumption of homogeneity of variance; no patterns were observed in model residuals. All data were analyzed using R v.4.0.3 in RStudio v.1.3.1093. An all-pair comparison table of the ANOVA is presented in **Supplementary Table 1**.

RESULTS

We found a significant effect of habitat and refuge (**Table 1**). Overall, mice ate 29% less food at the forest edge (Mean GUD = 0.936 g) compared to the forest interior (Mean GUD = 0.286 g; $t_{117,\ 1} = 11.834$, $p < 0.0001$). Mice ate 7% less food at trays in patches without refuges (Mean GUD = 0.598 g) compared to those enclosed in refuges (Mean GUD = 0.448 g; $t_{117,\ 1} = -2.931$, $p = 0.004$; **Figure 1**). Moon illumination and day were not significant (**Table 1**), although food eaten tended to decrease with increasing moon illumination (**Figure 2**). While treatment only tended to be significant ($p = 0.064$), the interaction between treatment and habitat was significant (**Table 1**). The fixed effects explained 48% of the variation in GUDs (marginal R^2; R package MuMIn v.1.43.17; Nakagawa et al., 2017) while the full model explained 76% of the variation in GUDs (conditional R^2).

When we compared different predator effects within a single habitat, we found that mice at the forest edge reduced foraging by 51% when exposed to fox playbacks (Mean GUD = 1.518 g; $t_{117,\ 2} = -3.398$, $p = 0.009$) and 31% when exposed to owl playbacks (Mean GUD = 1.106 g; $t_{117,\ 2} = -2.366$, $p = 0.0553$;

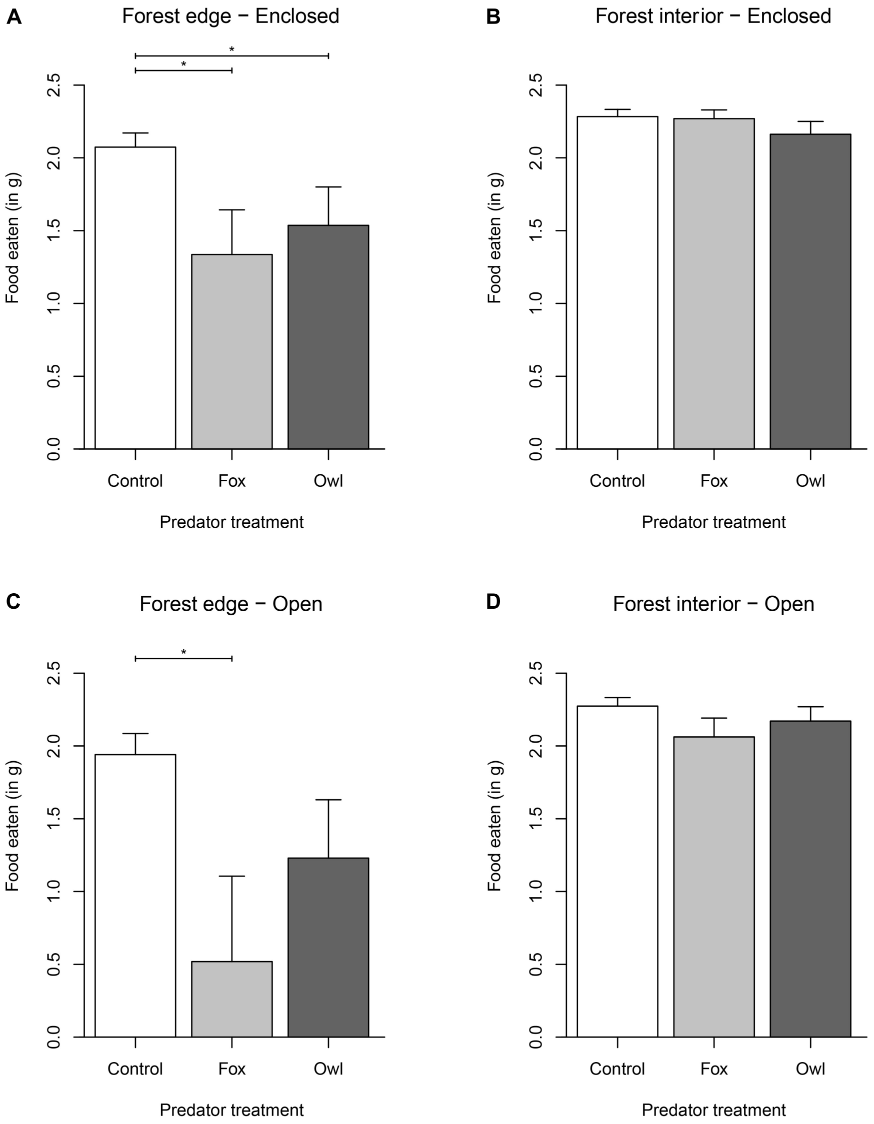

FIGURE 1 | Effect of habitat [forest edge **(A–C)** vs. forest interior **(B–D)**], refuge [open **(C,D)** vs. enclosed **(A,B)**], and treatment [control (white) vs. fox (light gray) vs. owl (dark gray)] on the amount of food eaten (2.5 g – GUD) by free-living white-footed mice. Upper 95% confidence interval is shown as whiskers. Asterisks indicate significant differences ($p < 0.05$) among groups.

Figure 1) compared to the control (Mean GUD = 0.488 g). However, in the forest interior, predator playbacks of either fox or owl had no effect on foraging. Mice ate 20% less food at trays in refuge-less patches at the forest edge (Mean GUD = 1.121 g) in comparison to the amount they ate in enclosed trays at the forest edge (Mean GUD = 0.782 g; $t_{117,1} = -2.529$, $p = 0.0132$), but there was no effect of refuge in the forest interior.

DISCUSSION

In this study, we examined foraging behavior in free-living white-footed mice to test the hypothesis that anti-predator responses depend on the environmental context under which they occur. Our results supported this hypothesis; in general, mice reduced foraging when exposed to predation risk, but the degree of this response differed based on habitat, refuge use, and predator type. Moonlight tended to reduce small mammal foraging, but not significantly as has been shown in other studies (Guiden and Orrock, 2019; Loggins et al., 2019; **Figure 2**). Overall, our results support the hypothesis that risk-induced trait responses of prey depend upon the context in which they occur.

Habitat has been shown to be a major factor influencing prey responses to predation risk. Studies have shown that prey will forage less in risky habitats and that, when exposed to

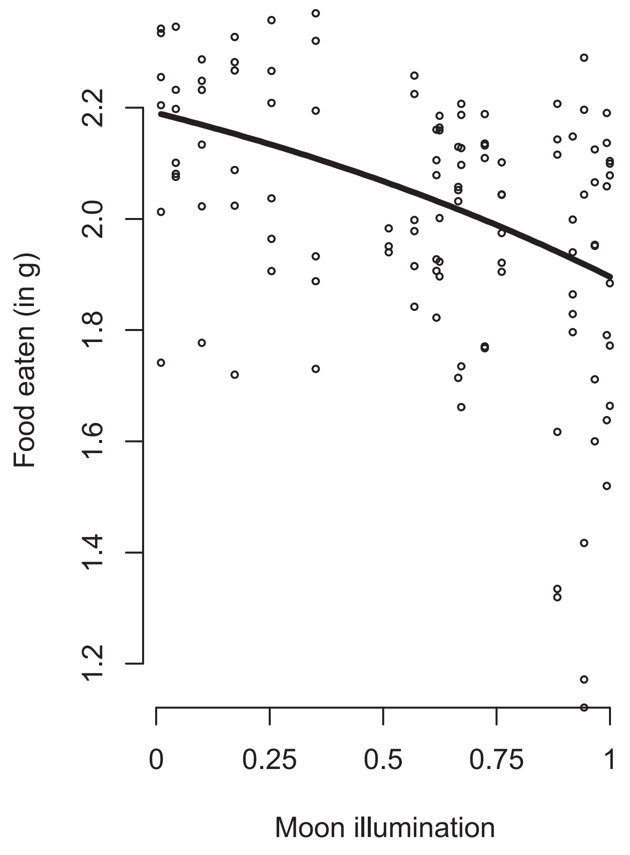

FIGURE 2 | The effect of relative moon illumination (black line; 0 at new moon, 1 at full moon) on the amount of food eaten by free-living white-footed mice. 95% Confidence interval shown as gray area. Dots represent datapoints. Moon illumination was based on date of data collection and accessed through the R package lunar.

predation risk, they will move from riskier to safer habitats (Lima and Dill, 1990). For example, granivorous rodents have been shown to preferentially forage in microhabitats that provide more cover (Brown, 1988). Similar effects have been found across taxa. In insects, for example, Kohler and McPeek (1989) found that *baetis* larvae avoided foraging in the top level of substrate (where predation risk is higher) when predator cues were present. Here, we found that mice ate significantly less at the forest edge as compared to the forest interior, regardless of refuge use or what predator type they were exposed to. In our study area there was a very distinct forest edge, delineated by sparse shrub and meadow habitat. Within the forest at the edge there was also less understory cover. Overall, this likely results in a riskier habitat with far less natural cover. Our finding that mice forage less in the risky habitat (forest edge) as compared to the safer habitat (forest interior) therefore corroborates previous research on prey anti-predator responses.

We also found that mice ate significantly more under refuges than they ate in patches without refuge. However, this result was confounded by habitat type—mice ate more under refuges only in the riskier forest edge habitat, while, in the safer forest interior habitat, there was no significant difference between the amount of food eaten under refuges in comparison to the amount of food eaten in patches without refuge. This is likely explained by the dense understory cover in the forest interior, which may have rendered the additional cover provided by refuges redundant. A similar effect has been found in some previous studies. Orrock et al. (2004), for example, showed that deer mice used refuges significantly more when exposed to predation risk. However, this effect was confounded by various aspects of environmental context which indirectly affect predation risk, such as foraging microhabitat, moon illumination, and precipitation. Donelan et al. (2017) found that snails increased the use of refuge and avoided foraging in open habitats in response to increases in predation risk. Thus, while studies have shown that prey utilize refuges in response to predation risk (Werner et al., 1983), the importance of such refuges in prey risk responses may be a consequence of the general habitat structure, increasing in importance as the habitat becomes riskier.

We also found that predator type, associated with different hunting modes, influenced prey foraging, and that this effect was also confounded by habitat type. Although it is generally assumed that sit-and-wait, ambush predators have a greater effect on prey, we found the opposite. At the forest edge, where we predicted owls to have the largest effect, we found mice reduced foraging by 51% in response to the fox treatment, whereas they only reduced foraging by 31% in response to the owl treatment. We found no effect of predation risk in the safer forest interior—however, fox playbacks overall (across both habitat types) reduced mouse foraging by much more than owls. We also found mice had a 3× increase in foraging under refuges when they were exposed to foxes at the forest edge, but refuges had little effect on mouse foraging when they were exposed to owls at the forest edge. This finding corroborates previous studies which have found differential prey responses to predation risk depending on predator identity. For example, Preisser et al. (2007) found that some predators elicit stronger non-consumptive effects than others, and Embar et al. (2014) found that prey use refuge differently in the presence of owls when compared to their refuge use in the presence of snakes.

Overall, the cursorial predator treatment clearly had a larger effect as compared to the ambush predator treatment. The greater effect of foxes (a terrestrial cursorial predator) as compared to owls (an avian ambush predator) may be driven by the possibility that foxes consume more mice as compared to owls and, thus, induce greater anti-predator responses. Our experimental design was unable to test whether foxes or owls are more lethal. However, it may not be as simple as the predator with the greater lethality inducing the greater response. Mice may have greater relative increases in proportional fitness with greater responses to foxes as compared to owls (Sheriff et al., 2020a); i.e., increasing the response to owls may provide no additional fitness benefits, whereas increasing the response to foxes does provide additional fitness benefits. Similarly, the greater response may be influenced by local densities and familiarity with each predator. Although we do not know the exact densities of either predator, prevalent signs of both predators have been seen and heard in the area.

Differences in predator hunting mode may also contribute to the differences in the responses of mice. Most studies that have examined the effects of predator hunting mode compare systems where ambush predators set up hotspots of high predation risk (creating a landscape of fear; Laundré et al., 2001; Gaynor et al., 2019; Smith et al., 2019) and prey are highly mobile. In such systems, prey can make spatiotemporal decisions across the landscape on where and when to forage. This contrasts systems where cursorial predators actively pursue prey (and as such do not set up hotspots of risk across the landscape in the same manner as ambush predators; Sih et al., 1998; Schmitz, 2008). In our system, owls often hunt in a given area over a certain number of nights, an area that is as large if not larger than a mouse's entire home range. Thus, while owls are ambush style hunters swooping down to attack their prey unaware, owls do not set areas of localized hotspots that mice can avoid. Rather, a mouse's entire home range becomes very risky when an owl is nearby. In contrast, foxes' cursorial, search-and-pursue hunting style may result in them creating acute temporal periods (minutes to hours) of high risk when they are within a small mammal's home range; i.e., we suggest that hearing a fox may indicate an acute localized threat, but hearing an owl may indicate a more constant unlocalized threat. Thus, small mammals may respond to predators as predicted by the risk allocation hypothesis, which predicts stronger risk responses during acute high-risk scenarios and reduced risk responses as those high-risk scenarios become prolonged (Lima and Bednekoff, 1999), rather than as predicted by predator-hunting-mode hypotheses (Schmitz, 2008).

In our study, we examined how prey responses were influenced by the interactive effects of habitat, refuge availability, and predator type. We found that, while mice generally foraged less when exposed to predation risk, the degree of this response depended upon an interaction among habitat, refuge use, and type of predator. Prey foraged the least and used refuges the most when exposed to cursorial-hunting foxes at the open forest edge. The risk-induced reduction in foraging and the preferential use of refuges was much weaker in the forest interior. Interestingly, we found that cursorial hunting foxes elicited a stronger effect than ambush hunting owls regardless of habitat type or refuge availability. We suggest that the temporal nature of cursorial predators (i.e., an acute localized threat as they move through an area) as compared to ambush predators in our system drives these results. More work needs to be done examining the effects of temporal hotspots on prey risk responses and what role these may play in hypotheses related to the landscape of fear. Together, our results offer support for the risk allocation hypothesis, and show that wild small mammal prey species can have considerably different foraging behavior in response to predation risk depending upon environmental context. This study, thus, addresses an important yet understudied aspect of predator-prey interactions—the effects of different interacting environmental factors on prey responses to predation risk.

Our findings have important implications for the way prey respond to predation risk in varying contexts. Our results may be explained by behavioral changes that individual mice express in response to cues of predation risk. However, it is possible that these findings could result from another mechanism. For example, individual mice may consistently occupy different microhabitats (i.e., niche specialization) rather than foraging both at the forest edge and at the forest interior (Schirmer et al., 2019). If this is the case, it is possible that habitat use may be correlated to foraging behavior (trait-related habitat matching; Edelaar et al., 2008). Some individuals may preferentially forage in open habitats and take less risks when foraging, while others may preferentially forage in closed habitats and take more risks when foraging. Therefore, although our results may be explained by flexibility in the behavior of individual mice, this conclusion would require further research with the added element of tracking the identities of individuals.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the IACUC, University of Massachusetts Dartmouth.

AUTHOR CONTRIBUTIONS

MS and VK designed the study. VK carried out the study. LH and VK did the statistical analysis. VK wrote the manuscript with contributions from LH and MS. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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ORIGINAL RESEARCH
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Pre- and Postnatal Predator Cues Shape Offspring Anti-predatory Behavior Similarly in the Bank Vole

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Prey animals can assess the risks predators present in different ways. For example, direct cues produced by predators can be used, but also signals produced by prey conspecifics that have engaged in non-lethal predator-prey interactions. These non-lethal interactions can thereby affect the physiology, behavior, and survival of prey individuals, and may affect offspring performance through maternal effects. We investigated how timing of exposure to predation-related cues during early development affects offspring behavior after weaning. Females in the laboratory were exposed during pregnancy or lactation to one of three odor treatments: (1) predator odor (PO) originating from their most common predator, the least weasel, (2) odor produced by predator-exposed conspecifics, which we call conspecific alarm cue (CAC), or (3) control odor (C). We monitored postnatal pup growth, and we quantified foraging and exploratory behaviors of 4-week-old pups following exposure of their mothers to each of the three odour treatments. Exposure to odors associated with predation risk during development affected the offspring behavior, but the timing of exposure, i.e., pre- vs. postnatally, had only a weak effect. The two non-control odors led to different behavioral changes: an attraction to CAC and an avoidance of PO. Additionally, pup growth was affected by an interaction between litter size and maternal treatment, again regardless of timing. Pups from the CAC maternal treatment grew faster in larger litters; pups from the PO maternal treatment tended to grow faster in smaller litters. Thus, in rodents, offspring growth and behavior are seemingly influenced differently by the type of predation risk perceived by their mothers.

Keywords: alarm pheromone, conspecific alarm cue, predation risk, odor cues, cross-generational effects, rodents

INTRODUCTION

Lethal and non-lethal effects of predators influence the life history of prey (Sih, 1994; Ylönen and Ronkainen, 1994; Werner and Peacor, 2003; Nelson et al., 2004; Ylönen and Brown, 2007; Sheriff et al., 2009). Over the last two decades, increasing attention has been paid to non-lethal effects that relate to fear in the face of high predation risk. Overall, this research suggests that non-lethal effects of predators mediated by fear in prey can have population level effects that are similar in magnitude to the direct effects of predators killing prey (Schmitz et al., 1997; Nelson et al., 2004; Preisser et al., 2005; Pangle et al., 2007; Creel and Christianson, 2008).

Predation risk can be detected by prey in several ways: visually, acoustically, olfactorily, or tactilely. Via olfaction, prey can either detect odors produced directly by a predator (predator odor, PO) or odors produced by conspecifics that engaged in non-lethal interactions with predators (conspecific alarm cue, CAC) (Haapakoski et al., 2018). Conspecific alarm cues are also sometimes referred to as "alarm secretions" (Brand et al., 1989) or "alarm pheromones" (Breed et al., 2004; Gomes et al., 2013). Irrespective of terminology, mechanisms and consequences associated with CAC production and release are well-documented (Gomes et al., 2013), including in anthozoa (Howe and Sheikh, 1975), insects (Bowers et al., 1972; Beale et al., 2006), amphibians (Hempel et al., 2009), and mammals (Boissy et al., 1998; Gutiérrez-García et al., 2007). In several social species, CAC serve as signals to protect at-risk families, colonies, or other groups (Breed et al., 2004; Kiyokawa et al., 2004; Gomes et al., 2013). While the structure of CAC remains unresolved for most mammals, it has been identified in, for example, aphids (Bowers et al., 1972), sea anemones (Howe and Sheikh, 1975), and several insects (Heath and Landolt, 1988; Kuwahara et al., 1989). Work in laboratory rodents has led to analyses of CAC from Wistar rats (Inagaki et al., 2014) and C57BL/6J and OMP-GFP mice (Brechbühl et al., 2013).

After encountering PO or CAC, prey are expected to adjust behaviorally in ways that minimize their chances of being preyed upon. Such changes include increased vigilance (Périquet et al., 2012) and freezing (Wallace and Rosen, 2000; Sundell and Ylönen, 2004), avoidance of risky areas (Kikusui et al., 2001), and altered space use and activity peaks (Jędrzejewska and Jędrzejewski, 1990; Jędrzejewski and Jędrzejewska, 1990; Sundell et al., 2008). In addition to the foraging-related repercussions of the behavioral changes listed above (Brown, 1999), decreased foraging can also be a direct result (Ylönen, 1989; Sundell and Ylönen, 2004; Bleicher et al., 2018). Ultimately, behavioral adjustments that enhance short-term survival of predators may lead to trade-offs with future reproduction (Ylönen and Ronkainen, 1994; Fuelling and Halle, 2004; Love and Williams, 2008; Haapakoski et al., 2012; Sheriff and Love, 2013; Voznessenskaya, 2014).

Stress responses, and in particular corticosterone production, represent another mechanism through which PO or CAC can affect prey reproduction and fitness. Exposure to either type of olfactory cue of predation risk can be transmitted from mother to offspring, influencing offspring growth (Berghänel et al., 2017), development (Hayward and Wingfield, 2004), behavior (St-Cyr et al., 2017; Sievert et al., 2020), and survival (Chin et al., 2009). A mother and fetus are connected prenatally via the placenta and postnatally via lactation; both serve as transmission routes for hormones, and thus information about the environment (Sullivan et al., 2011; Sheriff et al., 2017; Kuijper and Johnstone, 2018). Since maternal care can play a role in programming the hypothalamic-pituitary-adrenal axis (HPA-axis) of offspring, the stress responsiveness of offspring may itself be altered (Liu, 1997). Mothers might also communicate information about predation risk and stress via odors they produce (Koyama et al., 2015).

While predation risk in small mammals can affect the behavior and future reproduction of offspring (Ylönen and Ronkainen, 1994; Ylönen, 2001; Bestion et al., 2014; Sheriff et al., 2015), the role of the *timing of exposure* to predation risk remains unclear. Prenatal and postnatal exposure, which are governed by different mechanisms, might differ in their consequences. In this study, we exposed mothers to predator odor, CAC, or a control cue during pregnancy or during lactation. We observed the growth rate of their respective offspring and, once weaned, their offspring were tested in an experimental set-up where they encountered these odors again. Exploratory behavior as well as foraging behavior was then observed. We made several specific predictions. First, we predicted that the offspring of mothers exposed prenatally to either PO and CAC would grow more slowly than those of mothers exposed postnatally or to mothers exposed to neither, due to either increased stress metabolites *in utero* or a reduced maternal investment as an reaction to the perceived increased predation pressure (Bian et al., 2005; Dunn et al., 2010; Coslovsky and Richner, 2011). Second, we predicted that the offspring of mothers exposed postnatally to either PO or CAC would show greater avoidance of the odor to which their mother was exposed compared to offspring exposed prenatally (Dias and Ressler, 2014). This assumes that the odors are carried into the nest in the fur of the mother and the combination of the odor and potential changes in maternal care and/or increased stress hormone levels in the milk will trigger aversive behavior. Third, we predicted that offspring would forage less in the presence of PO and CAC, regardless of their mother's treatment (Brown, 1988; Sievert et al., 2019). Fourth, we predicted increased latencies to investigate foraging options, reduced time spent in foraging chambers, and fewer foraging chamber visits in chambers with PO or CAC compared to the control chamber (Apfelbach et al., 2015; Sievert and Laska, 2016; Parsons et al., 2018; Sievert et al., 2019). Prediction three and four assume that PO and CAC carries information about an increased risk and therefore these compartments will be largely avoided and not used for foraging (Brown, 1988).

MATERIALS AND METHODS

Study Species

The bank vole (*Myodes glareolus*) is a small granivorous rodent that is common in forested areas in boreal and temperate regions from Europe to Siberia. It is often used as a model species that is easy to keep and breed in captivity. The bank vole is killed by various predators, including the least weasel (*Mustela nivalis nivalis*). Vole populations are known to cycle multi-annually in Scandinavia, and the least weasel and other specialist predators play an important role in driving these cycles (Hanski et al., 2001). In central Finland, bank vole breed from April to September. During this time, female bank voles are strictly territorial, and each male's territory overlaps with several female territories (Bujalska, 1973). After a gestation of about 20 days, 3–6 pups are born. Pups mature after 30 days.

The predator-prey interaction between boreal voles and their specialist predators has been well studied (Korpimäki et al., 1991; Norrdahl and Korpimäki, 1995; Ylönen et al., 2019). Least weasels and bank voles share the same habitat. The weasel is able to hunt in tunnels and burrows throughout the year, due to its small size, leaving only a few areas safe for voles (Norrdahl and Korpimäki, 1995, 2000; Sundell et al., 2008). Like all mustelids, the least weasel uses strong odors for intraspecific communication (Brinck et al., 1983), giving the prey a cue to detect the predator's presence.

Experimental Animals

The experiments were conducted at the Konnevesi Research Station in Central Finland (62°37′ N, 26°20′ E). Bank voles were trapped from the wild and brought into captivity, where they were allowed to acclimatize for 3 weeks before the experiment commenced. All individuals were ear tagged for identification (#1005-1L1, National Band and Tag Company, Newport, Kentucky, United States). In the laboratory, the voles are kept in climate- and light-controlled (16L:8D daily cycle) husbandry rooms. Voles were housed individually in 42 × 26 × 15 cm transparent cages with wire mesh lids and wood shavings and hay as bedding, and they were provided with *ad libitum* water and food. Males and female voles were maintained in the same room.

Weasels for the odor treatment were housed individually in 60 × 160 × 60 cm cages in an outdoor shelter. Each cage had a nest box and wood shaving and hay as bedding. During the experiment, weasels were fed only with dead bank voles.

Experimental Design

Voles were weighed and divided randomly into three treatments: control (C), CAC and predator odor (PO). These three groups were then divided into prenatal (early) and postnatal (late) treatment groups, so that the mass distribution was similar across treatments (mean ± standard deviation: C early 17.7 g ± 4 g; C late 17.4 ± 3.2 g; CAC early 17.8 g ± 4.1 g; CAC late 17.6 g ± 3.9 g; PO early 17.2 g ± 3.4 g; PO late 16.7 g ± 4.2 g). The prenatal and postnatal control groups each consisted of 40 individuals; the other four treatment groups each consisted of 60 individuals. In all groups, the sex ratio was 50:50. Voles were randomly paired within treatment groups for mating; sibling couples were avoided. To allow for reproduction, the couples were kept in the same cage for 7 days.

During the treatment phase, females were exposed to one of the three odor treatments. The control treatment consisted of clean wood shavings. The CAC treatment consisted of wood shavings used by predator-exposed male voles. Specifically, four males that were not otherwise used in the experiment were placed individually in a weasel cage every weekday (Monday to Friday) for 3 min per day. During this exposure, the weasel was unable to actually attack the vole. The bedding from the cages of the four exposed voles was collected each day after exposure. Males were used for CAC production to avoid possible confounding effects of estrus. The PO treatment consisted of weasel bedding materials, including feces.

Female voles were exposed to the short bursts of the treatments to mimic encountering the odors outside the nest, for example during foraging trips, both pre- and postnatally. Females in the prenatal group were treated from mating until parturition (i.e., for 18–25 days depending on the timing of parturition); females in the postnatal group were treated from parturition until the pups were 21 days old. All the females received the treatment following the same protocol: three times per week (Monday, Wednesday, and Friday) for 3 min per session, females were placed individually in a small cage with a separate compartment holding the treatment materials (**Supplementary Figure 1**). The pups remained in the home cage during the treatments. The separation from the mothers took place in all six treatment groups to exclude it as a confounding factor.

Eighteen days after initiating mating, females were checked for signs of pregnancy, and the cages were checked twice a day (in the morning and in the evening) for the presence of pups. On the seventh day after birth, pups were toe marked for individual recognition. Pups were weighed three times a week until the 21 days of age. During this same 21 day period, the mothers in the postnatal group received the treatment. In total, 113 experimental pups were produced in 29 litters across the three treatment groups. After 21 days, the pups were separated, ear tagged, and placed in individual cages.

One week after separation, pup behavior was assayed in a foraging choice arena with three arms (**Supplementary Figure 2**). Four hours before being tested, pups had their food removed to incentivize foraging in the novel environment. The test arena was used to measure giving up density, GUD, as an indicator of foraging effort (Brown, 1988). The test arena was Y-shaped with one central compartment and three surrounding boxes. Each of the surrounding boxes contained one of the treatment materials (control (C) bedding, CAC bedding, or predator odor (PO) bedding). Each of the three boxes also contained a GUD tray with 1 liter sand mixed with 3.85 g of millet seeds. The experiment started by releasing a pup in the central compartment. The orientation of release was consistent over the different trials, but the orientation of the treatment boxes varied to avoid spatial bias. All trials, which were recorded from a birds-eye-view with Go Pro cameras, lasted 3 h. Upon trial completion, the remaining seeds were collected and quantified and GUD was determined for each treatment box. The sand from GUD boxes was sieved to collect the remaining millet. The sand was dried and aired for at least 5 days before it was reused in the experiments in order to minimize odor contaminations. To control for variation in moisture content, the millet was dried for 12 h at 30°C and then equilibrated to ambient indoor humidity for 24 h before weighing.

Data Analyses

A single observer (KB) analyzed all videos by recording (1) the time intervals the voles spent in each GUD tray with the millet seeds, (2) the latency to enter each treatment box, (3) and the latency to enter each GUD tray. A vole was defined as being in a treatment box upon first sight of movement in the box and as being in a GUD tray when wholly in the tray, ignoring its tail. We used the latencies to each GUD tray to determine the first choice (C, CAC or PO GUD tray) of each vole, and we counted the number of visits to each treatment box.

It was not possible to record data blind because the three different treatments (C, CAC, and PO) are visually and olfactorily easily distinguishable.

Statistical analyses were conducted using R version 3.5.1 (R Core Team, 2020). We used linear mixed models (Bates et al., 2015) to assess differences among treatments in terms of giving up density (GUD), foraging speed (grams foraged per second), time spent in boxes, number of box visits, and the two latency variables (**Supplementary Appendix 1** provides all models and summary tables). To analyze first choice (i.e., non-numeric: C, CAC, or PO) data, we used the multinomial function (Venables and Ripley, 2002). Offspring body mass data were log-transformed prior to fitting linear mixed models. We used Akaike information criterion (AICc) for purposes of model selection: all models < 2 AICc units away from the best model were averaged by AICc weight to determine model estimates (Barton, 2020). A Tukey HSD *post hoc* test was carried out for the number of offspring between the treatments. No other *post hoc* tests were used in the analyses. The fixed variables in the full starting model were maternal treatment, timing of the maternal treatment (i.e., prenatal or postnatal), box treatment, box entry latency, sex, and age on test day. The variables maternal treatment and box treatment were retained in all of the compared models. To account for repeated measures within individuals and for possible effects of the litter, the random effects pup-identity vs. mother-identity over pup-identity were fitted in a full starting model for each response variable and compared by AICc. The better performing random effect was then used in all the other models for a given response variable. In the case of significant interactions between timing and either maternal treatment or box treatment, data were split to further analyze the effect. The same was done with interactions between sex and any other categorical variable.

RESULTS

Birth Mass and Growth Rate

There were no differences in litter size between maternal treatments [$n = 35$ ($n_C = 9$, $n_{CAC} = 16$, $n_{PO} = 10$ and $n_{early} = 14$, $n_{late} = 21$), early vs. late $p = 0.680$, C vs. CAC $p = 0.664$, C vs. PO $p = 0.801$, **Table 1** and **Supplementary Table 1**]. An additional Tukey HSD *post hoc* test did not show any significant differences either ($p > 0.05$ for all comparisons). The treatments did not affect birth mass, but litter size did: each additional pup in a litter decreased the individual pup birth body mass by 0.07 g ($n = 141$, $p < 0.001$, weighted average, **Supplementary Table 2**).

TABLE 1 | Distribution of pups over the six different maternal treatments.

	Prenatal			*Postnatal*		
Treatment	***C***	***CAC***	***PO***	***C***	***CAC***	***PO***
Litters (n)	4	5	3	5	8	4
Pups (n)	13	20	13	26	25	16

Three prenatal groups: C, CAC and PO. Three postnatal groups: C, CAC, and PO. C, Control, CAC, Conspecific Alarm Cue, PO, Predator Odor.

Pup body mass was influenced by a three-way interaction between age in days, litter size, and maternal treatment, while the timing of the treatments (pre- or postnatal) had no significant effect on its own nor showed significant interactions with the treatments ($n = 35$ litters and $n = 1316$ observations, day*litter size*maternal CAC $p = 0.023$, day*litter size*maternal PO $p = 0.00048$, **Figure 1**, **Table 2**, **Supplementary Table 3**). A subset analysis showed that pups from the maternal C treatment from larger litters started at a disadvantage compared to smaller litters from this treatment, but this was compensated for over the first 21 days of growth (log values: day = 0.08, litter size = –0.03, day*litter size = 0.001, **Figure 1**). In contrast, pups from the maternal CAC treatment started heavier and showed slightly faster growth in larger litters (log values: day = 0.08, litter size = 0.04, day*litter size = 0.0005, **Figure 1**). In the maternal PO treatment, pups from larger litters were smaller at birth and grew more slowly than pups from smaller litters (log values: day = 0.08, litter size = –0.03, day*litter size = –0.002, **Figure 1**).

First Choice of the Treatment Box and the Giving Up Density Tray

Pups from CAC-treated mothers were more likely to enter first into the CAC-treated box compared to the control box (54% vs. 14%; $n = 113$, $p = 0.005$, weighted average, **Supplementary Table 4**). Similarly, pups from CAC-treated mothers were more likely to enter first into the CAC GUD tray compared to the control GUD tray (50% vs. 22%; $n = 113$, $p = 0.0194$, weighted average, **Supplementary Table 5**). No other treatment showed significant preferences. Treatment timing and sex did not have a measurable effect in either case.

Visits to the Treatment Boxes

Overall, pups visited the PO treatment box less frequently than the C treatment box (ca. 7 fewer visits, $n = 113$, $p < 0.001$, weighted average, **Figure 2** and **Supplementary Table 6**). Additionally, pups from CAC-treated mothers made fewer visits to any treatment box compared to pups from control mothers (ca. 10 fewer visits, $n = 113$, $p = 0.002$, weighted average, **Figure 2** and **Supplementary Table 6**). The number of visits to any treatment box decreased by 0.43 per day of age ($n = 113$, $p = 0.026$, weighted average, **Supplementary Table 6**). Female pups made more visits to any treatment box compared to males (ca. 7.8 visits more, $n = 113$, $p = 0.014$, weighted average, **Supplementary Table 6**). Visit frequency was unaffected by the timing of the maternal treatments.

Latency to Enter the Treatment Box and the Giving Up Density Tray, and the Time Spent in the Giving up Density Tray

The results for the differences in latency to enter the treatment box are inconclusive as all models produce singular fit errors. There were no significant differences in latency times between PO, CAC, or control GUD trays (**Supplementary Table 7**).

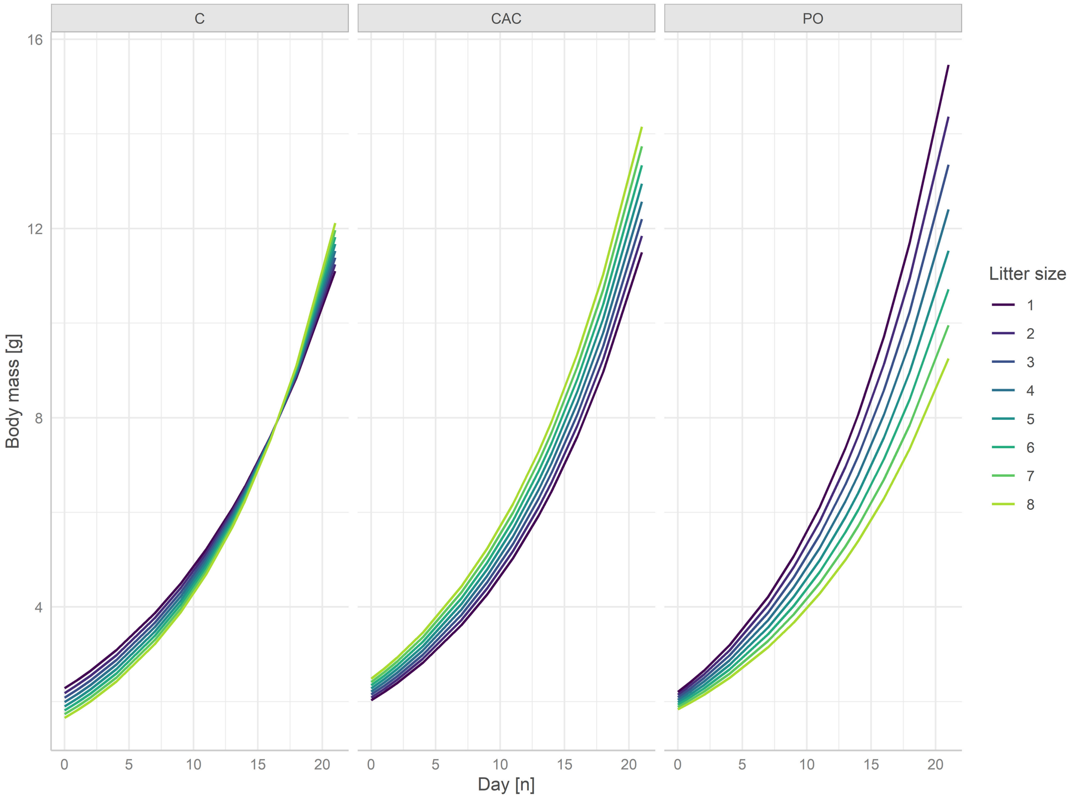

FIGURE 1 | Individual pup mass per day and litter size. Different treatments are in facets.

Overall, pups spent less time in the PO GUD tray compared to the control tray (ca. 492 s less, $n = 113$, $p < 0.001$, **Figure 3** and **Supplementary Table 8**). Pups from PO-treated mothers spent more time in any GUD tray compared to pups from control mothers (ca. 310 s, $n = 113$, $p = 0.039$, **Figure 3** and **Supplementary Table 8**). Time spent in any GUD tray decreased by 18 s per day of age ($n = 113$, $p = 0.004$, **Supplementary Table 8**). Treatment timing and sex did not have statistically significant effects on time spent in GUD trays.

Giving-Up-Densities and Foraging Speed

The interaction between maternal treatment and treatment timing significantly influenced GUD in the CAC and PO treatment, although not in the controls (**Figure 4**). Pups from prenatal control mothers left 3.65 g of millet per liter of sand. Compared to pups from mothers exposed prenatally to the same treatment, pups from mothers treated postnatally with CAC foraged 0.11 g more, and pups from mothers treated postnatally with PO foraged 0.10 g more (i.e., lower GUD following post-natal exposure, **Figure 4**, $n = 113$, $p = 0.006$ and $p = 0.029$, respectively, weighted model average, **Supplementary Table 9**). Among pups from postnatally-treated mothers, pups from CAC-treated mothers foraged significantly more (by ca. 0.09 g) than pups from control mothers, which left ca. 3.69 g of millet per liter of sand ($n = 67$, $p = 0.021$, weighted model average, **Figure 4** and **Supplementary Table 10**) while no difference was observed for pups from PO-treated mothers ($n = 67$, $p = 0.021$, weighted

TABLE 2 | Model summary of the offspring growth.

	Growth (g) of the offspring (Model averages)		
Predictors	**Estimates (log)**	**Std. error**	***p***
(Intercept)	0.881	0.098	**<0.001**
Treatment (CAC)	−0.192	0.129	0.136
Treatment (PO)	−0.055	0.136	0.684
Day	0.073	0.004	**<0.001**
Litter size	−0.046	0.019	**0.015**
Day: Treatment (CAC)	0.010	0.006	0.076
Day: Treatment (PO)	0.022	0.007	**0.001**
Litter size: Treatment (CAC)	0.075	0.027	**0.005**
Litter size: Treatment (PO)	0.020	0.026	0.451
Day: Litter size	0.003	0.001	**0.001**
Day: Litter size: Treatment (CAC)	−0.003	0.001	**0.023**
Day: Litter size: Treatment (PO)	−0.005	0.001	**<0.001**
Timing (postnatal)	−0.017	0.046	0.716
N_{mother}	35		
Observations	1,316		

Results represent averaged (by AICc) estimates on the log-scale.
P-values in bold are significant at p < 0.05.

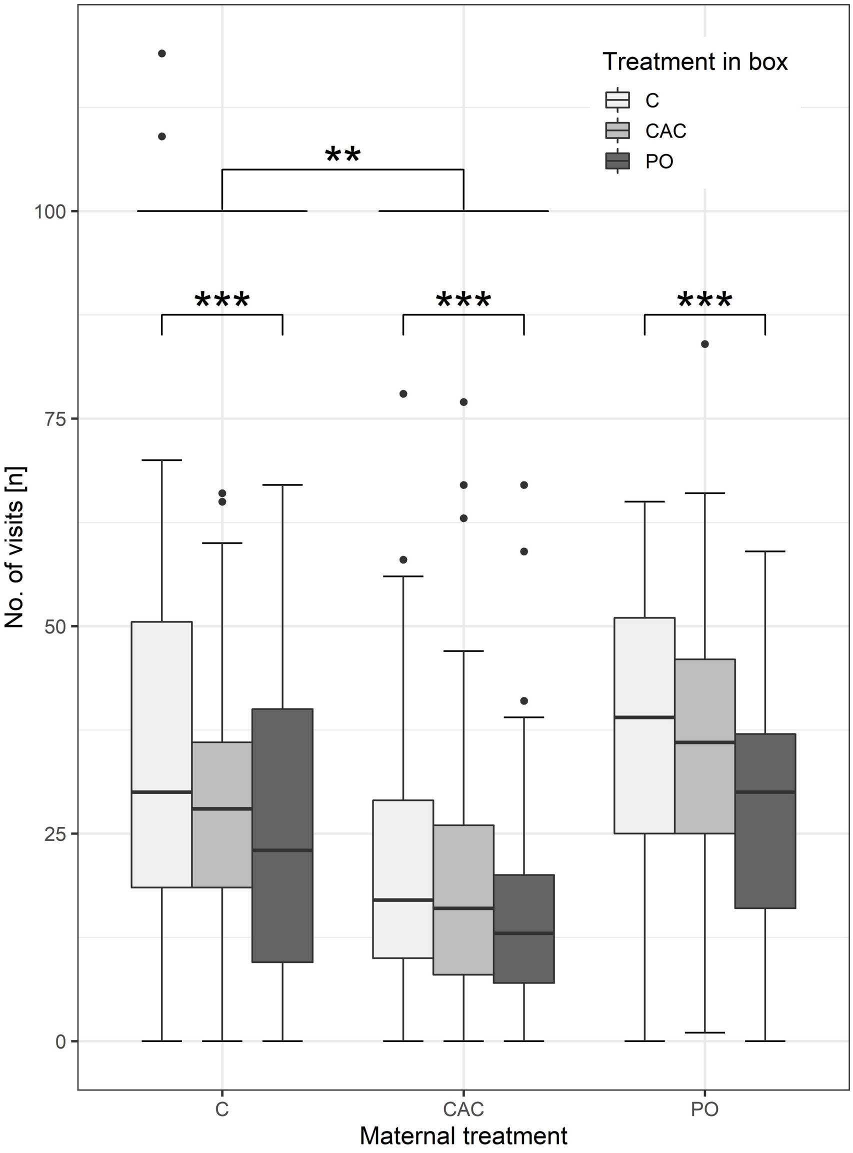

FIGURE 2 | Number of visits in the treatment boxes, split by maternal treatments. Asterisks indicate a significant difference from C at $p < 0.01$ (**) and $p < 0.001$ (***).

model average, **Figure 4** and **Supplementary Table 10**). Among pups from prenatally-treated mothers, no significant differences in GUD were observed between the maternal treatments ($n = 46$, $p > 0.05$, **Supplementary Table 11**). Overall, foraging box treatment did not significantly influence GUD. The foraging speed, grams per second, does not differ significantly between any treatments ($n = 64$, $p > 0.05$ for all comparisons, **Supplementary Table 12**).

DISCUSSION

The current study contributes to a growing understanding about how olfactory cues related to predation risk can have transgenerational impacts on behavior and physiology (Sievert et al., 2019, 2020). Our main focus here was to investigate to what extent pre- vs. postnatal exposure to predator odor and CAC affects offspring differently. While we found only limited evidence for such differential effects in bank voles, our study suggests that odor cue origin (i.e., from predators vs. from conspecifics) matters in ways that interact with other factors that are unrelated to exposure timing. For example, pups from predator-odor-treated mothers grew faster in smaller litters, while the opposite (faster growth in bigger litters) was found for pups from mothers treated with CAC. Additionally, we found that vole pups showed an innate avoidance of predator odor regardless of maternal exposure, but pups from mothers treated with CAC showed signs of preference for that cue. This apparent mismatch shows that both physiological and behavioral adaptations need to be

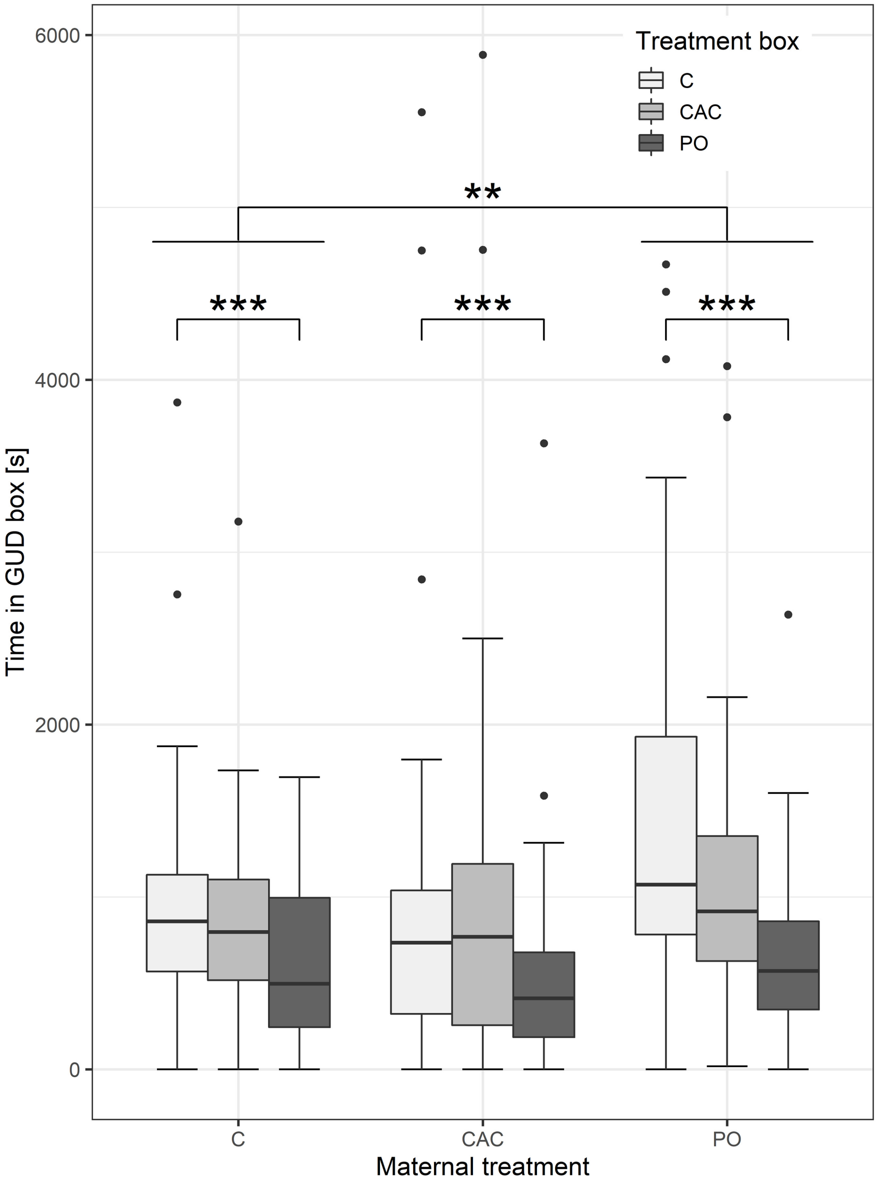

FIGURE 3 | Time spent in the GUD trays, split by maternal treatments. Asterisks indicate a significant difference from C at $p < 0.01$ (**) and $p < 0.001$ (***).

considered together as only looking at one aspect might skew the interpretations.

We found limited differences between in anti-predator behavior in relation to the timing of predation odor cue exposure of mothers. In fact, only foraging was affected by treatment timing. However, the increased consumption by pups from postnatally exposed mothers compared to those from prenatally treated mothers, ca. 19 millet seeds (ca. 0.0052 g per seed), likely represents an ecologically meaningful change in the foraging behavior. Interestingly we did not see a similar pattern when it comes to the time spent in the GUD trays. There pups from PO-treated mothers spent about 310 s more time in them, compared to pups from control-treated mothers. We further found that foraging speed is independent of any of our treatments. Previous studies have explored different aspects of early life exposure to a potential stressor, highlighting possible mechanisms such as maternal care (Bauer et al., 2015) and differences in the milk composition (Brummelte et al., 2010), or the adaptive aspects of the resulting changes in offspring physiology and behavior (Patacchioli et al., 2002; Dias and Ressler, 2014). However, the timing during which the early life stressor has to occur to trigger these changes remains largely obscure.

Pups in bigger litters from mothers treated with CAC and pups in smaller litters from mothers treated with predator odor both gained mass faster compared to the control. These drastically opposite result hint that different reproductive strategies are associated with different perceived risks that are communicated by different odor cues, as previously suggested (Sievert et al.,

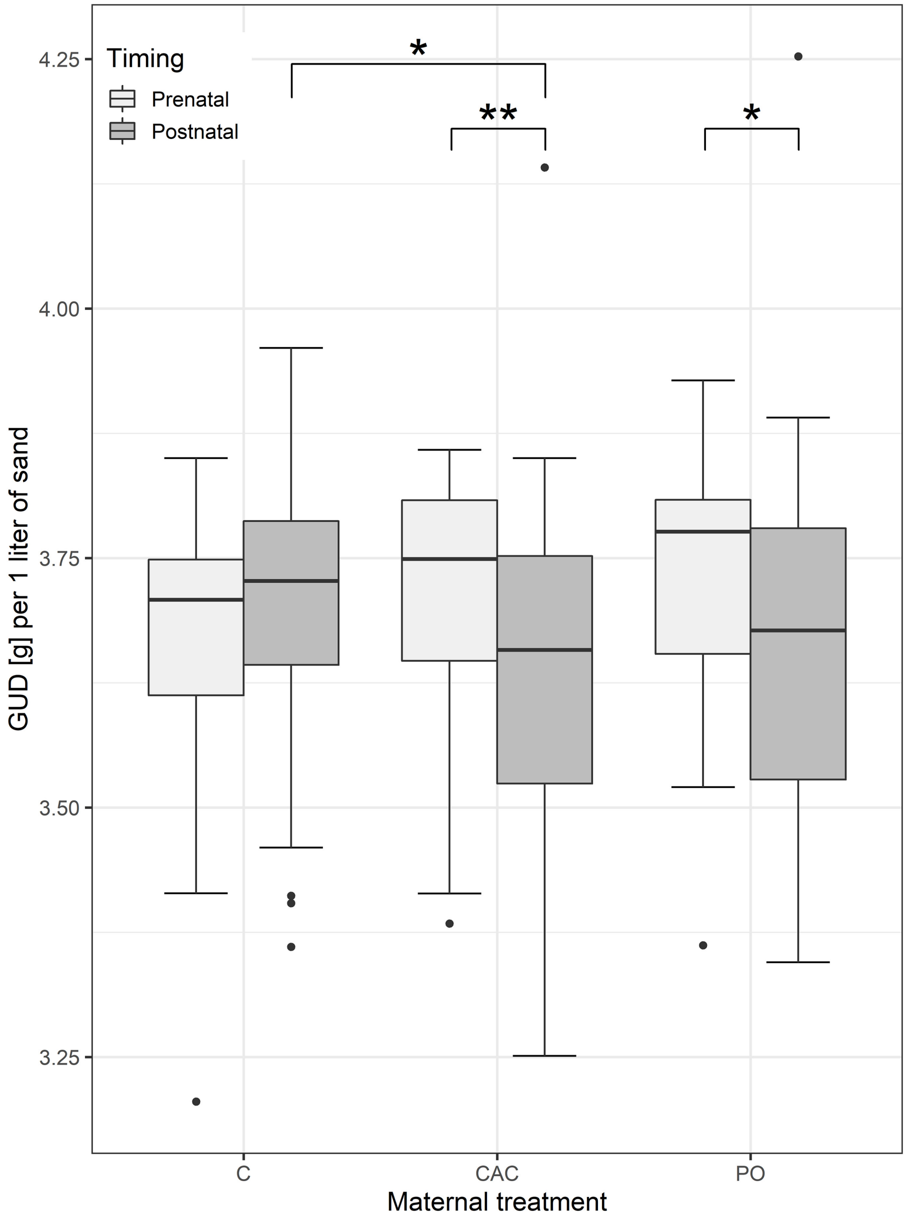

FIGURE 4 | Boxplots showing the GUD in gram split by the different maternal treatments and treatment timing (pre- and postnatal). Asterisks indicate a significant difference at $p < 0.05$ (*) and $p < 0.01$ (**).

2019). CAC exposure might trigger a form of terminal investment with a focus on larger litters; predator odor might trigger investment in a few high quality offspring. Specifically, we saw that offspring in the largest litters from the maternal CAC treatment weighted on average 14 g, while individuals from the smallest litters were only about 11 g (**Figure 1**). This difference was even more pronounced in the maternal predator odor treatment where now individuals from the smallest litters were about 15.5 g and pups in the biggest litters weighted only 9 g. Only male voles were used to generate the CAC, and this sex specificity may underlie the cue's mechanism of action. For example, exposure might communicate more about the unfamiliar conspecific male (Eccard et al., 2017) than about the predator that male encountered. While this possibility cannot be ruled out in the current study, previous work in bank voles revealed different reactions to the CAC produced by predator exposed male voles and the "normal" odors produced by unexposed male voles (Haapakoski et al., 2018; Sievert et al., 2019). Furthermore, we have recently analyzed possible molecular candidates for alarm substances in the bank vole (Sievert, 2020) showing that differences in chemical odor composition are only caused by the treatments (i.e., no handling, handling, and weasel exposure) and show no significant differences based on the sex of the individual.

A strong tendency to avoid boxes treated with predator odor compared to boxes treated with the CAC was found for pups from both maternal treatments (i.e., PO and CAC) at both time points (i.e., pre- and postnatally). This avoidance took the form of fewer visits to and less time spent in boxes treated with predator odor. Because this effect was independent of the maternal treatments, voles seemed to innately avoid areas that were possibly dangerous. Other species exhibit similar avoidance responses when predator-naïve individuals are confronted with predator odors, e.g., fathead minnows (*Pimephales promelas*) (Mathis and Smith, 2008), European rabbits (*Oryctolagus cuniculus*) (Monclús et al., 2005), mice (Sievert and Laska, 2016), and *Microtus* voles (Calder and Gorman, 1991; Bolbroe et al., 2000; Borowski, 2011). However, these studies and our current results contradict the conclusions that areas with predator odor only are not avoided by foraging individuals because odor alone might not be a sufficiently reliable cue (Orrock et al., 2004; Powell and Banks, 2004). Furthermore, some studies suggest that predator odor cues are actually attractive and trigger intensive investigative behavior (Garvey et al., 2016; Parsons et al., 2018). These contradictory outcomes might result from mismatched predator odors and prey species, as not all predator odors might evoke the same reaction in prey species, or from differences in individual sensitivity to the odor (Apfelbach et al., 2005); however, in general odors are important and reliable chemosensory cues for assessing predation risk as predator odors not only reveal predator presence but can also signal predator activity patterns and diet (Kats and Dill, 1998). However, for this study a mismatch between predator and prey can be excluded.

Opposite to avoidance of predator odor, pups from mothers treated with CAC tended to go first into CAC-treated box. Early exposure to odors can alter development of the olfactory bulb and induce preferences for biologically important odors (Todrank et al., 2011). For example, mice exhibit enhanced sensitivity to olfactory cues experienced by their parents (Dias and Ressler, 2014), and a variety of young mammals (e.g., mice, rats, and humans) show preferences for individually distinctive odors associated with their mothers and their mother's diet (Todrank et al., 2011). While these studies did not consider odors related to predation, they identify potential mechanisms linking maternal olfactory experience with offspring perception and response. Our study hints that these mechanisms might show a degree of odor specificity when it comes to risk cues, thereby highlighting differences between odors produced directly by predators and those odors produced by conspecifics having experienced predator threat (i.e., "second-hand" cues; Bian et al., 2005). While the predator-produced odor may carry a direct signal of danger, odors produced by conspecifics may need to be inspected more thoroughly, perhaps due to the complexity of their information content composition. Despite these differences, exposure to either odor may well influence the fitness of cue recipients (Bestion et al., 2014; Koyama et al., 2015).

Our study documents the relative strength of postnatal cues; this strength may result from the fact these cues can work via several mechanisms that may be additive. For example, if a mother is exposed to cues indicating predator presence (i.e., either predator odor or CAC) while outside of her nest (e.g., while foraging), she communicates that risk to her pups through changes in her body odors, her behavior, her milk, or some combination of these. Both the direct predator odor cue and the indirect CAC seem to shape the behavior of offspring, but the timing of exposure, pre- vs. postnatal, played only a minor role. While our results are mixed, our work highlights the need for more fine-tuned approaches to investigate how and when cross-generational effects are triggered in wild animals and in what ways these effects influence fitness. Moreover, the conditions under which the CAC becomes attractive, rather than repellant, warrants further study.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: doi: 10.6084/m9.figshare.11185868 and doi: 10.6084/m9.figshare.11186600.

ETHICS STATEMENT

The animal study was reviewed and approved by the Etelä-Suomen aluehallintovirasto (ESAVI/6370/04.10.07/2014).

AUTHOR CONTRIBUTIONS

TS, MH, and HY designed and planned the study. TS and KB collected the data and analyzed it. TS, KB, MH, KM, and HY wrote the manuscript. All authors contributed to the article and approved the submitted version.

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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.2021.709207/full#supplementary-material

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