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Cue reduction or general cue masking do not underlie generalized chemical camouflage in pirate perch

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Abstract

Avoiding detection is perhaps the ultimate weapon for both predators and prey. Chemosensory detection of predators via waterborne or airborne cues (predator-released kairomones) is a key prey adaptation in aquatic ecosystems. Pirate perch, *Aphredoderus sayanus*, a largely insectivorous mesopredatory fish, are considered to be chemically camouflaged because they are unavoided by all colonizing organisms tested, including treefrogs and aquatic insects, despite stronger predatory effects on target taxa than several avoided fish. To address the mechanism behind camouflage we used aquatic insect colonization as a bioassay to test (1) whether increasing pirate perch density/biomass leads to increased avoidance, and (2) whether pirate perch mask heterospecific fish kairomones. Insect abundances, species richness, and community structure showed no response to pirate perch density. Last, pirate perch did not mask the kairomones of heterospecific predatory fish. Results support the idea that fish kairomones are species-specific, and chemical camouflage is driven by a unique chemical signature that is either undetectable or has no negative associations for colonists.

KEYWORDS

chemical camouflage, chemoreception, colonization, crypsis, habitat selection, oviposition site choice, predation, predator-released kairomones, predator-risk effects, semiochemicals

INTRODUCTION

Predation is a fundamental top-down force that shapes life history, population dynamics, and community structure (Kerfoot & Sih, 1987; Wellborn et al., 1996). Prey utilize a bewildering array of mechanisms to avoid predation (Edmunds, 1974; Ruxton et al., 2004), and organisms lacking morphological or chemical defenses often use behavioral mechanisms, such as flocking, mobbing, group vigilance, reduced activity, and spatial or temporal habitat shifts (avoidance), to coexist with predators (Lima & Dill, 1990; Lima, 1998). In patchy environments, predator avoidance through habitat selection can be a critical mechanism in regional coexistence and in spatiotemporal patterns of community and metacommunity structure (Leibold et al., 2004; Resetarits et al., 2005; Abrams, 2000). However, for habitat selection to be effective, prey must

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BOX 1 **Key definitions modified from Stevens and Merilaita (2009) and Ruxton (2009)**

camouflage—all strategies of deception involved in concealment, including prevention of detection and recognition:

crypsis—organism is not detected and/or not recognized as discrete entity of interest.

mimicry—organism is detected but misidentified; it is recognized as a specific entity of interest—requires identification of a model.

cloaking—organism does not produce, or somehow masks, signals that might register with potential receivers (possible for chemosensory and auditory systems).

first be able to detect, identify, and localize predators (Ferrari et al., 2010).

Camouflage occurs when one organism hinders the ability of another to determine its presence, identity, or location (Ruxton, 2009; Stevens & Merilaita, 2009), and is manifested in three distinct ways (Box 1). While we think of camouflage as a visual phenomenon, there is increasing evidence for camouflage involving other sensory modalities (e.g., sound, motion, chemoreception; Dettner & Liepert, 1994; Ruxton, 2009; Stevens & Merilaita, 2009, 2011), which may manifest via different mechanisms. In aquatic environments, chemical camouflage may be prominent given the prevalence of predator-released kairomones (PRKs; Wisenden, 2000; Ferrari et al., 2010; Ruxton, 2011), semiochemicals produced by predators that benefit the receiver, but not the emitter (Brown et al., 1970). This hypothesis is contrary to the suggestion that chemical crypsis might be unlikely to evolve because of the central role of primary metabolites in predator detection (Conover, 2007), a perception that is rapidly changing. Chemical camouflage has been identified in multiple contexts in invertebrates (Akino et al., 2004; Breed et al., 1992; Brooker & Dixson, 2017; Fishlyn & Phillips, 1980; Longhurst et al., 1978; Portugal & Trigo, 2005; Raffa et al., 2007), as well as vertebrates (Brooker et al., 2014; Resetarits & Binckley, 2013a; Rödel et al., 2013; Miller et al., 2015). However, in most of these examples, camouflage works against a limited range of taxa, often a single species, unlike generalized camouflage.

Semiochemicals are important in aquatic systems and many organisms have evolved chemoreception abilities capable of detecting PRKs (Chivers & Smith, 1998; Kats & Dill, 1998; Silberbush et al., 2010; Wisenden, 2000). By detecting and localizing predators using kairomones, prey can behaviorally avoid habitats with high predation risk (Eveland et al., 2016; Silberbush & Blaustein, 2008). Thus, production of identifiable PRKs can reduce feeding opportunities for predators, and predators would benefit by reducing, concealing, or modifying PRKs.

In aquatic systems, fish are dominant predators (Wellborn et al., 1996) but are typically physically confined within habitat patches. This constraint allows organisms with aquatic life stages and terrestrial dispersal stages (e.g., insects, amphibians) to use habitat selection to avoid patches containing fish (Resetarits & Wilbur, 1989; Vonesh & Blaustein, 2010). For organisms with complex, multistage, life cycles, habitat selection at the oviposition stage can be critical to offspring success because it is the only form of parental care (Rausher, 1993; Resetarits, 1996; Singer, 1984), and re-dispersal to alternative habitats is often impossible (e.g., larval stages cannot disperse and for adult stages of aquatic insects secondary dispersal is energetically prohibitive, and impossible for some because of histolysis of flight muscles; Zalom et al., 1979; Zera & Denno, 1997).

PRKs of predatory fish generate strong, predation risk effects on a variety of taxa, including behavioral, morphological, and life history responses (Kats & Dill, 1998; Pohnert et al., 2007; Weiss et al., 2012), which were hypothesized to be driven by generalized fish kairomones conserved across fish taxa. Most aquatic insects with complex life cycles undergo dispersal and colonization upon reaching the adult stage (e.g., many coleopterans, hemipterans, and dipterans) and that dispersal occurs primarily nocturnally. Many of these taxa show avoidance of a wide breadth of fish diversity (Resetarits & Binckley, 2013a; Resetarits & Pintar, 2016; Silberbush & Resetarits, 2017; Vonesh & Blaustein, 2010). However, limited characterization of fish kairomones suggests they are volatile, difficult to identify, and may be taxonomically unique (Akkas et al., 2009), and work on other PRKs has suggested they are sender and receiver specific (Hahn et al., 2019; Poulin et al., 2018; Selander et al., 2015; Silberbush et al., 2010; Weiss et al., 2018). Recent work on habitat selection also supports the idea that fish PRKs may be species-specific (Resetarits & Pintar, 2016; Resetarits et al., 2021). Limited evidence suggests that adult insects detect fish via volatilized PRKs in the headspace above the water, rather than by sampling the water itself (Silberbush & Blaustein, 2008). Also, because colonization is nocturnal and fish most often occupy some sort of cover and/or are inactive at night, use of visual cues for detection is largely obviated.

The pirate perch (*Aphredoderus sayanus*) is a moderately sized (≤14.5 cm) nocturnal, largely insectivorous, freshwater fish widespread in the Mississippi River drainage and Atlantic Coastal Plain of the southeast United States (Gunning & Lewis, 1955; Lee et al., 1980; Monzyk et al., 1997; Parker & Simco, 2006; Shepherd & Huish, 1978), that is common and native at our field site. It is a sit-and-wait predator known to eat a wide array of invertebrate species, as well as small vertebrates (fishes, larval amphibians); diet varies with body size, but there is no evidence of an ontogenetic niche shift. (Albecker & Vance-Chalcraft, 2015; Benke et al., 1985; Flemer & Woolcott, 1966; Sheldon & Meffe, 1993; Shepherd & Huish, 1978; Silberbush & Resetarits, 2017). While colonizing and ovipositing species from diverse taxa show avoidance of kairomones from a diverse array of fishes, thus far no species of insect or amphibian detects/avoids pirate perch (see Figure 1), nor do pirate perch elicit typical plastic phenotypic responses in larval anurans or salamanders, despite strong consumptive effects (Bohenek et al., in review), reinforcing the idea that pirate perch possess a form of chemical camouflage (Resetarits & Binckley, 2013a). In addition, least killifish (*Heterandria formosa*) do not alter their own activity in response to pirate perch, despite pirate perch being their most common predator in some systems (Levell & Travis, 2018), and pirate perch are significantly underrepresented in the diet of a piscivorous predator, northern watersnakes (*Nerodia sipedon*) (Perkins & Eason, 2018). A critical question here is whether *A. sayanus* has as strong a selective effect on prey species as fish that are avoided, and whether weaker effects, rather than camouflage, are responsible for lack of avoidance. Body size and gape are of primary importance in determining effects of fish predators, and both are less limiting (they possess a relatively large gape) for *A. sayanus* than strongly avoided small fish such as *Pimephales*, *Notemigonus*, and even similar sized fish such as *Enneacanthus*. Neither is *A. sayanus* a prey specialist. Its common name derives from its voraciousness in consuming other fish in aquaria (Abbott, 1871), but *A. sayanus* is primarily insectivorous (Forbes, 1888; Forbes & Richardson, 1908; Goldstein & Simon, 1999; Gunning & Lewis, 1955; McLane, 1955), with diet as much as 80% (Flemer & Woolcott, 1966) to 90% (Benke et al., 1985) aquatic insects (including midges, mayflies, stoneflies, caddisflies, beetles, hellgrammites, and dragonflies), though it is also an effective predator of larval amphibians (Albecker & Vance-Chalcraft, 2015; Bohenek et al., in review). This is a greater proportion of aquatic insects than in the diet of *Lepomis macrochirus* (bluegill) (65% [Flemer & Wolcott, 1966], 78% [Benke et al., 1985]), and bluegill have strong effects on the distribution and abundance of aquatic insects (Smith et al., 1999) and can elicit strong avoidance responses in beetles (Resetarits, 2001). Other avoided species have significantly lower proportions of aquatic insects in the diet, with crustaceans making up 55% of the diet of

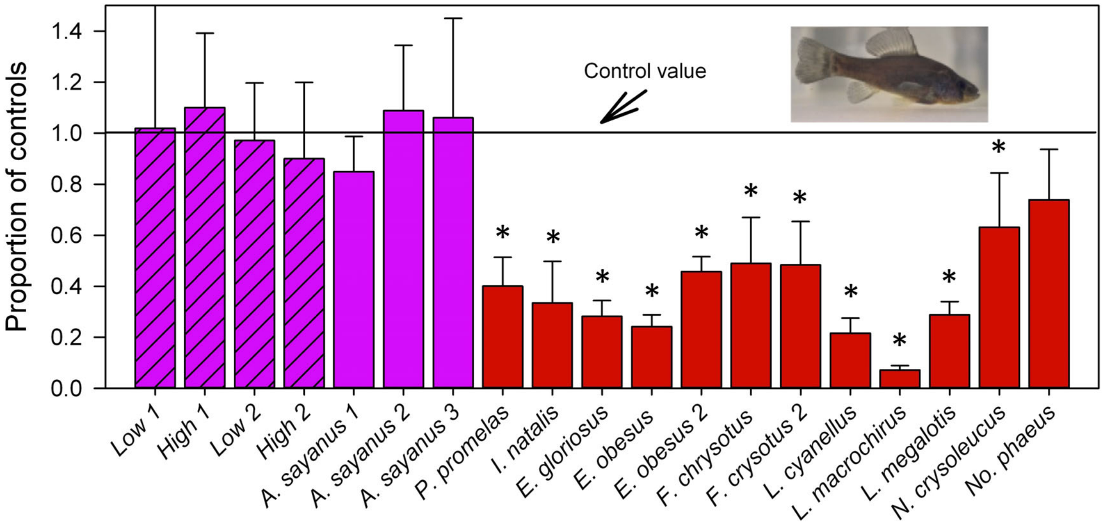

FIGURE 1 Responses of colonizing aquatic beetles (15–57 species) to 11 species of predatory fish in eight field experiments (Binckley, 2004; Binckley & Resetarits, 2005; Resetarits & Binckley, 2013a; Resetarits & Pintar, 2016). For comparison purposes, data are presented as mean number of beetles per mesocosm expressed as a proportion of controls in the respective experiments (+SE). Hatched bars show data from the present paper, solid bars show data from six additional field experiments. Purple bars show pirate perch (*Aphredoderus sayanus*), red bars show other species of fish. * Indicates values significantly different ($p \leq 0.05$) from controls in the respective experiments (Dunnett's). Other than pirate perch, only *Noturus phaeus* was not significantly avoided collectively by beetles, though it was avoided by several individual species; no individual species of any insect or amphibian taxa has been shown to avoid pirate perch. Fish species are all gape-limited generalist predators, and include small-gaped primary planktivores (*Pimephales*, *Notemigonous*) and small-gaped insectivores (*Fundulus*), which are of limited threat to beetles, but nonetheless are strongly avoided. Pirate perch have a relatively wide gape and are effective predators on prey ranging from small insects and invertebrates to larval frogs and salamanders and fish (summary in Resetarits & Binckley, 2013a, 2013b)

Enneacanthus gloriosus (Flemer & Woolcott, 1966), while *Pimephales promelas* and *Notemigonus crysoleucas* are primarily planktivorous, consuming few aquatic insects (Carter, 1949; Duffy, 1998). Considering that much less effective predators are strongly avoided, the most parsimonious explanation is that pirate perch are chemically camouflaged (Resetarits & Binckley, 2013a).

Pirate perch is the only extant species of Aphredoderidae, one of only three families in the order Percopsiformes, which includes nine extant species. Six of these, and the closest relatives of pirate perch, are amblyopsid cavefishes (Amblyopsidae) (Dillman et al., 2011). Pirate perch share a number of characteristics with cavefishes, such as metamorphic migration of the anus to the throat region and a highly developed lateral line system (Dillman et al., 2011; Mansueti, 1963; Moore & Burris, 1956; Poly, 2004; Poly & Proudlove, 2004). Cavefishes and other hypogean fishes also have decreased metabolic rates (Hüppop, 1986; Poulson, 2001), and pirate perch may share this trait (Parker & Simco, 2006). If kairomone production is tied to metabolism (i.e., kairomones are secondary metabolites), then chemical camouflage may be explained by slow ancestral metabolic rates shared with cavefishes; they simply produce less PRK. Alternatively, pirate perch may produce a secondary masking compound. Gulf toadfish (*Opsanus beta*) excrete urea to presumably mask their ammonia waste, which is utilized by gray snapper (*Lutjanus griseus*) to locate and detect prey (this hypothesis was never tested in predation trials, only with purified chemicals; Barimo & Walsh, 2006). Cephalopod ink is also hypothesized to work partly by jamming predator olfaction or mimicking a predator food source (phagomimicry) to cause redirection, but the visual and chemical effects of the ink cannot be teased apart (Derby et al., 2007; Wood et al., 2010). Red lionfish (*Pterois volitans*) are not detected by several species of prey fish, suggesting they are both visually and chemically camouflaged (Anton et al., 2016; Lönnstedt & McCormick, 2013). Pirate perch may produce a masking compound that either blocks reception of kairomones by chemoreceptors in the prey or degrades/alters the species-specific PRK compound, precluding chemoreception.

Thus, hypothesized mechanisms for chemical camouflage in pirate perch are (1) production of undetectable, or below threshold, levels of predator-released kairomone (crypsis), (2) production of an additional masking compound that interferes with prey chemoreception of fish kairomones (cloaking), (3) chemical structure modification of their (ancestral) predator-released kairomone to prevent binding to prey chemoreceptors or prey recognition of the PRK (crypsis or cloaking), or (4) mimicry of some innocuous or attractive entity (mimicry). The last two hypotheses cannot currently be tested directly because no functional fish kairomones have been chemically identified, despite hundreds of papers invoking fish PRKs, and the number of potential models for the mimicry hypothesis is astronomical.

Our goal here is to shed light on the mechanism underlying chemical camouflage in pirate perch. To that end, we tested the two most experimentally tractable hypotheses. Hypothesis 1: pirate perch simply produce less PRK than other fish; therefore, increasing pirate perch density/biomass should increase kairomone signal resulting in detection and avoidance whether the PRK is general or species specific. Hypothesis 2: pirate perch produce a general fish PRK, but also produce an additional compound that masks it. Thus, avoidance of other fish should be reduced by the presence of pirate perch. We cannot determine whether or not pirate perch produce a species-specific PRK, plus a masking compound that works specifically on that PRK. This is a more complex mechanism, but operationally still constitutes a unique, species-specific, chemical signature.

METHODS

We conducted experiments at the 318-ha University of Mississippi Field Station (UMFS) in Lafayette County, Mississippi, USA (34°25′ N, 89°23′ W). Located within the Northern Hilly Gulf Coastal Plain ecoregion of the southeastern United States, UMFS contains >200 permanent and temporary ponds and multiple small streams, wetlands, fields, and mixed forests, with >26 species of fish, >132 species of aquatic beetles, and >40 aquatic hemipterans (Pintar & Resetarits, 2020a, 2020b). All fish used in the experiments were wild caught at UMFS.

Density/biomass experiments

To test whether increased pirate perch density/biomass diminished the camouflage effect, we manipulated pirate perch densities in a pair of mesocosm experiments. Because of the ubiquitous avoidance of most predatory fish by colonizing species (e.g., Figure 1; Resetarits & Binckley, 2013a), these experiments focused only on pirate perch and did not contain any avoided fish as a positive control. The second experiment built on the first by increasing the range of fish density/biomass, and increasing the size of the mesocosms, which increased the total number and diversity of colonists. Increasing the number of fish may actually increase the amount of PRKs produced relative to increasing just biomass because of metabolic rates and surface/volume ratios (Chalcraft & Resetarits, 2004).

Density/biomass Experiment 1

On 4 July 2015, six mesocosms (~450 L, 1.13 m diameter) in a rectangular array (2 × 3) were set up at three sites ($N = 18$) (Experiment 1). Each mesocosm was 1 m from the nearest neighbor, and each array was near the forest edge. Six mesocosms at each site were split into two rows of three because fish size distribution required the density/biomass gradient to be established in two different manners. Mesocosms were randomly assigned (within rows) with three treatments that varied in number and biomass of pirate perch: control (fishless), two small pirate perch (low biomass), four small pirate perch (high biomass). Mesocosms in the second row were assigned control (fishless), one medium-sized pirate perch (low biomass), one large pirate perch (high biomass). Extreme temperatures compelled us to assess fish size by eye to reduce fatal handling stress. In both rows, mean final biomass in low treatments was 8.9 g, while high was 14.75 g, (overall fish survival was 96%; final mass range: small 3.2–5.4 g, one outlier at 7.6 g; medium 7.4–8.8 g; large 12.75–16.0 g). Mesocosms were filled with well-water, received 1 kg of leaf litter for nutrient base and structure, and covered with window screen (1.3 × 1.13 mm openings), which was submerged below water level to allow colonization but prevent access to colonists by fish. The prey base for the fish in both density/biomass experiments (and all prior experiments using other fish species) consisted of taxa that can colonize through the screens, primarily chironomids, ephemeropterans, odonates, and abundant zooplankton. None of the taxa that we assayed were represented below the screens, so any prey consumption cues were nonspecific. The experiment was open to insect colonization from 8 July to 1 September. Insects were exhaustively collected weekly from atop the submerged screens, which reset the initial state. Insects were preserved in ethanol, sorted, and identified to species (based on Pintar & Resetarits, 2020a, 2020b), with a few taxa identified to genus (Appendix S1: Table S1).

Density/biomass Experiment 2

On 12 June 2016, 12 mesocosms ($N = 12$, 1200 L, 1.83 m diameter) were arranged linearly along forest edge in four blocks with three mesocosms each (Experiment 2). Each mesocosm was ≥1.5 m from forest edge and 1 m from the nearest mesocosm, with blocks separated by 4.5 m. Three treatments were randomly assigned within blocks: control (fishless), 2 pirate perch (low density), and 12 pirate perch (high density). Low density tanks had a mean of 16.3 g initial total biomass (range 15.4–17.4 g) while high density had a mean of 58.2 g initial total biomass (range 55.5–60 g) (initial individual size range 1.7–9.4 g, most individuals were between 3 and 7 g). Pirate perch survival was 82% over the duration of the experiment, 88% in low density and 81% in high density. Because both mortality and growth were density dependent, the difference between low and high densities was smaller by the end of the experiment, with a low density mean of 17.3 g (range 12.1–21 g) and a high density mean of 38.3 g (range 28.7–53.7 g). Mesocosms were filled as in Experiment 1 and were open to colonization from 14 June–17 August. Adult aquatic insects were exhaustively collected weekly and processed as in Experiment 1 (see Appendix S1: Table S1).

Data analysis

Total beetle abundance (Experiment 1), total insect abundance (Experiment 2), insect species richness, abundances of Hydrophilidae, Dytiscidae, and Hemiptera (Experiment 2), and abundance of species with total $N > 90$ (Table 2) were square-root transformed ($\sqrt{X+0.5}$) and analyzed using general linear mixed-model ANOVA in PROC MIXED. The absence of any positive response to fish by colonizing organisms in prior experiments informed our hypothesis that any fish effects would take the form of reduced abundance and richness, hence we used one-tailed Dunnett's procedure to compare all treatments to controls (Dunnett, 1955; Rice & Gaines, 1994; Ruxton & Neuhäuser, 2010). We assessed the entire insect assemblage and beetle assemblage composition using permutational multivariate analysis of variance (PERMANOVA), and beta diversity using homogeneity of dispersion (PERMDISP), all on square-root-transformed abundance data (Bray-Curtis). We also regressed total pirate perch biomass per mesocosm against total number of colonists. Analyses used SAS v. 9.4 (SAS Institute, Cary, NC) with Type III sums of squares and $\alpha = 0.05$. PRIMER v 7.0.13 and the PERMANOVA+ add-on were used for PERMANOVA and PERMDISP analyses and to generate the NMDS results (Anderson et al., 2015; Clarke & Gorley, 2015).

Masking experiments

To test whether pirate perch mask PRKs from heterospecific fish, we conducted two colonization experiments comparing patches with single fish species to patches with two species. On 9 October 2016, we constructed six blocks of four wading pools (mesocosms; 0.91 m diameter, ~110 L; $N = 24$) to compare pirate perch, golden topminnows (*Fundulus chrysotus*), and their combination to controls. Blocks were linearly arranged parallel to, and 1 m

TABLE 1 Fixed-effects results (Type III) from mixed-model ANOVAs on square-root-transformed ($\sqrt{X+0.5}$) values for species richness of all insects and abundance of species with > 90 colonists in each experiment

Parameter or taxon	Source	*F*	*p*
Density/biomass Experiment 1 (df = 2,10)			
Species richness	biomass	0.64	0.5468
Abundance			
All beetles	biomass	0.52	0.6102
Hydrophilids	biomass	0.48	0.6339
Dytiscids	biomass	0.28	0.7632
Species			
Berosus infuscatus	biomass	0.03	0.9719
Copelatus glyphicus	biomass	0.48	0.631
Laccophilus fasciatus	biomass	2.55	0.1272
Laccophilus proximus	biomass	0.14	0.8736
Paracymus	biomass	1.86	0.2059
Tropisternus lateralis	biomass	0.55	0.5917
Density/biomass Experiment 2 (df = 2,6)			
Species richness	biomass	0.37	0.7082
Abundance			
All insects	biomass	0.32	0.7361
All beetles	biomass	0.25	0.7831
Hydrophilids	biomass	0.62	0.5707
Dytiscids	biomass	0.62	0.5671
Hemipterans	biomass	1.17	0.3737
Species			
Berosus infuscatus	biomass	1.1	0.3908
Laccophilus fasciatus	biomass	0.32	0.7345
Paracymus	biomass	0.91	0.4524
Peltodytes sexmaculatus	biomass	0.05	0.9545
Tropisternus collaris	biomass	0.84	0.4777
Tropisternus lateralis	biomass	1.82	0.2413
[a]*Hesperocorixa vulgaris*	biomass	1.39	0.3183
[a]*Notonecta irrorata*	biomass	0.16	0.8528
[a]*Sigara*	biomass	0.32	0.7345

Note: See Table 2 for individual species results. None of the Dunnett's tests were close to significant (Figure 2).

[a] Indicate Hemiptera, others are Coleoptera.

from, the forest edge in an open field. Mesocosms within blocks were separated by 2.5 m center to center, and blocks separated by ≥15 m, edge to edge. Mesocosms were filled with well-water and received 100 g of leaf litter and two holding cages (lidded plastic plant pots, 28 cm height × 32 cm diameter) with two 800-cm^2 mesh windows (1.3 × 1.13 mm mesh) that allowed chemical communication with the mesocosm proper. Cages housed predators and prevented any predation on colonists.

Mesocosms in a spatial block were randomly assigned one of four treatments: fishless Control, one pirate perch, one golden topminnow, or one of each. Previous experiments have shown that one golden topminnow causes significant reduction of mosquito oviposition (Pintar et al., 2018). Fish were held separately to prevent interspecific antagonism. Mosquito egg rafts were collected and removed daily. All identified mosquitoes were *Culex restuans*: ~99% of egg rafts collected in mesocosms at UMFS are *C. restuans* (Bohenek et al., in review). This experiment concluded after 6 days. On 20 October, the same experimental design was repeated in the same location using another species with known repellant effects, mosquitofish (*Gambusia affinis*) (Eveland et al., 2016), and using pirate perch in sets of three: controls, three pirate perch alone, one *G. affinis* alone, and three pirate perch with one *G. affinis*. Because we saw no effect in the prior experiment using a single pirate perch, we increased the relative density for the second experiment to further test the masking hypothesis.

Each egg raft was considered a single oviposition event and we used generalized linear mixed model ANOVA with a Poisson distribution (PROC GLIMMIX) on square-root-transformed ($\sqrt{X+0.5}$) total egg rafts per treatment. Dunnett's procedure (one-tailed) was again used to compare fish treatments to controls. Analyses used SAS v. 9.4 (SAS Institute, 2016) with Type III sums of squares and $\alpha = 0.05$.

RESULTS

Density/biomass experiments

In the first experiment, we collected 961 individuals of 28 beetle taxa and 38 individuals of three Hemiptera taxa (Appendix S1: Table S1). Initial analysis revealed that mode of achieving biomass differences (density/size) had no effects, so was eliminated in the final analyses. Mean total number of all beetles, dytiscids, hydrophilids, and insect species richness showed no differences among treatments (Table 1; Figure 2a). Species-level responses for the six most abundant taxa also showed no differences (Table 1; Figure 2b). Analysis of the entire insect assemblage revealed no differences in community composition (treatment, $F_{2,10} = 1.16$, $p = 0.339$; block, $F_{5,10} = 4.04$, $p = 0.001$) or

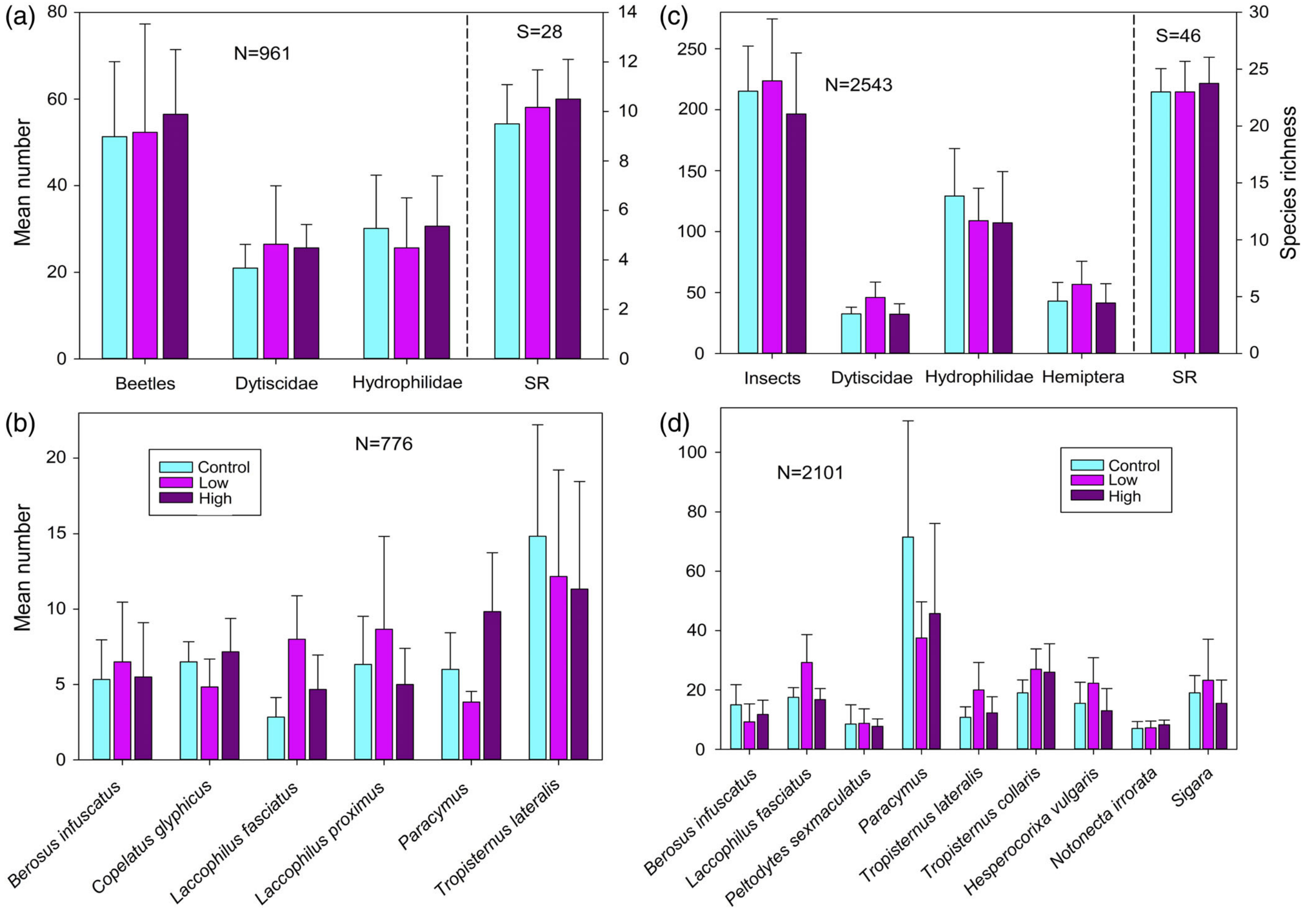

FIGURE 2 (a, left) Mean number of beetles collected per mesocosm in higher taxonomic groups (+SE) from each three treatment levels (control, low, high) of varying density/biomass in Experiment 1. (a, right) Mean species richness (SR) per mesocosm (+SE). (b) Mean number of beetles collected per mesocosm (+SE) of the six most abundant species (>90 colonists). Neither the main effect of density nor Dunnett's procedure (one-tailed) were significant for higher taxonomic groups, individual species, or species richness. (c, left) Mean number of insects collected per mesocosm in higher taxonomic groups (+SE) from three treatment levels (control, low, high) of varying density/biomass in Experiment 2. (c, right) Mean species richness per mesocosm (+SE). (d) Mean number of beetles collected per mesocosm (+SE) of the nine most abundant species (>90 colonists). Neither the main effect of density nor Dunnett's procedure (one-tailed) were significant for higher taxonomic groups, individual species, or species richness

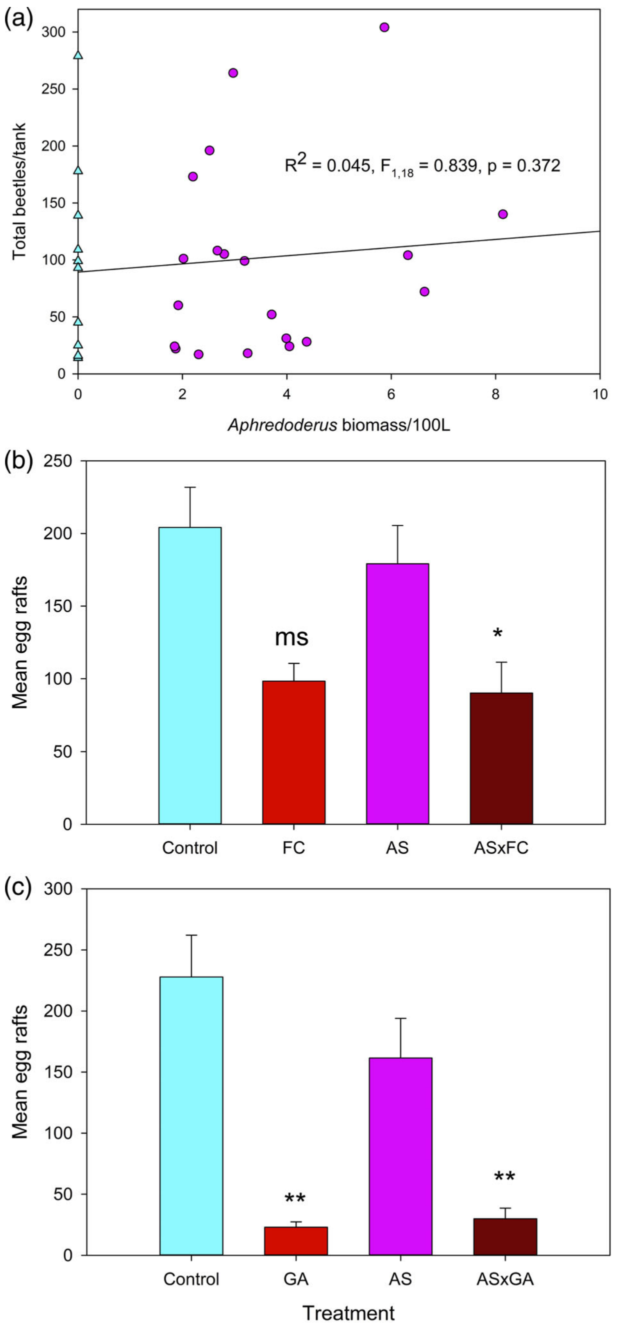

FIGURE 3 (a) Relationship between pirate perch biomass (g/100 L) and the total number of beetle colonists per mesocosm (+SE) for both density/biomass experiments combined. Control values (cyan triangles) for reference, not included in regression. Minimum biomass density that typically elicits avoidance of other fish species by colonizing beetles is <2 g/100 L (Resetarits & Binckley, 2013a; Resetarits et al., 2019). There was no relationship between pirate perch biomass and the number of beetle colonists. (b, c) Mosquito oviposition responses across each predator species treatment in the two masking experiments. Mean total mosquito egg rafts per mesocosm (+SE) in each predator treatment (b) (FC, one *Fundulus chrysotus* (golden topminnow); AS, one *Aphredoderus sayanus* (pirate perch); AS × FC, one pirate perch and one golden topminnow). (c) (GA, one *Gambusia affinis* (mosquito fish); AS, three pirate perch; AS × GA, three pirate perch and one mosquitofish). * Significantly different from control ($p \leq 0.05$); ms, marginally nonsignificant (Dunnett's one-tailed test) (Table 2)

beta diversity ($F_{2,15} = 0.54$, $p = 0.625$), with similar results for the beetle assemblage (treatment, $F_{2,10} = 1.01$, $p = 0.439$; block, $F_{3,10} = 3.74$, $p = 0.001$) and beta diversity ($F_{2,15} = 0.27$, $p = 0.774$) (Appendix S1: Figure S1a,b).

In the second experiment, we collected 1978 individuals of 39 beetle taxa and 565 individuals of seven Hemiptera taxa (Appendix S1: Table S1). There was no difference in the total number of insects, dytiscids, hydrophilids, hemipterans, or their species richness among density treatments (Table 1; Figure 2a). All individual species followed the same pattern as higher taxonomic groups with no differences in colonization rates among density treatments (Table 1; Figure 2b). Analysis of the whole insect assemblage revealed no differences in community composition (treatment, $F_{2,6} = 0.83$, $p = 0.613$; block, $F_{3,6} = 3.85$, $p = 0.001$) or beta diversity ($F_{2,9} = 0.32$, $p = 0.75$), with similar results for the beetle assemblage for community composition (treatment, $F_{2,6} = 0.91$, $p = 0.546$; block, $F_{3,6} = 3.79$, $p = 0.001$) and beta diversity ($F_{2,9} = 0.52$, $p = 0.598$) (Appendix S1: Figure S1c,d). There was also no relationship between pirate perch biomass per mesocosm and the total number of colonists per mesocosm (combined data from both density/biomass experiments; Figure 3a).

Masking experiments

In the golden topminnow experiment, there were 2654 *Culex restuans* egg rafts deposited over six days. There was a strong, significant treatment effect on mean total egg rafts

TABLE 2 Fixed-effects results (Type III) and Dunnett's (one-tailed) comparison of all treatments to the control from mixed-model ANOVAs on square-root transformed ($\sqrt{X+0.5}$) number of *Culex restuans* egg rafts in each experiment

***Culex* egg rafts**	***F***	***t***	***p***
Pirate perch (PP) vs. golden topminnow (FC)			
Treatment (df = 3,20)	3.05		*0.0523*
Dunnett's			
PP vs. control		0.43	0.5952
FC vs. control		2.13	*0.0583*
Both vs. control		2.49	**0.0288**
Pirate perch vs. mosquitofish (GA)			
Treatment (df = 3,20)	15.31		**<0.0001**
Dunnett's			
PP vs. control		1.2	0.2823
GA vs. control		5.31	**<0.0001**
Both vs. control		5.03	**<0.0001**

Note: Boldface type indicates significant effects ($p \leq 0.05$), italic type marginally nonsignificant.

per mesocosm. Dunnett's procedure showed significantly or marginally nonsignificantly fewer egg rafts with golden topminnows, and pirate perch and golden topminnows, but not pirate perch alone, than in controls (Table 2, Figure 3b).

There were 3432 *Culex restuans* egg rafts deposited in the mosquitofish masking experiment. Results mirrored the first experiment, with a strong treatment effect on mosquito egg rafts per mesocosm. Significantly fewer egg rafts were deposited in mesocosms containing mosquitofish, and pirate perch and mosquitofish, but not pirate perch alone, than in controls (Table 2, Figure 3c).

DISCUSSION

We addressed two hypotheses regarding chemical camouflage in pirate perch. First, that lack of detection and avoidance is a dose response, resulting from a lower production of PRKs (either general or species-specific), and a second, alternative hypothesis, that pirate perch produce PRKs at a comparable rate to avoided fish, but also produce a second chemical (or chemicals) that affect detection of those PRKs, a masking chemical. We used this bioassay approach because of the long history of difficulties and lack of success identifying fish kairomones, and because it directly addresses the functioning of chemical camouflage. Even now, very few compounds involved in aquatic chemical signaling systems have been identified, most involving responses of specific prey to specific predators (Brönmark & Hansson, 2000; Hahn et al., 2019; Poulin et al., 2018; Selander et al., 2015; Silberbush et al., 2010; Weiss et al., 2018). None of these involve a generalized response by diverse prey, and the only one involving fish specifically affects the diel vertical migration of *Daphnia* (Hahn et al., 2019).

We can effectively eliminate lower production of PRKs, as pirate perch biomass densities ~10 times the biomass densities that induce beetle avoidance of other fish species (Resetarits & Binckley, 2013a), densities that are on the extreme end of natural pirate perch densities (W. J. Resetarits, personal observation), generate no avoidance of pirate perch. This holds for all higher taxa and all 11 of the most abundant insect species across two density experiments. Results for insect community composition mirror those for abundance; neither species richness, community structure, nor beta diversity were affected by pirate perch at any density, contrasting with effects of most other fish species (Resetarits & Binckley, 2013a; Resetarits & Pintar, 2016; Resetarits et al., 2019). Thus, on an operational basis, pirate perch are not producing meaningful levels of a recognizable PRK. Our data also demonstrate that pirate perch are not producing and masking a generic PRK signal, as pirate perch do not reduce avoidance of other fish species when in combination, even at increased relative pirate perch densities. However, this does not eliminate the possibility that pirate perch produce a species-specific cue and a species-specific cue masking compound.

There are two remaining hypothetical mechanisms for chemical camouflage in pirate perch. Chemical mimicry (in contrast to crypsis; Box 1) is well-documented in insects for highly coevolved interactions, such as brood parasites, specialist parasitoids, and other highly specialized interactions, but there are no examples of generalized chemical mimicry (Ruxton, 2009, 2011). Pirate perch could be chemically mimicking some innocuous or attractive scent, but this is impossible to test without guidance as to the model. In our numerous experiments with pirate perch, we have no compelling evidence for attraction, excepting a significant positive response by *Uvarus granarius* (Resetarits & Pintar, 2016). The more likely scenario for mimicry is that of something innocuous, thus largely equivalent to crypsis. Thus, the most parsimonious explanation is that fish PRKs are, at least at some level, species or taxon specific, and pirate perch are not producing detectable or identifiable PRKs, calling into question the view that fish produce a generalized "fish cue" common to most freshwater fish (Binckley & Resetarits, 2003; Resetarits & Binckley, 2013a). Recently we have seen variation in which specific fish species, or combinations of species, colonizing prey avoid, as well as variation in which prey species respond to which fish predator or predator combination (Resetarits & Pintar, 2016; Resetarits et al., 2019, 2021), potentially explaining why the hypothesized fish PRK has proven so elusive. Species avoiding only certain combinations of fish species, while not avoiding the individual species at the same densities, further argues against a simple, generic fish PRK (Resetarits et al., 2021). Perhaps this should not be surprising, as prey can differentiate the chemical cues of different mammalian predators (Thaker et al., 2011), but the sheer number of freshwater fish species (e.g., 280 in Mississippi alone) and the ubiquity of prey responses to fish chemical cues has always argued against species-specific chemical cues in fish.

While camouflage has long fascinated biologists within the realm of animal behavior and predator–prey interactions, camouflage in the context of demographic habitat selection (in contrast to transient habitat selection), has the potential to impact community assembly on both a local and landscape scale. These community-level consequences of camouflage have been largely overlooked, primarily because studies of camouflage have overwhelmingly focused on camouflage in prey (Pembury Smith & Ruxton, 2020). Most fish dramatically alter the abundance and species composition of colonizing/ovipositing organisms in specific habitat patches and cause redistribution of colonists among patch types at the landscape scale, thus reducing local consumptive effects (Resetarits & Pintar, 2016; Vonesh et al., 2009). In contrast, a chemically camouflaged species increases local colonization relative to other fish species and increases local consumptive effects: individuals are removed from the population, not redistributed, thus creating an ecological trap (Delibes, Ferreras, et al., 2001; Delibes, Gaona, et al., 2001), which has potentially dramatic consequences for populations and communities at both the local and landscape scales (Resetarits et al., 2005). As a result of impacts on demography and community assembly, the importance of chemical camouflage extends well beyond simple predation rates.

Chemical signals in aquatic environments remain poorly studied (Brönmark & Hansson, 2000; Ferrari et al., 2010; Ruxton, 2011). Chemical mimicry and camouflage have yet to benefit from the explosion in studies of camouflage (Stevens & Merilaita, 2011), and reviews of camouflage overwhelmingly focus on vision (Cuthill, 2019; Pembury Smith & Ruxton, 2020). Thus, there are likely numerous cases of chemical mimicry and camouflage yet to be discovered (Brönmark & Hansson, 2000; Ruxton, 2009). It has been suggested that chemical camouflage may be as widespread as visual camouflage, but we lack the tools to identify it (Ruxton, 2009, 2011; Stevens & Merilaita, 2011). Humans have better visual acuity than most animals (Caves et al., 2018), thus it is not surprising that we have identified thousands of examples of visual camouflage and mimicry. However, many other taxa, such as insects, are more dependent on chemosense and far superior in discriminating and identifying chemical cues (Crespo, 2011). Thus, it is not surprising we cannot routinely identify chemical camouflage, and we should expect it to be common, especially in environments and with species that depend heavily on chemosense. The early objections to the idea of widespread chemical camouflage were based on the idea that PRKs were largely derived from highly conserved metabolic processes that were less accessible to selection (Conover, 2007). Recent work has largely dispelled that theory (Akkas et al., 2009; Brönmark & Hansson, 2000; Hahn et al., 2019; Poulin et al., 2018; Selander et al., 2015; Silberbush et al., 2010; Weiss et al., 2018), which lessens the potential costs of chemical camouflage. If PRKs are simply species-specific pheromones upon which prey eavesdrop, then making them less informative to prey while maintaining their primary function is certainly more feasible than modifying conserved metabolic processes.

The most parsimonious explanation is that chemical camouflage in pirate perch is achieved via production of unique, undetectable or unidentifiable PRKs. While pirate perch undoubtedly release myriad chemical substances (i.e., waste material), the cues produced are either not detectable or are not associated with increased risk: pirate perch are not identified as a predator. Thus, species react to patches containing pirate perch as if the habitat was predator free. Is this phenomenon unique to pirate perch among freshwater fish? The idea that, out of the first 15 species of fish that we have extensively tested, we chanced upon the only instance of chemical camouflage out of >40,000 extant species of freshwater fish seems improbable at best. As we include more fish species and species combinations, and assay responses of an expanding number of colonizing/ovipositing species, considerable variation in the responses of prey seems the norm. While pirate perch remain the only species that has generated no avoidance in any of species tested, another species, the brown madtom (*Noturus phaeus*) generated avoidance in only 2 of the 15 most abundant insect colonists (Resetarits et al., 2021), and other fish species generate avoidance by colonizing insects at varying rates (Binckley & Resetarits, 2003, 2005; Resetarits & Silberbush, 2016). Thus, chemical camouflage can be thought to exist on a continuum, as with visual camouflage, from near invisibility to mild disruption of identification and/or location, and we should expect it to be widespread in the animal kingdom.

The myriad competing weapons in the predator–prey arms race have been a central focus in behavior and ecology since Darwin (1859) and Wallace (1870) presented visual camouflage as a singular exemplar of the power of natural selection to shape animal morphology. We have long understood the importance, and sometimes dominance, of other sensory modalities in the ecology of many species, but only recently have we have begun to identify and catalogue examples of camouflage of both predators and prey in these other modalities (Ruxton, 2011) and realize its parallel potential for shaping animal physiology. Pirate perch are the best example to date of generalized chemical camouflage, being chemically invisible to a large proportion of your prey that use chemosense to identify and locate predators is perhaps the ultimate weapon in a predator's arsenal.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher’s website.

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