

Complex multi-predator effects on demographic habitat selection and community assembly in colonizing aquatic insects

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Abstract. Running the gauntlet of predators consumes critical time and energy resources, as all species are vulnerable to one or, typically, more predators at some life stage. Prey employ a vast array of mechanisms to avoid predation, and predators, likewise, come in a bewildering variety. Thus, defensive adaptations are rarely one size fits all. Considerable work has addressed multi-predator consumptive effects, but we now know that non-consumptive effects of predators can dramatically impact individuals, (meta)populations, and (meta)communities. However, little is known regarding the community-wide dynamics of non-consumptive effects generated by multiple predators. Predator avoidance by choosing a patch that is free of a particular predator or predators can be the most effective strategy if conditions at colonization are a reliable predictor of absence, which is often true for fish in freshwater systems. We experimentally manipulated composition of the predator assemblage in aquatic mesocosms in a substitutive design, with zero, one, two, or three caged predatory fish species (one benthic, one pelagic, and one surface fish) at constant density and biomass, and assayed responses of naturally colonizing aquatic insects. We addressed three related questions; first, how do members of a diverse assemblage of colonizing aquatic insects respond to this variation in species and species combinations, second, do individual species (and higher taxa), respond differently to single vs. multiple predator species (species richness), and third how do any responses to fish species and species combinations, and effects on species richness, translate into community-wide changes in the composition of colonists. Prey had varied responses to specific predators or combinations of predators, resulting in distinct community composition across treatments and higher β -diversity with predators. Prey showed emergent multi-predator effects, where certain species only responded to predator species combinations, but not to any individual predator, and stronger effects of multiple predator vs. single-predator treatments, despite strong responses to individual predators in many taxa. Habitat selection effects can range from the individual to the metacommunity, and the dynamics of habitat selection in response to predators is a complex function of predator identity, density, richness, species composition, and patch spatial context.

Key words: β -diversity; Coleoptera; colonization; community assembly; demographic habitat selection; Hemiptera; immigration; metacommunities; multi-predator effects; non-consumptive effects; patch quality; predation risk.

INTRODUCTION

The world is a dangerous place, and running the gauntlet of predators is a critical time- and energy-consuming occupation, as all organisms are vulnerable

to one or, typically, more predators at some life stage (Polis 1991). Animals use a vast array of mechanisms to avoid predation, from morphological adaptations for speed, weaponry, camouflage, or distastefulness, to behavioral mechanisms of reduced activity, flocking, temporal activity shifts, and habitat use patterns (Edmunds 1974, Taylor 1984, Caro 2005). Predators also come in a bewildering variety of shapes, sizes, offensive weaponry, hunting behaviors, sensory abilities, etc., and defensive adaptations are rarely one size fits all (Van Buskirk 2001, Chalcraft and Resetarits 2003, Relyea 2004, Preisser et al. 2007, Resetarits and Chalcraft 2007, Botham et al. 2008, Morosinotto et al. 2010). Specialist predators are the exception rather than the rule in natural systems, thus most prey are vulnerable to a variety of

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predators, while most predators have rather diversified diets (Polis 1991, Sih et al. 1998).

A classic example is the bullfrog (*Rana catesbiana*), an anuran species that thrives in the presence of fish. Bullfrog larvae are distasteful because of compounds in the skin, thus, they are protected against fish and other vertebrate predators. However, this highly effective adaptation is useless against many predatory insects that simply remove the skin, or use piercing mouthparts to consume the insides. Thus, bullfrogs are the dominant anuran in ponds with fish, but play a minor role in many fishless ponds (Werner and McPeck 1994, Hecnar and M'Closkey 1997). Similarly, two speciose genera of damselflies, *Enallagma* and *Ischnura*, each have species that specialize on fish and fishless ponds, and cannot persist in the opposite habitat. Fish pond specialists have behavioral adaptations (reduced activity, reduced flight response) allowing persistence with fish, but are vulnerable to a dominant predator in fishless ponds, larval dragonflies, whereas fishless pond specialists have behavioral adaptations against dragonflies (greater activity, strong flight response), but are highly vulnerable to fish (McPeck 1990, 2008). Integration of variable responses and vulnerabilities to different predators across prey taxa generates much of the characteristic variation we see, not just between predator and predator-free habitats, but among habitats dominated by different types of predators (Wellborn et al. 1996, Wilbur 1997, Preisser et al. 2007, Van Allen et al. 2017), or even different size predators of the same species (Krenek and Rudolf 2014).

Observed variation among communities with differing predation regimes was long ascribed to consumptive effects of predators: prey colonize, predators remove the vulnerable, and the community we see is the end result. However, non-consumptive effects of predators can have dramatic impacts on individuals and populations, and by extension, wide-ranging effects on communities (Schmitz et al. 1997, 2004, Werner and Peacor 2003, Preisser et al. 2005, Peckarsky et al. 2008, McCauley et al. 2011, Resetarits and Pintar 2016, Trekels and Vanschoenwinkel 2016, Breviglieri et al. 2017, Resetarits et al. 2019). Surprisingly little is known regarding the dynamics of non-consumptive effects of multiple predator species on multiple interacting prey species and communities, especially as regards colonization. We know that predator induced changes in colonization in response to specific predators can generate effects on both populations and communities (Kraus and Vonesh 2010, Resetarits and Pintar 2016, Trekels and Vanschoenwinkel 2016, Resetarits et al. 2019, Turner et al. 2020) and Staats et al. (2016) showed that colonization rate decreases with predator richness for several ovipositing dipterans, but we know little else.

True matching habitat selection, where decisions accomplish matching of phenotype to environment to maximize fitness, is considered rare because of the diversity and complexity of environmental cues that determine expected fitness (Edelaar et al. 2017). However, for binary

situations that have very strong fitness effects, such as predator/no predator, it can be expected to be more prevalent. What happens when that binary situation increases in complexity with risk from multiple simultaneous predators? Organisms must be able to assess risk and “respond” appropriately, either long-term as a population via the evolution of anti-predator adaptations, or more immediately as individuals via morphological and behavioral plasticity. In any system of relatively discrete habitat patches, the most effective anti-predator strategy is avoidance of patches containing predators, as long as the absence of that predator at the time of colonization is a reliable predictor of long term absence (Resetarits and Wilbur 1989, Blaustein 1999, Kershenbaum et al. 2012). However, for habitat selection to be effective, prey must first be able to detect, identify, and localize predators (Ferrari et al. 2010), or utilize surrogate mechanisms, such as choosing ephemeral ponds to reduce potential exposure to fish (Wellborn et al. 1996).

Historically, work on habitat selection has focused on what can be called transient habitat selection, typically in the context of foraging behavior and foraging theory, including predator avoidance behavior. This includes the large body of work using giving up densities (GUDs), and recent work on the “ecology of fear” (Schmitz 1997, Preisser et al. 2005, Preisser and Bolnick 2008, Beckerman et al. 2010, Clinchy et al. 2013). In transient habitat selection, prey organisms are in a near-continuous interaction with perceived risk and reward, and can, in theory, change habitat at any time. However, another widespread form of habitat selection is more episodic, as exemplified by nest site selection in birds (Schmidt 2004, Emmering et al. 2018), denning in mammals (Fernández and Palomares 2000, Davies et al. 2016), oviposition site choice in amphibians (Resetarits and Wilbur 1989, Rudolf and Rödel 2005, Touchon and Worley 2015), colonization/oviposition by phytophagous and aquatic insects (Sadeh et al. 2009, Berger et al. 2012, Friberg et al. 2016, Resetarits and Pintar 2016), and even settlement in sessile marine organisms (Price 2010, Dumas et al. 2014). These decisions are enduring and may even be permanent, and thus might be termed demographic habitat selection because they directly affect population sizes within and among habitat patches. Matching demographic habitat selection plays a potentially critical role in evolutionary processes (Fry 1997, Edelaar et al. 2008), and while less prevalent and flexible than adaptive plasticity (Edelaar et al. 2017), modeling suggests that habitat selection successfully matching phenotype to environments (directed gene flow) has greater adaptive potential than either adaptive plasticity or divergent natural selection in generating local adaptation and preventing local maladaptation (Nicolaus and Edelaar 2018). Thus, demographic habitat selection can reinforce local adaptation, or adaptation to a habitat type, as well as limit future niche shifts.

Multiple predator effects on colonization can occur in at least two ways; predator cues could interact in

additive, synergistic, or antagonistic fashion to affect perceived risk level, or, alternatively, behavioral algorithms used to assess and respond to predation risk could interact in additive, synergistic, or antagonistic fashion. Either require the ability to detect, integrate, and respond appropriately to multiple simultaneous environmental cues. What is the expectation for combining non-consumptive effects of predators on colonization? For consumptive effects, expectations are shaped by the fact that a single individual can only be eaten once, so any prediction regarding multi-predator effects must at least consider effects of prey depletion. Debate has often focused on whether consumptive multi-predator effects are additive or multiplicative, and whether they generate predictable or unpredictable emergent effects (Wilbur and Fauth 1990, Soluk 1993, Sih et al. 1998, Vance-Chalcraft and Soluk 2005, McCoy et al. 2012). Consumptive effects are quantified and compared by determining survival rates with individual and combined predator species, but non-consumptive effects present a more complex problem. Non-consumptive effects can be manifested as behavioral, morphological, or life history responses, and each generates their own set of complexities when it comes to predicting the combined effects of multiple predators (Preisser and Bolnick 2008).

Our focus here is predator effects at the colonization stage; direct, non-consumptive effects, which, when they accomplish predator avoidance, obviate consumptive effects (Resetarits et al. 2005). Consumption and colonization share the characteristic of directly affecting number of individuals in a particular habitat patch, consumption by removal and colonization by redistribution. However, demographic habitat selection is unlike most other non-consumptive effects, in that it affects multiple habitat patches simultaneously, and thus, is also a metapopulation and metacommunity process. Colonization is largely binary; an individual can colonize only a single habitat patch. This is true for oviposition, where larvae have no ability to disperse, but also for many adult insects, especially beetles, that rarely undergo secondary dispersal because dispersal is energetically costly and many actually begin to lose wing muscles once they enter the water (Zera and Denno 1997). Thus, the questions are whether an individual is more likely to avoid a patch with multiple predator species than with a single predator species, how do organisms combine information from multiple predators into an adaptive response, and what is the limit to that ability? Colonization responses likely involve very different processes not amenable to simple predictions based on individual predator effects, but similarly involve saturation of responses, or emergent effects of species combinations (Sih et al. 1998, Schmitz 2007, Preisser and Bolnick 2008, McCoy et al. 2012, Davenport and Chalcraft 2013).

In aquatic systems, lentic habitat patches are often discrete and have either no surface aquatic connection to

other patches, or have connections that vary seasonally or temporally (Semlitsch and Bodie 1998). Thus, presence/absence of the dominant predator in freshwater habitats, fish, is relatively predictable and can inform decisions on colonization and/or oviposition that can facilitate behavioral avoidance (Wellborn et al. 1996, Wilbur 1997). However, both presence/absence and, especially, fish species composition, can vary greatly over time and space. Thus we were interested in three related questions. First, how does a diverse assemblage of colonizing aquatic insects respond to a variety of predatory fish species and species combinations? Second whether, and in what manner, do species respond differently to the non-consumptive presence of single vs. multiple predator species (species richness)? And third, how does variation in responses to fish species and species combinations translate into community-wide changes in the diversity and composition of colonists? This latter question has rarely been addressed for either consumptive or non-consumptive effects of diverse species assemblages in response to multiple, simultaneous predators in the same trophic level.

To address these questions we experimentally manipulated composition of the predator assemblage in aquatic mesocosms in a substitutive design (de Wit 1960), holding total predator density and biomass constant while manipulating species composition. Treatments consisted of either zero, one, two, or three predatory fish species at a constant total density and biomass, and we assayed the response of naturally colonizing aquatic insects to this variation in predator composition. We chose three fish species, representing the same trophic level, that covered a range of habitats; one benthic, one pelagic, and one surface feeding species. We hypothesized that predators within a trophic level with different habits and habitats were more likely to coexist in a three species assemblage, and would also be more likely to have synergistic effects on prey.

MATERIALS AND METHODS

Our experiment was conducted in a large old field at the University of Mississippi Field Station (UMFS), Lafayette County, Mississippi, USA. There is a diverse assemblage of aquatic insects at UMFS, including 132 species of aquatic beetles and over 40 species of aquatic hemipterans (Pintar and Resetarits 2020a, b). We set up five arrays (blocks), each with nine, 1,300-L (surface area 2.54 m²) cylindrical mesocosms ($N = 45$) laid out in isosceles trapezoids (Fig. 1a; Appendix S1: Fig. S1). We crossed the presence/absence of three species of fish: golden topminnows (*Fundulus chrysotus*), golden shiners (*Notemigonus crysoleucas*), and brown madtoms (*Noturus phaeus*) (Fig. 1b), which are among the most commonly encountered species at UMFS. Each species represents a different habitat/foraging strategy. *Notemigonus crysoleucas* is a small, pelagic, omnivorous-planktivorous, gape-limited fish that is widespread and

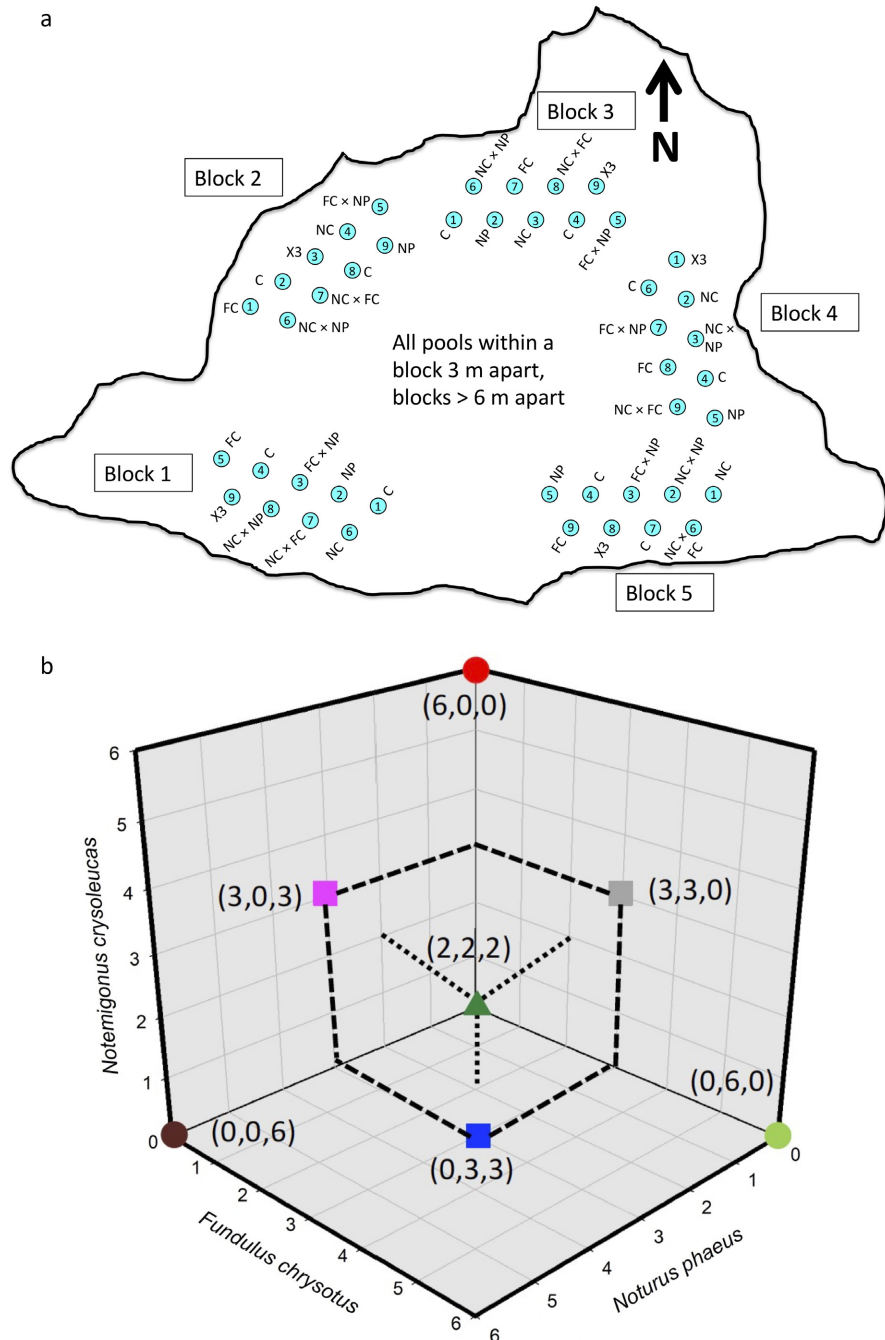


FIG. 1. (a) Schematic of experimental layout (not precisely to scale), indicating location of treatments within each block. Enclosing line indicates edge of an old field surrounded by forest. All patches within a block are separated by 3 m (see photos Appendix S1: Fig. S1). (b) Experimental design, de Wit replacement series crossing the presence/absence of three different fish species in a substitutive design, holding total fish density (6 fish/patch) and initial biomass constant (see Materials and Methods). Each of the seven fish treatments were compared individually to the control (not shown, falls at far apex, behind green triangle) using Dunnett's procedure. Three contrasts were conducted among classes of predator richness. Contrasts were (1) single-predator (circles) vs. multi-predator treatments (squares and triangle), (2) single predator (circles) vs. predator pairs (squares), and (3) single predator species (circles) vs. all three predator species (triangle). Key: *Notemigonus crysoleucas* (NC, red circle), *Fundulus chrysotus* (FC, light green circle), *Noturus phaeus* (NP, dark red circle), NC \times NP (pink square), NC \times FC (gray square), FC \times NP (blue square), X3 (all three predators, dark green triangle).

abundant in both lentic and lotic habitats (Lee et al. 1980). *Fundulus chrysotus* is a small, gape-limited, surface-feeding topminnow and is also widespread and abundant (Lee et al. 1980). *Noturus phaeus* is a small, benthic-foraging catfish typically found in lotic habitats (Lee et al. 1980, Chan and Parsons 2000), though is also common in overflow pools, backwaters, slow moving wetlands, and ponds that flood, including those at UMFS (W. J. Resetarits, M. R. Pintar, and J. R. Bohenek, *personal observation*); it is least gape-limited of the three.

We began filling mesocosms with well water on 11 May 2017, one block at a time, completing filling on 13 May, at which point 1-kg aliquots of mixed, dried, leaf litter (mixed hardwoods) were added to each mesocosm. Mesocosms were very quickly colonized by zooplankton and numerous small dipterans whose adults and/or eggs/larvae can pass through the screens (1.3×1.13 mm mesh), providing the fish a resource base. The high overall survival rate and positive growth of all three fish species supports the presence of an adequate food base. On 14 May, each patch received six fish: six per species in single-predator treatments, three each of two species in two-predator treatments, and two of each of three species in three-predator treatments, plus fishless controls. Density is on the lower end of biomass density in previous experiments and natural ponds, but above the threshold eliciting avoidance in many aquatic insects and treefrogs (Rieger et al. 2004, Resetarits and Binckley 2013). To equalize biomass among fish treatments within blocks, we created complementary (one “large,” one “small”) pairs within each of the three species for each block (by eye to minimize stress), and randomly assigned the appropriate number of pairs to each fish treatment patch within that block, maintaining the same density, approximate biomass, and size structure across patches within blocks. On 15 May, the experiment was begun by submerging screen lids to allow efficient collection of insects, and to separate fish from colonists, obviating consumptive effects (Appendix S1: Fig. S1). Dead fish (18/210) were replaced until 20 May, after which there was no observed mortality. Insects were collected weekly from 22 May until 20 November, with one final collection on 8 December, and preserved for later identification. Insect colonization is not responsive to either intra- or interspecific density, even when insects are not removed weekly (Pintar and Resetarits 2020c), and predation among adult insects is limited to predation by *Notonecta irrorata* on certain beetles, which always results in decapitation (Pintar and Resetarits 2021). There was no evidence of predation in this experiment, and *N. irrorata* occurred in only 77/1,260 samples (97 individuals). We have not observed any terrestrial or avian predators at our cattle tanks across dozens of experiments.

Insect identification followed Pintar and Resetarits (and sources therein) (Pintar and Resetarits 2020a, b). Only members of the genus *Sigara* (primarily

S. pectenata at UMFS) and *Paracymus* (primarily *P. subcupreus* at UMFS) were not all identified to species. Fish survival from 20 May to 8 December was 91% across all three species: 89% for *F. chrysotus*, 94% for *N. phaeus*, and 91% for *N. crysoleucas*. Distribution of mortality was such that only two patches did not hold their full treatment until the end of the experiment; one *F. chrysotus* \times *N. phaeus* (FC \times NP) patch had no surviving *F. chrysotus*, and one three predator patch (X3) also had no surviving *F. chrysotus*. We could not track fish survival during the experiment, so we cannot determine when mortality occurred. Since neither tank was an outlier or otherwise out of the ordinary, our assumption was that these tanks held their respective treatments for much of the experiment. Given the overall low fish mortality, this is not unreasonable. Thus, we included both in the final analyses to maintain the balanced design required for independent estimates of multivariate community location and dispersion using PERMANOVA and PERMDISP (Anderson and Walsh 2013).

Data analysis

We used a randomized complete block design crossing the presence/absence of three species of fish in a de Wit replacement series design (de Wit 1960) (Fig. 1b) with two replicates of fishless controls per block. Controls are hyper-replicated to facilitate use of Dunnett’s procedure for comparing all treatments to a control (Dunnett 1955). Abundance and species richness were transformed ($\sqrt{X+0.5}$) to meet the assumptions of ANOVA. We used general linear mixed-model ANOVA in PROC MIXED (SAS; SAS Institute, Cary, North Carolina, USA) on abundance for all insects, component higher taxa, and species with >150 colonists, as well as species richness and evenness, with treatment as a fixed effect and block as a random effect. Treatment means were compared using a one-tailed Dunnett’s procedure (with Dunnett-Hsu correction) for abundance and richness, and a two-tailed Dunnett’s for evenness, comparing all treatments to the control to examine specific preferences (Dunnett 1955, Hsu 1992). Absence of observed positive responses to fish treatments by colonizing insects in prior experiments (Binckley and Resetarits 2005, Resetarits and Binckley 2013, 2014, Resetarits and Pintar 2016, Resetarits et al. 2019) informed our hypothesis that fish effects would manifest as reduced abundance and richness, hence the more powerful one-tailed test (Rice and Gaines 1994, Ruxton and Neuhauser 2010). We had no a priori expectation for species evenness in response to fish. We also used three, one-tailed (two-tailed for evenness), a priori non-orthogonal contrasts to examine specific hypotheses relating to multi-predator effects. Contrasts were (1) single predator vs. multiple predator species, (2) single predator vs. predator species pairs, and (3) single predator species vs. all three predator species (Fig. 1b). All ANOVA-based analyses used SAS v. 9.4 (SAS Institute, Cary, North Carolina, USA) with Type III sums of squares and $\alpha = 0.05$.

To examine community composition for higher taxa (Dytiscidae, Hydrophilidae, Hemiptera), we performed a MANOVA on the relative abundances of the three groups, exclusive of the uncommon beetle families. We used PERMANOVA to test for differences in multivariate centroid location across treatments, and PERMDISP to examine differences in multivariate dispersion (β -diversity), using the lowest taxonomic level we could resolve, typically species. As a measure of location, PERMANOVA is robust to variation in dispersion for balanced designs, so we can examine both aspects of community composition (Anderson and Walsh 2013). We conducted analyses using the Bray-Curtis index, with both abundance and species composition, then visualized data using shade plots, a bubble plot, and nonmetric multidimensional scaling (NMDS). As in the ANOVA analyses we assessed both individual treatments to examine community responses to different fish species and combinations of fish species, and contrast groups to examine specific hypotheses regarding multi-predator effects. PERMANOVA, PERMDISP, NMDS plots, and shade plots used PRIMER 7 with PERMANOVA add-on (Clarke and Gorley 2015); bubble plot was created in Sigmaplot 14 (Inpixon HQ, Palo Alto, California, USA).

RESULTS

Our experiment was colonized by 5,961 insects of 66 species: 5,175 individuals of 57 species of aquatic beetle (Coleoptera, seven families) and 786 individuals of nine species of aquatic true bugs (Hemiptera, six families; Table 1). Hydrophilidae ($S = 24$) and Dytiscidae ($S = 23$) were the dominant beetles, comprising 82% of total species, and 97% of total individuals, with 158 individuals total from 10 species in five other families. The 57 species represents 43% of the 132 species of aquatic beetles known from UMFS (Pintar and Resetarits 2020b), the nine hemipterans represent 21% of the 43 species of aquatic hemipterans at UMFS (Pintar and Resetarits 2020a). Corixidae ($S = 2$) and Notonectidae ($S = 3$) were the dominant hemipterans, comprising 55% of total species, and 86% of total individuals, with 113 individuals total from 4 other families.

Responses to specific fish species and combinations

Insect abundance was highly responsive to fish treatments, with all fish treatments receiving significantly fewer colonists than controls, with the exception of the *N. phaeus* (NP) treatment (Table 2, Fig. 2). Results for Coleoptera were essentially the same, as beetles largely drove overall insect responses, thus we focus on all insects and specific component taxa going forward. Relative abundance of the three abundant higher taxa (Dytiscidae, Hydrophilidae, Hemiptera) differed significantly among treatments (Wilks' $\lambda = 0.100$, $F_{21,89.6} = 5.24$, $P < 0.0001$), driven largely by reduced

TABLE 1. List of the 66 taxa of Coleoptera and Hemiptera collected.

Taxon	Abundance
Coleoptera ($S = 57$)	5,175
Dytiscidae ($S = 23$)	2,015
<i>Copelatus glyphicus</i>	917
<i>Hydroporus rufilabris</i>	289
<i>Laccophilus fasciatus</i>	275
<i>Laccophilus proximus</i>	270
<i>Thermonectus basillaris</i>	97
<i>Uvarus lacustris</i>	29
<i>Neoporus undulatus</i>	27
<i>Hydaticus bimarginatus</i>	21
<i>Uvarus granarius</i>	17
<i>Desmopachria</i> sp.	14
<i>Copelatus chevrolati</i>	9
<i>Hydroporus pseudoniger</i>	9
<i>Agabus disintegratus</i>	8
<i>Meridiorhantus calidus</i>	8
<i>Bidessonotus inconspicuus</i>	7
<i>Hydrocolus oblitus</i>	6
<i>Platambus flavovittatus</i>	4
<i>Acilius mediatius</i>	2
<i>Thermonectus nigrofasciatus</i>	2
<i>Celina angustata</i>	1
<i>Celina hubbelli</i>	1
<i>Coptotomus longulus</i>	1
<i>Hydrovatus pustulatus</i>	1
Haliplidae ($S = 4$)	104
<i>Peltodytes sexmaculatus</i>	100
<i>Peltodytes dunavani</i>	2
<i>Haliplus triopsis</i>	1
<i>Peltodytes muticus</i>	1
Helophoridae ($S = 1$)	7
<i>Helophorus linearis</i>	7
Hydraenidae ($S = 1$)	5
<i>Hydraena marginicollis</i>	5
Hydrochidae ($S = 2$)	18
<i>Hydrochus</i> sp.	12
<i>Hydrochus rugosus</i>	6
Hydrophilidae ($S = 24$)	3,002
<i>Tropisternus lateralis</i>	1,226
<i>Berosus infuscatus</i>	780
<i>Tropisternus collaris</i>	342
<i>Paracymus</i> sp.	275
<i>Tropisternus blatchleyi</i>	167
<i>Enochrus ochraceus</i>	97
<i>Helochaes maculicollis</i>	55
<i>Enochrus pygmaeus</i>	16
<i>Berosus peregrinus</i>	8
<i>Berosus exiguus</i>	6
<i>Derallus altus</i>	5
<i>Hydrobiomorpha casta</i>	4
<i>Berosus sayi</i>	3
<i>Tropisternus natator</i>	3
<i>Cymbiodyta chamberlaini</i>	2
<i>Enochrus consors</i>	2
<i>Enochrus hamiltoni</i>	2
<i>Enochrus fimbriatus</i>	2

TABLE 1. Continued.

Taxon	Abundance
<i>Hydrochara soror</i>	2
<i>Berosus aculeatus</i>	1
<i>Berosus pantherinus</i>	1
<i>Cymbiodyta vindicata</i>	1
<i>Enochrus consortus</i>	1
<i>Helocombus bifidus</i>	1
Noteridae (<i>S</i> = 2)	24
<i>Hydrocanthus oblongus</i>	20
<i>Hydrocanthus antripennis</i>	4
Hemiptera (<i>S</i> = 9)	786
Belostomatidae (<i>S</i> = 1)	1
<i>Belastoma lutarium</i>	1
Corixidae (<i>S</i> = 2)	551
<i>Sigara</i> sp.	446
<i>Hesperocorixa</i> sp.	105
Gerridae (<i>S</i> = 1)	108
<i>Limnoporus canaliculatus</i>	108
Mesoveliidae (<i>S</i> = 1)	2
<i>Mesovelia</i> sp.	2
Nepidae (<i>S</i> = 1)	1
<i>Ranatra buenoi</i>	1
Notonectidae (<i>S</i> = 3)	123
<i>Notonecta irrorata</i>	97
<i>Buenoa</i> sp.	20
<i>Notonecta indica</i>	6

Note: *S* is the number of species, or in a few cases, genera.

relative abundance of dytiscids ($F_{7,33} = 26.10$, $P < 0.0001$) and increased relative abundance of hydrophilids ($F_{7,33} = 6.59$, $P < 0.0001$), but not hemipterans ($F_{7,33} = 1.50$, $P = 0.2003$), in fish treatments (Fig. 2).

For dytiscids, treatment had a highly significant effect on abundance, and all treatments significantly differed from controls, but the response to NP was weaker, with all other treatments generally similar (Table 2, Fig. 3a). Among the most abundant dytiscids, both *Copelatus glyphicus* and *Hydroporus rufilabris* showed a significant main effect of treatment (Table 2) and had reduced numbers in all fish treatments, with a weaker response in the NP treatment (nonsignificant for *H. rufilabris*; Fig. 3b, c). *Laccophilus fasciatus* showed a significant main effect of treatment (Table 2) and had significantly reduced abundance in all treatments except *Notemigonus crysoleucas* (NC), NP, and *F. chrysotus* × *N. phaeus* (FC × NP), which was marginally nonsignificant (Fig. 3 d); *L. proximus* showed a significant main effect of treatment (Table 2) and significantly reduced abundance in all treatments except NP (Fig. 3e).

For hydrophilids, treatment had a significant effect on abundance (Table 2), but only the FC × NP and X3 treatments were significantly different from Controls (Fig. 4a); hydrophilid responses to fish were limited and weaker overall. *Tropisternus lateralis* showed a significant main effect of treatment (Table 2), and along with *Berosus infuscatus* had significant reduction in

TABLE 2. Main effect of treatment for mixed-model ANOVA on square-root ($x + 0.5$)-transformed abundance data for higher taxa and species with >150 colonists.

Taxon	$F_{7,33}$	Pr > F
All insects	9.07	<0.0001
Coleoptera	8.66	<0.0001
Dytiscidae	24.98	<0.0001
<i>Copelatus glyphicus</i>	18.15	<0.0001
<i>Hydroporus rufilabris</i>	7.98	<0.0001
<i>Laccophilus fasciatus</i>	5.25	0.0004
<i>Laccophilus proximus</i>	8.71	<0.0001
Hydrophilidae	2.65	0.0271
<i>Tropisternus lateralis</i>	2.58	0.0309
<i>Berosus infuscatus</i>	1.72	0.1395
<i>Tropisternus collaris</i>	0.88	0.5337
<i>Paracymus</i>	5.36	0.0004
<i>Tropisternus blatchleyi</i>	1.01	0.4441
Hemiptera	2.13	0.0675
Corixidae		
<i>Sigara</i>	4.28	0.0018

Notes: Results of Dunnett's procedure comparing all treatments to controls are presented in Figs. 2–4, with additional information in Appendix S1: Table S1. Significant effects are highlighted in boldface type and marginally nonsignificant effects in italic type.

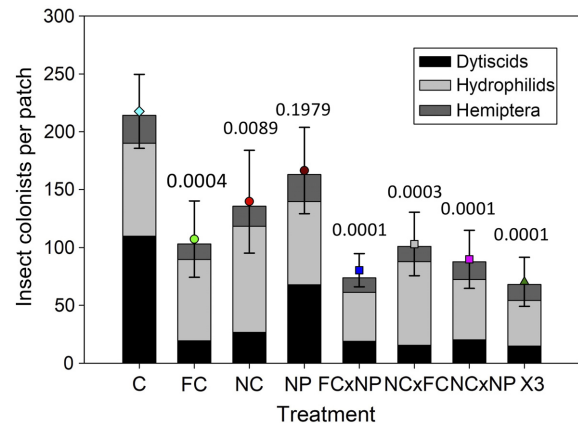


FIG. 2. Untransformed treatment means (\pm SE) and P values for one-tailed Dunnett's procedure (with Dunnett-Hsu correction) comparing each treatment to the control for abundance of all insects, illustrating absolute and relative abundance of the three dominant taxonomic groups. *N* is total abundance. Treatment codes: *Fundulus chrysotus* (FC), *Notemigonus crysoleucas* (NC), *Noturus phaeus* (NP), *F. chrysotus* × *N. phaeus* (FC × NP), *N. crysoleucas* × *F. chrysotus* (NC × FC), *N. crysoleucas* × *N. phaeus* (NC × NP), X3 (all three predators). Main effects in Table 2.

abundance in only the X3 treatment (Fig. 4b, c). *Tropisternus collaris* and *T. blatchleyi* showed no significant main effect of treatment (Table 2) and did not respond to any of the fish treatments (Fig. 4d, f). In striking contrast, *Paracymus* sp. showed a significant main effect of treatment (Table 2) and significantly reduced

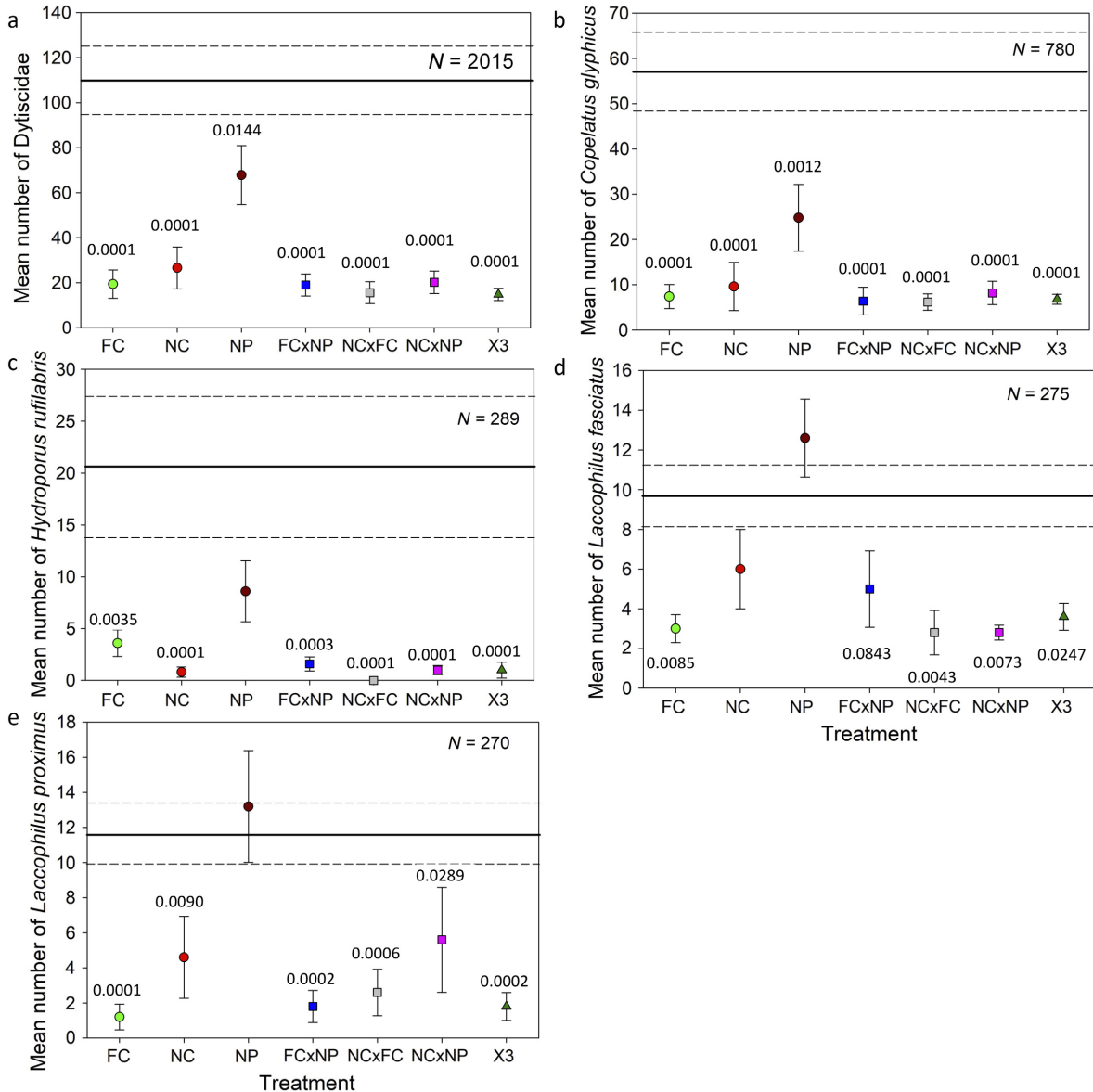


FIG. 3. Results of Dunnett's procedure (one-tailed) comparing each treatment to the control for untransformed abundance of (a) all dytiscids, (b) *Copelatus glyphicus*, (c) *Hydroporus rufilabris*, (d) *Laccophilus fasciatus*, (e) *Laccophilus proximus*, showing means \pm SE and P values with Dunnett-Hsu correction. Solid and dashed lines represent the mean value for control patches \pm SE, N is total abundance. Treatment codes as in Fig. 2. Full table of P values is in Appendix S1: Table S1. Main effects in Table 2.

colonization of all treatments except NC (marginally nonsignificant), and NC \times NP (Fig. 4e).

Total abundance of Hemiptera showed a marginally nonsignificant main effect of treatment (Table 2), a significant reduction in the FC \times NP treatment, and a marginally nonsignificant reduction in NC \times FC and X3 treatments, although abundances were reduced in most fish treatments relative to controls (Fig. 4g). This was driven largely by members of the genus *Sigara*, which comprised well over half the total number of hemipterans. *Sigara* sp. showed a significant main effect of

treatment (Table 2), and abundance was significantly reduced relative to Controls for the FC, FC \times NP and X3 treatments, with a marginally nonsignificant reduction in the NC \times FC treatment (Fig. 4h).

Community-level metrics and individual treatments

In the results below, we specifically focus on species richness as the metric of interest, but the observed variation in richness was driven by significant variation in abundance for all higher taxa. Including abundance as a

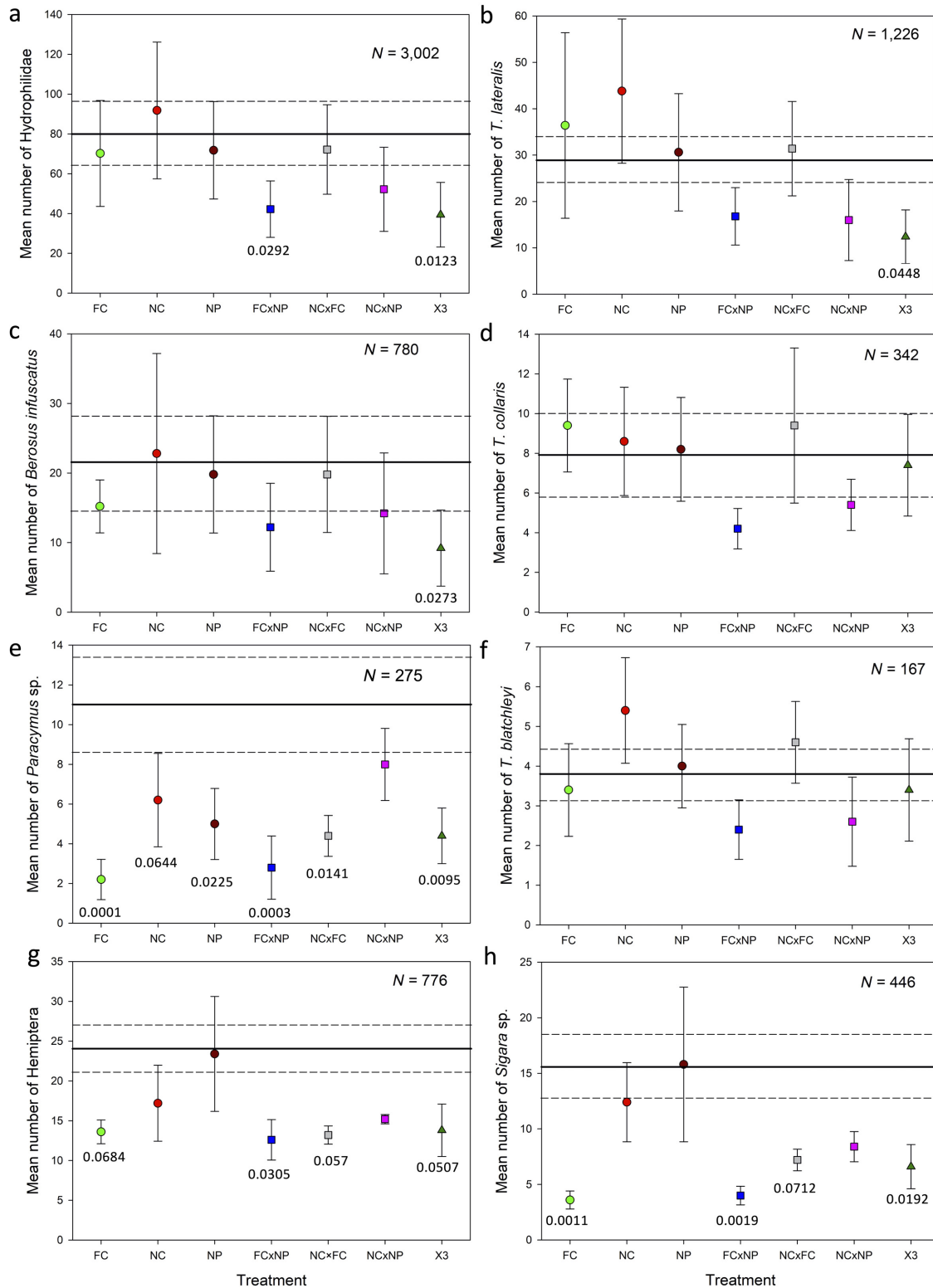


FIG. 4. Results of Dunnett's procedure (one-tailed) comparing each treatment to the control for untransformed abundance of (a) all hydrophilids, (b) *Tropisternus lateralis*, (c) *Berosus infuscatus*, (d) *Tropisternus collaris*, (e) *Paracymus* sp., (f) *Tropisternus blatchleyi*, (g) all hemipterans, (h) *Sigara* sp., showing means \pm SE and P values with Dunnett-Hsu correction. Solid and dashed lines represent the mean value for control patches \pm SE, N is total abundance. Treatment codes as in Fig. 2. Full table of P values in Appendix S1: Table S1. Main effects in Table 2.

covariate (Table 3) eliminated the significant richness differences for both all insects and dytiscids. Treatments differed highly significantly in species richness of all insects (Table 3); richness was significantly lower than controls in all multi-species treatments, and was marginally nonsignificant in FC and NC treatments (Fig. 5a). Evenness showed a marginally nonsignificant main effect of treatment (Table 3), and was significantly higher in the X3 treatment (Appendix S1: Fig. S2a). For dytiscids, there was highly significant main effect on both species' richness and evenness (Table 3). Richness was significantly reduced in all but the NP treatment compared to controls (Fig. 5b), while evenness was higher and either significant or marginally nonsignificant for all treatments (Appendix S1: Fig. S2b). Treatment did not have a significant main effect on hydrophilid species richness (Table 3), but species richness was significantly lower than controls for the FC \times NP and X3 treatments (Fig. 5c). There was a highly significant main effect of treatment on evenness (Table 3), though evenness was not significantly lower in any single treatment vs. controls (Appendix S1: Fig. S2c). Hemipterans showed no significant overall variation in species richness (Table 3), and no treatments were different from controls (Fig. 5d). There was significant variation in evenness (Table 3), with FC significantly higher and X3 marginally nonsignificantly higher than controls (Appendix S1: Fig. S2d).

Shade plots (Fig. 6) illustrate the variation in species composition among treatments for all insects (Fig. 6a) and the dramatic difference in the pattern for dytiscids (Fig. 6b) vs. hydrophilids (Fig. 6c). Dytiscids show

considerable variation across treatments both between controls and fish treatments, and among the fish treatments, while hydrophilids show much more consistent species composition and abundance overall. The pattern for hemipterans is largely driven by the most abundant taxon, *Sigara* sp. (Appendix S1: Fig. S4a).

For all insects, PERMANOVA revealed a significant effect of treatment on multivariate location (Table 4), with most of the variation occurring between controls and fish treatments, rather than among fish treatments, all of which were significantly different from controls and showed clear differences in the shade plot (Fig. 6a), and little or no overlap in the NMDS plot except in the C vs. NP comparison (Appendix S1: Fig. S6a); C vs. NP showed the greatest similarity, while the C vs. X3 comparison had the least (Table 4). Controls had the highest within treatment similarity (69.15), while FC \times NP had the lowest (48.78). Multivariate dispersion (β -diversity) was not significant for treatments overall, but FC \times NP was significantly different from Controls, with NC and NC \times NP marginally nonsignificant. Controls had the lowest dispersion, while FC \times NP had the highest (Table 4, Fig. 6a; Appendix S1: Fig. S6a).

Multivariate location was significantly different among treatments for dytiscids, with all treatments significantly different from controls (Table 4, Fig. 6b), however the NP treatment was much more similar to controls than other fish treatments and the only treatment overlapping Controls in NMDS plots (Appendix S1: Fig. S6b). C vs. NP showed the greatest similarity, while the C vs. X3 comparison had the least (Table 5, Fig. 6b; Appendix S1: Fig. S6b). Controls had the most

TABLE 3. Main effects of mixed-model ANOVA type 3 tests of fixed effects based on specific treatments for square-root transformed ($\sqrt{x + 0.5}$) species richness (Fig. 6) and untransformed evenness (Appendix S1: Fig. S2) for the major taxonomic groupings.

Factor	df		Species richness		Evenness	
	Numerator	Denominator	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Insects						
Treatment alone	7	33	4.98	0.001	2.00	<i>0.0851</i>
Abundance	1	32	75.65	<0.0001		
Treat w/cov	7	32	0.71	0.6614		
Dytiscidae						
Treatment	7	33	7.21	<0.0001	4.62	0.0011
Abundance	1	32	41.23	<0.0001		
Treat w/cov	7	32	1.69	0.1471		
Hydrophilidae						
Treatment	7	33	1.64	0.1596	2.97	0.0159
Abundance	1	32	10.84	0.0024		
Treat w/cov	7	32	0.77	0.6163		
Hemiptera						
Treatment	7	33	0.67	0.699	3.19	0.0112
Abundance	1	32	18.4	0.0002		
Treat w/cov	7	32	0.6	0.7519		

Notes: For each taxon, there are two species richness analyses: one with treatment effect only and one examining treatment with abundance included as a covariate (w/cov). Significant effects are highlighted in boldface type and marginally nonsignificant effects in italic type.

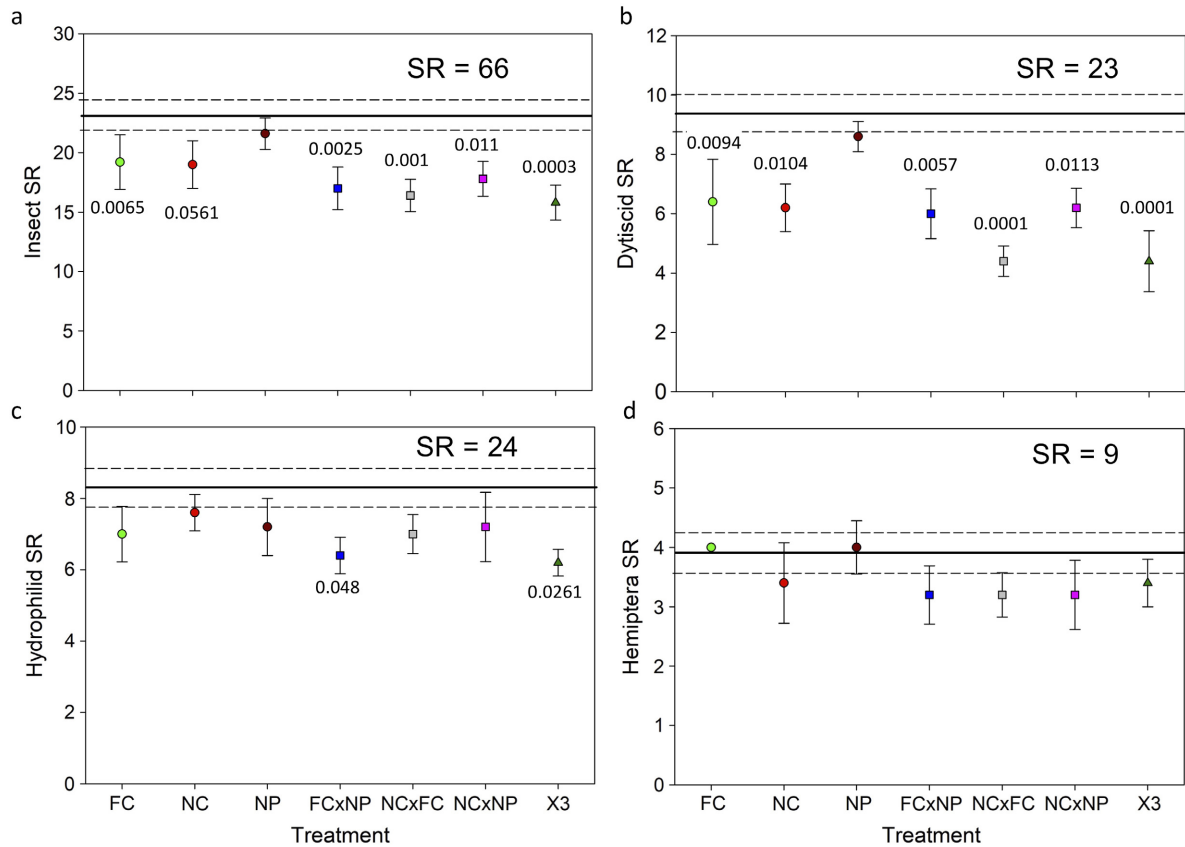


FIG. 5. Results of Dunnett's procedure (one-tailed) comparing each treatment to controls for untransformed species richness (SR) of (a) all insects, (b) dytiscids, (c) hydrophilids, (d) hemipterans, showing means \pm SE, and P values with Dunnett-Hsu correction. Solid and dashed lines represent the mean value for control patches \pm SE, SR is total species richness. Treatment codes as in Fig. 2. Main effects in Table 3.

internal similarity (71.86) while the FC \times NP had the least (40.85). Multivariate dispersion (β -diversity) was significant for treatment, with FC, NC, FC \times NP treatments showing significantly greater dispersion than controls, and NC \times FC marginally different (Table 4, Fig. 6b; Appendix S1: Fig. S6b). Controls had the lowest dispersion, while FC \times NP had the highest.

Multivariate location was not significantly different among treatments for hydrophilids, and no treatments were significantly different from controls (X3 was marginally nonsignificant); C vs. NC \times FC showed the greatest similarity, while the C vs. X3 comparison had the least (Table 4, Fig. 6c; Appendix S1: Fig. S6c). Controls had the greatest internal similarity (69.91) while the NC \times NP had the least (57.14). Multivariate dispersion (β -diversity) was also nonsignificant for treatments, with only X3 showing a marginal difference from controls; NC \times FC had the lowest dispersion, X3 had the highest (Table 4, Fig. 6c; Appendix S1: Fig. S6c).

Multivariate location was significantly different among treatments for Hemiptera, but only the FC treatment was significantly different from controls, with NC

\times FC and X3 marginally nonsignificant; C vs. NC \times NP showed the greatest similarity, while the C vs. FC \times NP comparison had the least (Table 4; Appendix S1: Figs. S4a, S6d). NC \times FC had the greatest internal similarity (68.99) while the NP had the least (54.16). Treatment was not significant for multivariate dispersion, with no treatments significantly different from controls; NC \times FC had the lowest dispersion, while FC \times NP had the highest (Table 4; Appendix S1: Figs. S4a, S6d).

Single vs. multi-predator effects: a priori contrasts

In the significant and marginally nonsignificant contrasts described below for abundance, multi-predator contrast groups were lower in all cases. For abundance, all three contrasts were significant for all insects, dytiscids and hydrophilids, while no contrasts were significant for hemipterans (Figs. 7, 8a, 9a, g; Appendix S1: Table S2). For dytiscids, contrasts were significant or marginally nonsignificant for all four species, except for the single vs. all three species (SvsX3) for *C. glyphicus*, though the means and standard error for the paired (X2)

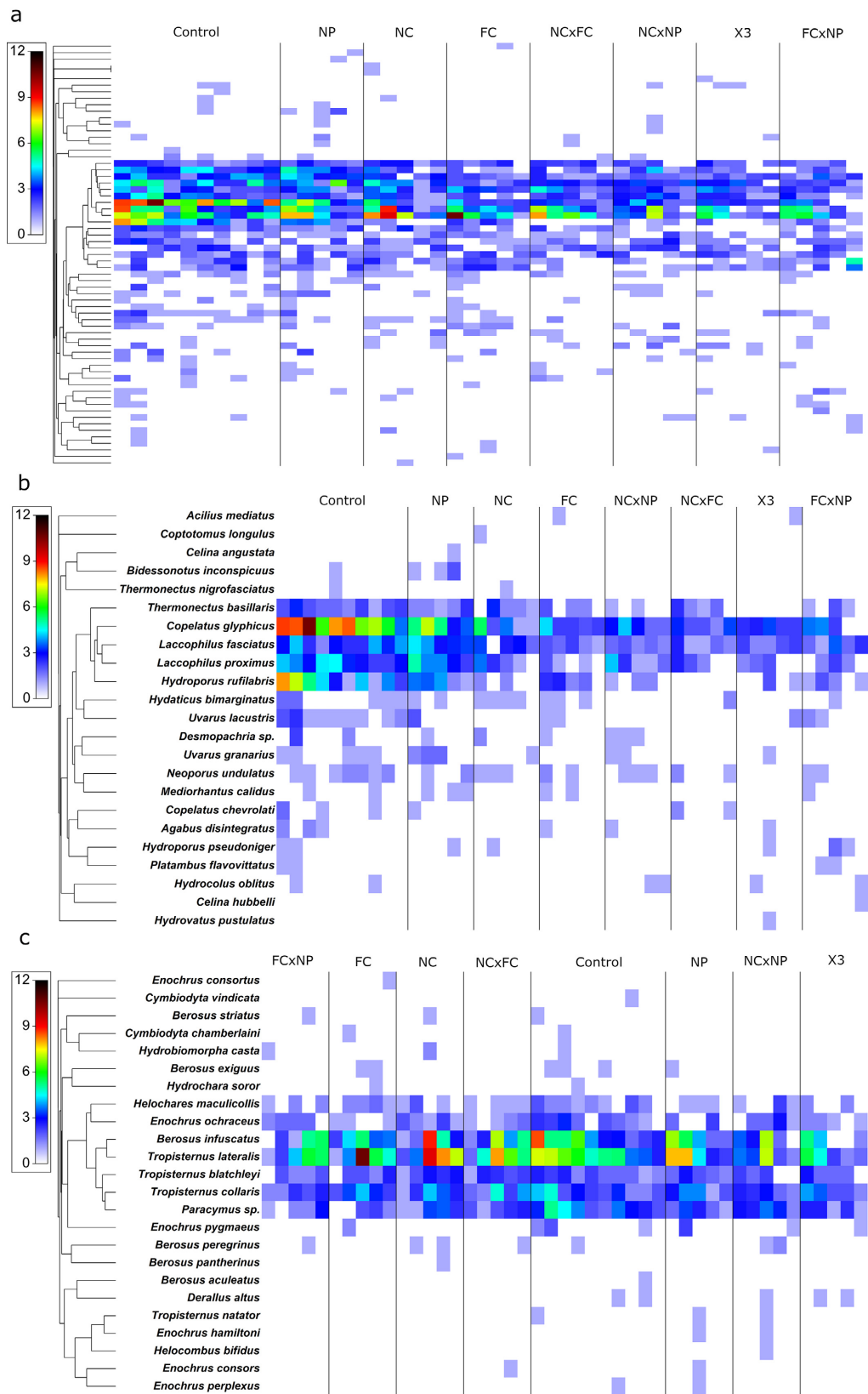


FIG. 6. Shade plot (heat map) showing square-root-transformed abundances by treatment for (a) all insects, (b) dytiscids, and (c) hydrophilids. Warmer colors indicate greater abundance. Treatments ordered by similarity, and species order on y-axis based on

(FIG. 6. *Continued*)

similarity of distribution (see Table 4 for PERMANOVA and PERMDISP results, species similarity key for all insects in Appendix S1: Fig. S3, NMDS plots in Appendix S1: Fig. S6). Note that treatment order differs slightly between panels a and b, and is very different in panel c. Treatment codes as in Fig. 2. Shade plot for hemipterans in Appendix S1: Fig. S4a.

and X3 treatments were almost identical (Fig. 8b–e; Appendix S1: Table S2). This is likely the result of reduced power with only five replicates for X3 in the SvsX3 contrast vs. 15 for S and X2 in the SxX2 contrast and 20 replicates for multi in the SvsM (single vs. multiple predator) contrast. For hydrophilids all three contrasts were significant, while for individual species all contrasts were significant for *T. lateralis*, SvsM and SvsX3 were significant for *B. infuscatus*, and SvsM and SvsX2 were marginally nonsignificant for *T. collaris* and *T. blatchleyi*; none of the contrasts were significant for *Paracymus* sp., which showed strong, similar avoidance of all single-predator and multi-predator treatments (Fig. 9b–f; Appendix S1: Table S2). Single vs. M and SvsX2 were marginally nonsignificant for *Sigara* sp. (Fig. 9h; Appendix S1: Table S2).

Multiple predators and community metrics

In the significant and marginally nonsignificant contrasts described here for species richness, multiple predator contrast groups were lower in all cases. For species richness of all insects and dytiscids, all three contrasts were significant, while for hydrophilids the X3 was marginally nonsignificant and for hemipterans both the SvsM and SvsX2 contrasts were marginally nonsignificant (Fig. 10a–d; Appendix S1: Table S3). For evenness, only the SvsX3 contrast was significant for all insects, and all three contrasts were significant for Hydrophilidae, while dytiscids had only a marginally nonsignificant contrast for SvsX3, and hemipterans had no significant contrasts (Appendix S1: Table S3, Fig. S5). In the significant and marginally nonsignificant contrasts for

TABLE 4. PERMANOVA (multivariate community location) and PERMDISP (multivariate community dispersion— β -diversity) results based on Bray-Curtis similarity in square-root-transformed abundance for treatment level effects.

Taxon	Pseudo- <i>F</i>	<i>P</i> (perm)	C	FC	NC	NP	FC × NP	NC × FC	NC × NP	X3
PERMANOVA										
Insects	2.409	0.001								
Coleoptera										
Dytiscidae	3.778	0.001								
Hydrophilidae	1.154	0.305								
Hemiptera	1.766	0.028								
Average similarity between treatments and Control (pseudo-Dunnett's)										
Insects				56.82	58.07	65.25	53.57	57.01	56.53	52.36
Coleoptera										
Dytiscidae				47.94	48.71	67.52	43.91	42.32	47.99	40.28
Hydrophilidae				65.21	67.03	67.16	63.2	70.68	64.53	<i>61.87</i>
Hemiptera				57.45	62.72	60.67	57.13	<i>61.24</i>	63.27	<i>61.51</i>
PERMDISP										
Taxon										
Insects	1.54	0.444								
Coleoptera										
Dytiscidae	3.948	0.022								
Hydrophilidae	0.745	0.845								
Hemiptera	0.469	0.966								
Comparison of average deviation from centroid between treatments and control (pseudo-Dunnett's)										
Insects			21.34	23.85	27.77	24.30	31.61	23.02	<i>26.74</i>	26.09
Coleoptera										
Dytiscidae			18.87	27.24	31.98	20.29	37.73	26.83	24.17	24.13
Hydrophilidae			20.37	24.31	24.17	25.09	26.33	19.45	26.93	<i>27.09</i>
Hemiptera			24.58	20.67	27.23	28.64	28.76	20.39	23.66	21.24

Notes: Treatment codes are *Fundulus chrysotus* (FC), *Notemigonus crysoleucas* (NC), *Noturus phaeus* (NP), *F. chrysotus* × *N. phaeus* (FC × NP), *N. crysoleucas* × *F. chrysotus* (NC × FC), *N. crysoleucas* × *N. phaeus* (NC × NP), X3 (all three predators). Significant effects are highlighted in boldface type and marginally nonsignificant effects in italic type. Hyper replication of controls (2×) should slightly bias PERMDISP against finding differences in dispersion, given dispersion is lowest in controls, so results for dispersion should be viewed as conservative. See shade plots in Fig. 6; Appendix S1: Fig. S4 and NMDS plots in Appendix S1: Fig. S6.

TABLE 5. PERMANOVA (multivariate community location) and PERMDISP (multivariate community dispersion— β -diversity) results based on Bray-Curtis similarity in square-root-transformed abundance for contrast-level effects.

Taxon	PERMANOVA, contrasts						PERMDISP, pseudo-contrasts					
	Single vs. Multi		Single vs. X2		Single vs. X3		Single vs. Multi		Single vs. X2		Single vs. X3	
	Pseudo-	<i>P</i>	Pseudo-	<i>P</i>	Pseudo-	<i>P</i>	<i>t</i>	<i>P</i>	<i>t</i>	<i>P</i>	<i>t</i>	<i>P</i>
	<i>F</i>	(perm)	<i>F</i>	(perm)	<i>F</i>	(perm)		(perm)		(perm)		(perm)
Insects	4.558	0.017	2.976	<i>0.053</i>	3.589	0.012	0.280	0.818	0.249	0.821	0.707	0.597
Coleoptera												
Dytiscidae	8.998	0.002	4.690	0.013	5.817	0.005	0.228	0.832	0.437	0.699	1.525	0.209
Hydrophilidae	2.087	0.172	1.462	0.282	2.190	0.110	0.292	0.798	0.006	0.996	0.335	0.795
Hemiptera	2.093	0.130	1.702	0.202	2.862	0.037	0.788	0.511	0.675	0.583	1.142	0.396

Notes: Significant effects are highlighted in boldface type and marginally nonsignificant effects in italic type. Because of the strongly asymmetrical replication for the SvsX3 contrast, PERMDISP should be biased in favor of finding a difference in dispersion, yet none was detected. See shade plots in Fig. 11; Appendix S1: Fig. S4 and NMDS plots in Appendix S1: Fig. S7.

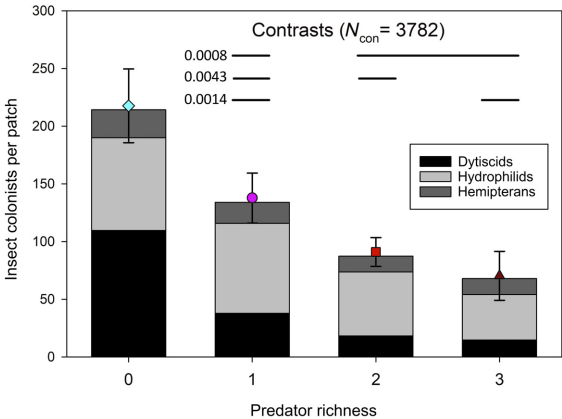


FIG. 7. Contrast group means (means \pm SE) and *P* values for one-tailed a priori contrasts for untransformed abundance of all insects, showing control values for comparison and the absolute and relative abundances of the three dominant taxa. N_{con} is the total number of individuals in the contrast treatments; bars above treatment groups indicate which treatment groups contribute to each contrast. Contrasts are (from top) single predator vs. multi-predator, single predator vs. paired predators, and single predator vs. all three predators. Main effects in are in Table 2.

evenness, multiple predator contrast groups had greater evenness in all cases. Relative abundance of the three abundant higher taxa (Dytiscidae, Hydrophilidae, Hemiptera) did not differ significantly among levels of predator richness (1–3; Wilks' $\lambda = 0.8503$, $F_{6,52} = 0.73$ $P = 0.6258$), as the bulk of the observed variation occurring between the controls and all fish treatments in dytiscids (Fig. 7).

Shade plots illustrate the variation in species composition across contrast groups (controls shown for comparison) for all insects (Fig. 11a), and illustrate the difference in the pattern for dytiscids (Fig. 11b), which show considerable variation across single vs. multiple predators groups as well as between controls and fish

treatments, and the hydrophilids (Fig. 11c), which show much more consistent species composition and abundance overall. The pattern for hemipterans is again largely driven by the most abundant taxon, *Sigara* sp. (Appendix S1: Fig. S4b). For measures of multivariate location the SvsM and SvsX3 contrasts were significant for all insects and dytiscids, with the SvsX2 also significant for the latter (Table 5, Fig. 11a, b; Appendix S1: Fig. S7a, b). Hydrophilidae showed no significant effects of multiple predators, and for Hemiptera only the SvsX3 was significant (Table 5, Fig. 11c; Appendix S1: Figs. S4b, S7c, d). No contrasts were significant for multivariate dispersion for any of the four higher taxa (Table 5, Fig. 11a–c; Appendix S1: Fig. S4b), but multiple predator treatments had greater dispersion, both collectively and individually, vs. controls for all but the hemipterans (Appendix S1: Fig. S7).

DISCUSSION

Potential prey responses to predators, predator combinations, and predator richness can take a variety of forms (Sih et al. 1998, Peckarsky et al. 2008, Preisser and Bolnick 2008, Kishida et al. 2010, Vonesh and Blaustein 2011, Buxton and Sperry 2017). Here we focus on colonization, where species may vary in their ability to detect relevant predator cue or cues, or may vary in whether and how they respond to cue(s). In studies of colonization, avoidance is evidence of detection, but lack of avoidance provides no evidence on detection: we cannot determine whether relevant cues register with receivers except by observing a response. In our system, if fish produce species-specific cues, insect colonists may vary in detection or response to each cue, and possibly to combinations of cues. Thus, we should accumulate more insect species showing avoidance as we increase fish species richness, and thus greater effects on species composition and community structure, so long as specific cue intensities are above the detection threshold of at least some colonists. Last, we may see emergent

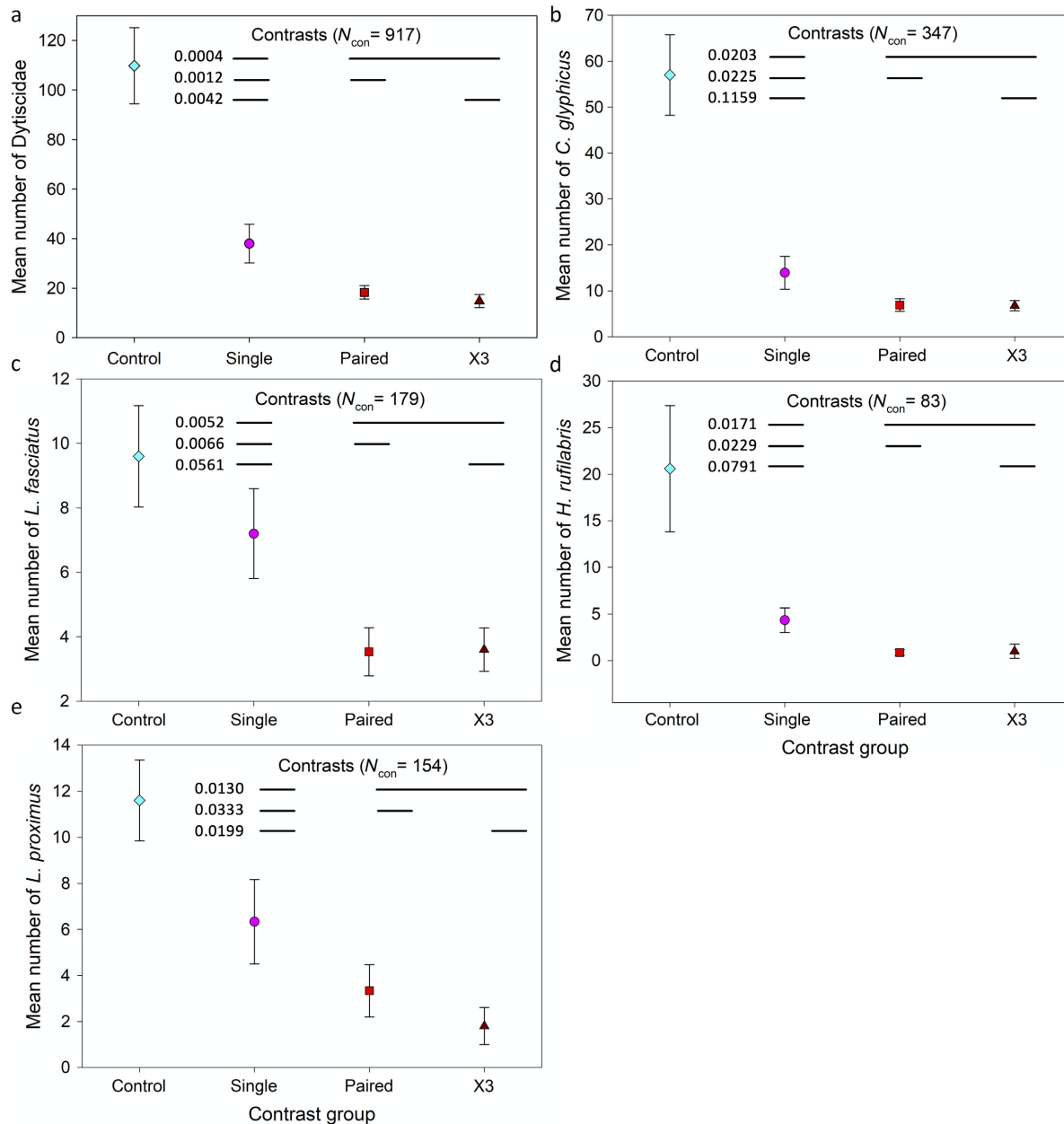


FIG. 8. Contrast group means (means \pm SE) and P values for one-tailed a priori contrasts for untransformed abundance of (a) all dytiscids, (b) *Copelatus glypticus*, (c) *Laccophilus fasciatus*, (d) *Hydroporus rufilabris*, and (e) *Laccophilus proximus*, showing control values (open circles) for comparison. N_{con} is the total number of individuals in the contrast treatments; bars above treatment groups indicate which treatment groups contribute to each contrast. Contrasts are (from top) single predator vs. multi-predator, single predator vs. paired predators, and single predator vs. all three predators. Main effects in Table 2.

synergistic or antagonistic multi-predator effects (Sih et al. 1998, Schmitz 2007, Preisser and Bolnick 2008, McCoy et al. 2012, Davenport and Chalcraft 2013), mediated either by interactions among the cues themselves, interactions among the predators, or by an interaction effect of simultaneous detection of multiple predators.

Behavioral responses to fish species and species combinations

On a proximate level, we observed significant variation in whether and how insect taxa responded to fish, as well as variation in which fish species and combinations of fish species generated responses. Based on early

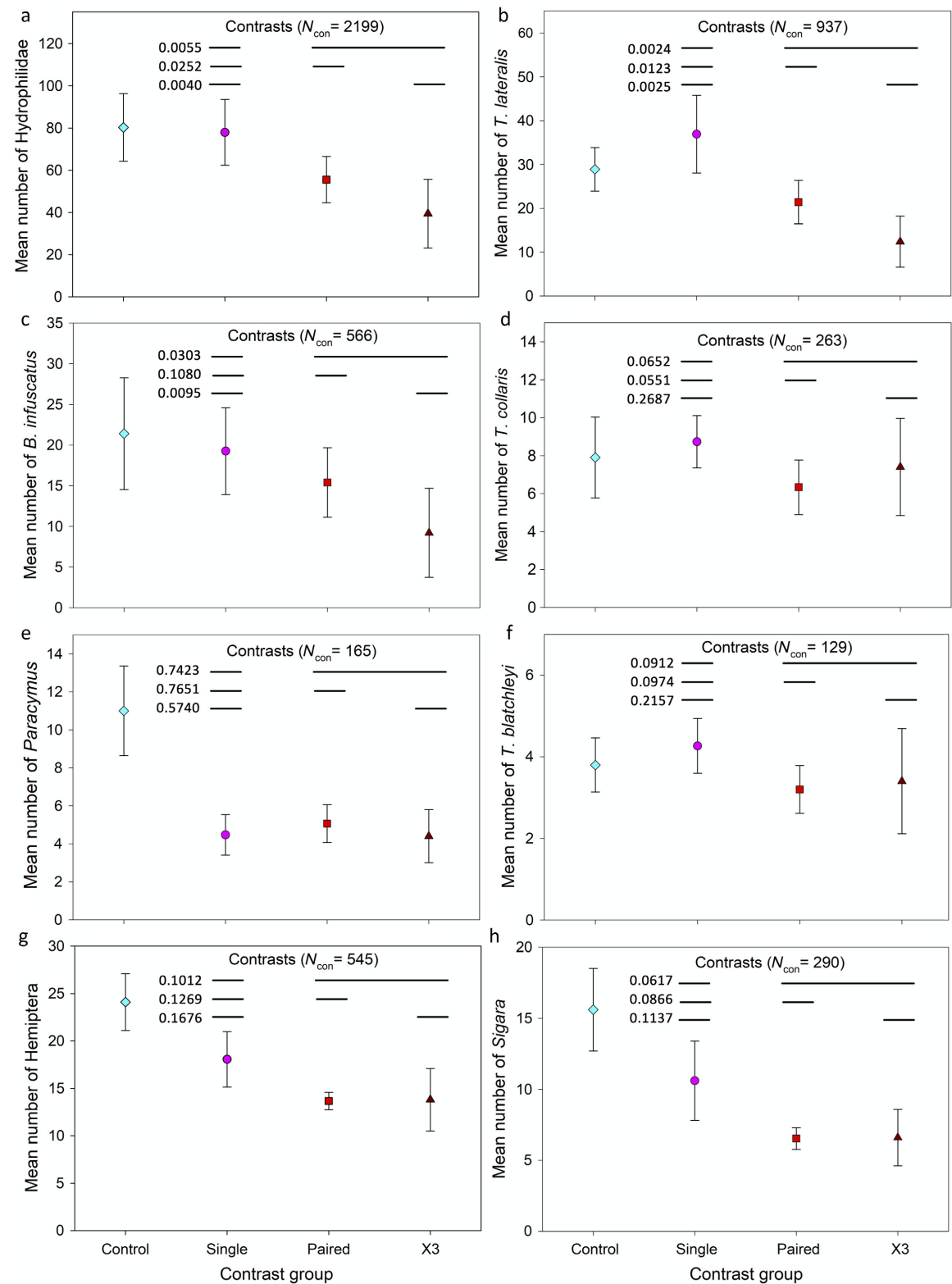


FIG. 9. Contrast group means (means \pm SE) and *P* values for one-tailed a priori contrasts for untransformed abundance of (a) all hydrophilids, (b) *Tropisternus lateralis*, (c) *Berosus infuscatus*, (d) *Tropisternus collaris*, (e) *Paracymus* sp., (f) *Tropisternus*

(FIG. 9. *Continued*)

blatchleyi, (g) all hemipterans, and (h) *Sigara* sp., showing control values (open circles) for comparison. N_{con} = total number of individuals in the contrast treatments; bars above treatment groups indicate which treatment groups contribute to each contrast. Contrasts are (from top) single predator vs. multi-predator, single predator vs. paired predators, and single predator vs. all three predators. Main effects in Table 2.

experiments, our working hypothesis has been that there is a generalized fish kairomone(s), and species responsive to this kairomone signature avoid all fish, though response strength varies. Limited characterization of fish kairomones now indicates that they are both very difficult to chemically identify and may be taxonomically unique (Akkas et al. 2009), and work on other predator-released kairomones suggests they are both sender and receiver specific (Silberbush et al. 2010, Selander et al. 2015, Poulin et al. 2018, Weiss et al. 2018, Hahn et al. 2019). This, along with variation observed here and elsewhere (Resetarits and Pintar 2016), now strongly supports species-specific cues. One paradox is that the presence and strength of avoidance often does not match

our perception of predation risk, though we rarely have a complete picture of predation risk across all life stages (Wilbur 1988, Krensek and Rudolf 2014, Rudolf et al. 2014). Variation observed here also reflects an emerging pattern of variation among insects in colonization responses to a variety of biotic and abiotic factors, including predators, patch size, temperature, and community assembly (Kraus and Vonesh 2010, Resetarits and Pintar 2016, Resetarits et al. 2019, McNamara et al. 2021).

Here, insect abundance was highly responsive to fish, with all but *N. phaeus* avoided, and species richness was significantly reduced in all but the *N. phaeus* treatment, resulting in it being most similar to the controls in both

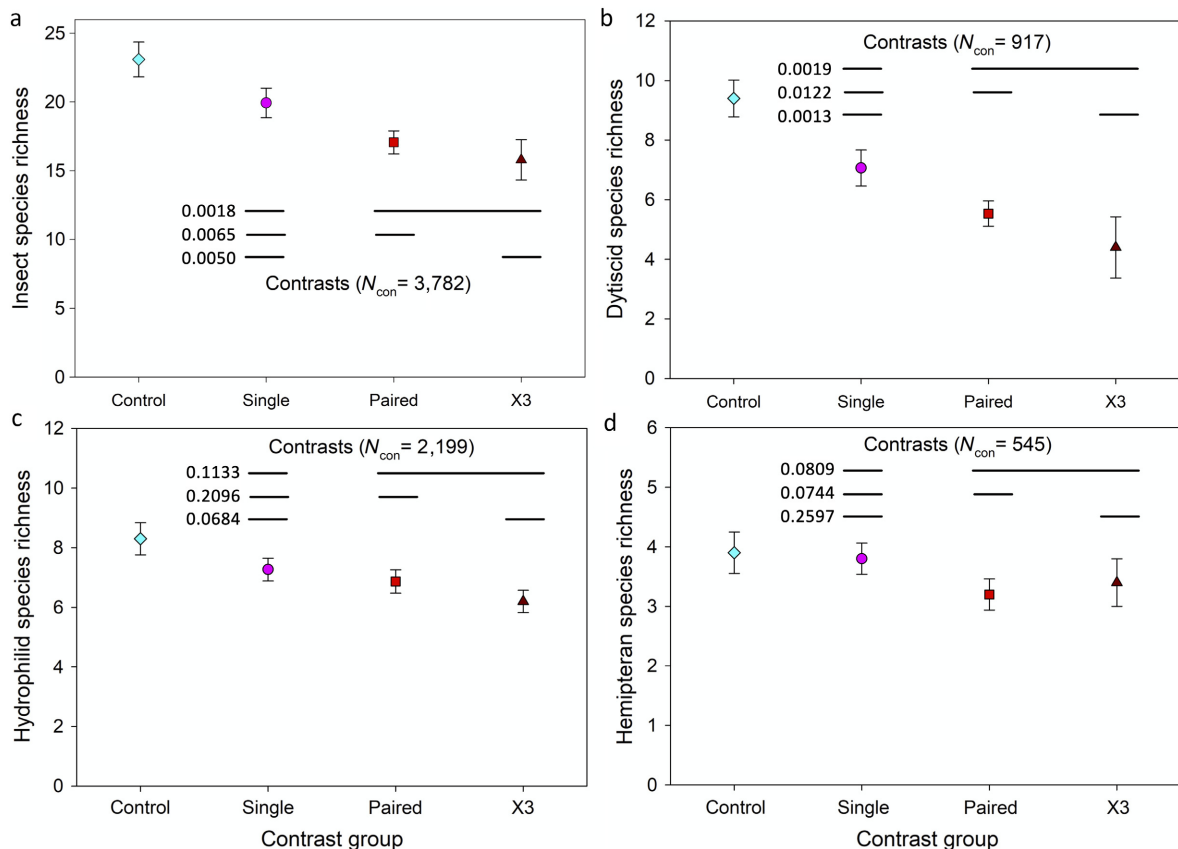


FIG. 10. Contrast group means (means \pm SE) and P values for one-tailed a priori contrasts for untransformed species richness (controls [open circles] shown for reference) for (a) all insects, (b) Dytiscidae, (c) Hydrophilidae, and (d) Hemiptera. N_{con} is the total number of individuals in the contrast treatments; bars above treatment groups indicate which treatment groups contribute to each contrast. Contrasts are (from top) single predator vs. multi-predator, single predator vs. paired predators, and single predator vs. all three predators. Main effects in Table 3.

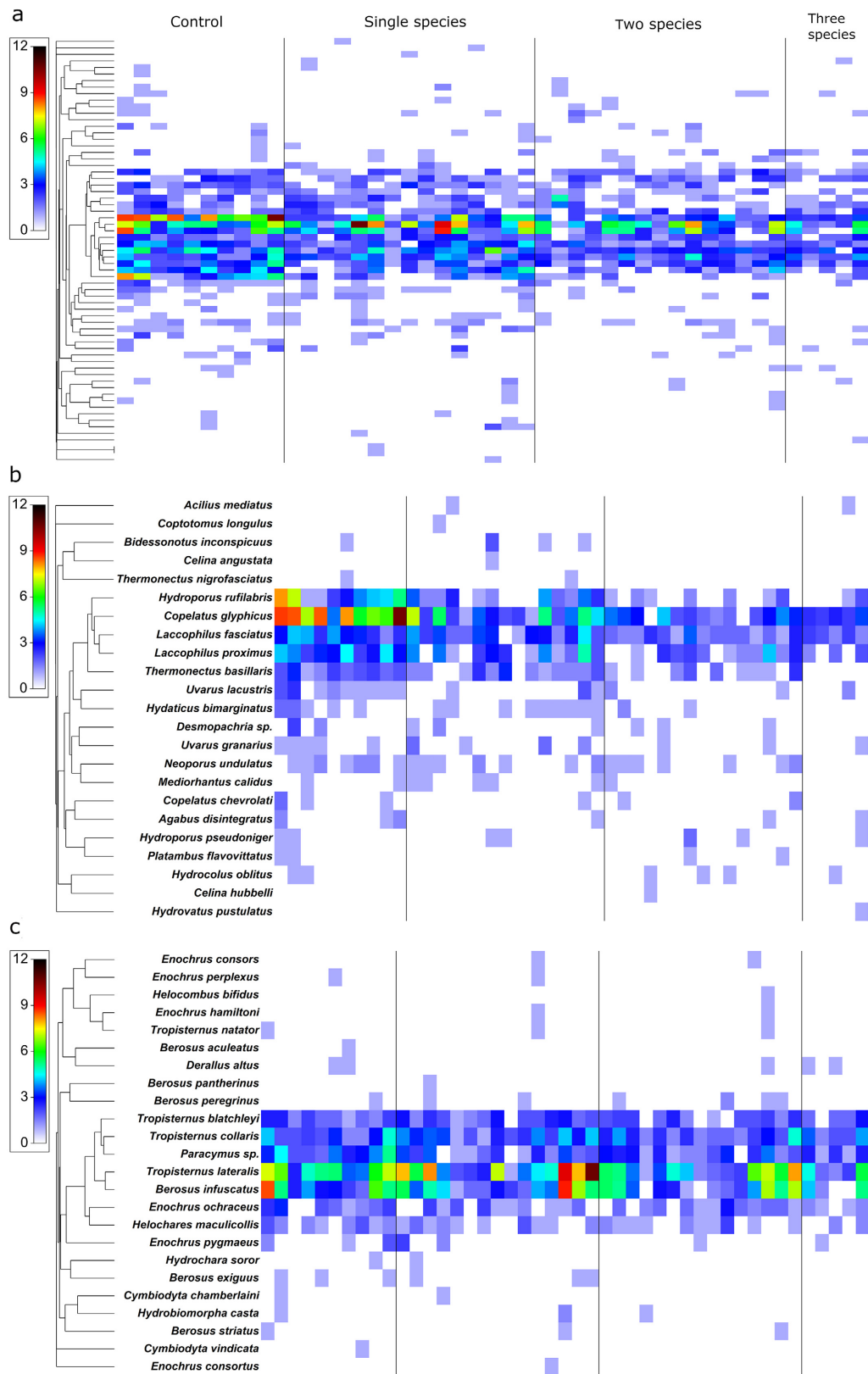


FIG. 11. Shade plot (heat map) showing square-root transformed abundances by contrast group (controls shown for reference) for (a) the entire insect assemblage, (b) dytiscids, (c) hydrophilids. Warmer colors indicate greater abundance. Contrast groups

(FIG. 11. *Continued*)

displayed in order of number of predator species, species order on y-axis is based on similarity of distribution. (See Table 5 for PERMANOVA and PERMDISP results, species similarity key for all insects in Appendix S1: Fig. S3, NMDS plots in Appendix S1: Fig. S7). Shade plot for Hemiptera in Appendix S1: Fig. S4b.

abundance and species composition. *Noturus phaeus* can be common in ponds and intermittent streams that seasonally form pools, and clearly can be detected, as it was avoided by one abundant dytiscid, and one abundant hydrophilid, but it is also either the least detectable or least avoided species. Overall, the strongest responses came from dytiscids, which largely avoid all fish species and combinations, though responses to *N. phaeus* were weaker, while avoidance of the other fish species and species combinations were roughly equivalent. We have previously observed strong responses to fish in dytiscids, and one possibility is that both adults and larvae of dytiscids are predaceous, thus dytiscids may respond to fish both as predators and competitors (Resetarits and Pintar 2016, Resetarits et al. 2019). Dytiscids are also attracted to higher zooplankton abundance (Pintar and Resetarits 2017), thus may be responding directly to both fish cues and to reduced zooplankton abundance characteristic of fish patches. Generally, for dytiscids, one species of avoided fish generates the full avoidance response, and this holds individually for *C. glyphicus*, *H. rufilabris*, and *L. proximus*. This suggests that either dytiscids are incapable of discriminating among fish species, though reduced responses to *N. phaeus* speaks against that, or that the response is saturated by effects of any strongly avoided fish. Dytiscids may detect and recognize different fish species, but a single species is sufficient to generate near complete avoidance; thus, the question may be ecologically moot, as well as operationally intractable, by our methods.

In contrast, hydrophilids, both as a group and individually, only avoided specific, multi-predator treatments, with exception of *Paracymus* sp. (the smallest of our common beetles), which both here, and in previous experiments, responded more like a dytiscid (Resetarits and Pintar 2016, Pintar and Resetarits 2017, Resetarits et al. 2019). *Paracymus* sp. avoided all except the NC × NP treatment, interesting because it avoided both NC and NP alone. The other hydrophilids responding to fish were *T. lateralis* and *B. infuscatus*, which only avoided the X3 treatment, while avoiding none of the single or two species treatments. *Tropisternus lateralis* and *B. infuscatus* do avoid several centrarchids, and one species pair that contained centrarchids, at similar densities (Resetarits 2001, Resetarits and Binckley 2014, Resetarits and Pintar 2016, Resetarits et al. 2019). Patterns for both species are interesting because lack of response to any single fish species demonstrates an emergent response to multiple predators, also seen in ovipositing treefrogs (Resetarits et al. 2021a).

Sigara sp., showed another unique pattern, essentially avoiding *F. chrysotus* and all multi-predator treatments

containing FC. This pattern, unlike that of *Paracymus* sp., is consistent, and FC, a surface feeding fish, could have stronger effects on *Sigara* sp. than the other two fish species. However, the cue threshold must be low, because of reduced densities of FC in multi-species treatments.

Predicting multi-predator effects on colonization from responses to single predators

A central question for species interactions is whether the interaction coefficients from pairwise interactions predict multi-species interactions: the community matrix approach (Vandermeer 1969, 1970, Novak et al. 2016). For predation, we generally ask whether individual predator effects predict multi-predator effects on prey (Soluk and Collins 1988, Wilbur and Fauth 1990, Soluk 1993, Sih et al. 1998, Vance-Chalcraft and Soluk 2005, McCoy et al. 2012). We used a substitutive, rather than the additive design used in many studies of consumptive multi-predator effects, because our interest was in effects of predator identity and richness (Vance-Chalcraft et al. 2004, Griffen 2006), and we set fish density above the typical observed threshold for detection and avoidance (Rieger et al. 2004, Resetarits and Binckley 2013). Because we could not include multiple densities of the individual predators, we have no rigorous estimate of absolute (additive) or relative (substitutive) per capita effects (Griffen 2006). Thus, we cannot use the typical approach, involving predictions from additive and multiplicative models, to combine the observed single-predator effects into meaningful, quantitative predictions of multi-predator effects.

Further, while both consumption and colonization share the characteristic of directly affecting number of individuals, consumptive effects by removal and colonization effects by redirection and redistribution (Resetarits et al. 2005, Orrock et al. 2010), colonization responses clearly involve information integration processes that do not lend themselves to simple predictions based on individual predator effects, but similarly can involve saturation of responses, or emergent effects of species combinations (Sih et al. 1998, Schmitz 2007, Preisser and Bolnick 2008, McCoy et al. 2012, Davenport and Chalcraft 2013). The specific responses of hydrophilids as a group, *Paracymus* sp., *T. lateralis*, *B. infuscatus*, and *Sigara* sp. all suggest some type of synergy either in the cues themselves or the responses to those cues, while for dytiscids as a group, and all the abundant species (*C. glyphicus*, *H. rufilabris*, and both *Laccophilus*), results suggest saturation of the avoidance response by the presence of any strongly avoided fish species.

Community-level metrics and individual treatments

Unlike studies involving predators at different trophic levels, where we have expectations driven by our understanding of trophic cascades, mesopredator release, etc. (Ripple et al. 2016, Van Allen et al. 2017), there is actually little or no theory or empirical work on which to base expectations for the non-consumptive, or even consumptive, effects of multiple predators at the same trophic level on community assembly. As pointed out above, increasing predator richness should elicit more avoidance responses. This effect is enhanced with species such as *T. lateralis*, that may only show avoidance responses to specific predators and combinations of predators. Richness patterns to the contrary suggest that predators must affect each other or affect the habitat in different ways when coexisting, so as to change its perception to colonists (Sih et al. 1998, Vance-Chalcraft and Soluk 2005, Griffen 2006, Griffen and Byers 2006, Davenport and Chalcraft 2013).

Fish treatments strongly affected the relative abundance of the three dominant higher taxa, dytiscids, hydrophilids, and hemipterans, largely as a result of the strong effects on dytiscid abundance (Fig. 2). Species richness for all insects was significantly reduced in all treatments except NP, driven largely by the effects on dytiscids, with some contribution by hydrophilids (Fig. 5). Evenness was generally higher with lower species richness across the taxa. Community structure was significantly different between predator treatments for all insects, again driven mainly by the differences between all fish treatments and controls for dytiscids, though the magnitude of the difference for NP was far less than other fish treatments. β -diversity (multivariate dispersion) was only significantly different for the dytiscids, with both the control and NP having similarly low β -diversity, thus strong effects of predators on colonization increased β -diversity in the colonists. Fish generate strong, community-level effects on dytiscids, as displayed in the shade plots (Fig. 6, see also NMDS plots Appendix S1: Fig. S6), as is the difference in the overall pattern for dytiscids (Fig. 6b), which show the strong, but somewhat variable, responses across predator treatments, and hydrophilids (Fig. 6c), whose responses were limited. Fish did not decrease spatial β -diversity across all taxa, unlike in previous work examining combined consumptive and non-consumptive effects (Chase et al. 2009, Van Allen et al. 2017), but actually increased β -diversity at the colonization stage, suggesting that the strong depressive effect of fish on β -diversity occurs post-colonization, while the converse is true at the colonization stage. This is likely a result of the contribution of rare species, which are selectively filtered by higher extinction rates in patches with fish, but are represented in the actual colonist pool (Chase et al. 2009, Ryberg et al. 2012).

Single vs. multi-predator effects: a priori contrasts

While the comparisons of specific single and multi-predator treatments revealed some predictable and unpredictable patterns across insect taxa, looking at single vs. multi-predator treatments (predator richness) allows us to probe the question more generally (Vance-Chalcraft et al. 2004, Staats et al. 2016). For dytiscids as a whole and the four most abundant species, despite the fact that the presence of any avoided fish came close to saturating the response space, the number of colonists decreased with increasing predator richness. However, the SvsX3 contrast was only significant for one species, despite the fact that effect sizes were as large or larger, as a result of the reduced power of that specific contrast, with 15 vs. 5 replicates as opposed to 15 vs. 15 for SvsX2 and 15 vs. 20 for SvsM (Fig. 8). All three contrasts were also significant for hydrophilids overall; *T. lateralis*, as mentioned earlier, did not respond to single species but only to species combinations, while for *Paracymus* sp., all of the variation occurred between controls and any predator treatment, but none within the contrast groups. *Berosus infuscatus* is interesting in that it had significant responses to none of the specific treatments, but the X3 treatment and the SvsM and SvsX3 contrasts were significant, so there is something unique about a combination of species (Fig. 9). None of the contrasts for all hemipterans or *Sigara* sp. were significant, but did follow the pattern of lower abundance in multi-predator treatments (Fig. 9).

Predator species richness and community metrics

Greater effects of multiple vs. single predators on species richness shows up in overall insect species richness, and dytiscid species richness, and is largely driven by variation in abundance, though the X3 treatment may show some independent effect on richness for dytiscids (Fig. 10a, b; Appendix S1: Table S3); insect and dytiscid assemblages are both distinct across different levels of predator richness (Fig. 11). Hydrophilids and hemipterans show a weak trend towards lower richness with increasing predator richness as well (Fig. 10c, d). Evenness has meaningful contrasts for all but Hemiptera, with evenness greater in specific multi-predator treatments (Appendix S1: Table S3, Fig. S5). Multivariate location (average community composition) is affected by predator species richness for all insects and dytiscids, with all contrasts meaningful, and for Hemiptera only in the SvsX3 contrast (Appendix S1: Fig. S5). Predators increase β -diversity vs. the controls, especially for dytiscids (see above and Fig. 11; Appendix S1: Fig. S7), but there are no clear evidence that increased predator richness beyond single species affects β -diversity of colonists. The general effects are most apparent in dytiscids, and can be seen in both the shade plots (Fig. 11) and NMDS plots (Appendix S1: Fig. S7). This is first documentation

of non-consumptive, multi-predator effects on aspects of community structure (relative abundance, richness, evenness, composition, and β -diversity) by species at the same trophic level on such a diverse, multi-species, prey assemblage.

CONCLUSIONS

What does the landscape of variation in the responses to different species and species combinations of predators look like across a diverse multi-species assemblage of colonizing insects? Prey species do not uniformly respond to specific predators or combinations of predators, and that variation in responses results in greater β -diversity in all predator treatments vs. controls and within most predator treatments. It is interesting that the variation does not reflect any obvious differences in predator traits (Chalcraft and Resetarits 2003, Schmitz 2017), though an even larger sampling of predators would be necessary to establish this. We can envision taxa like *Sigara* being more susceptible to surface feeding species like *F. chrysotus*, and adult beetles and hemipterans, in general, may be less vulnerable to benthic species represented here by *N. phaeus*, but as we have seen in the past, avoidance and expected predatory effect often do not match (Resetarits and Binckley 2013, Pintar and Resetarits 2021). There is no a priori reason

to expect hydrophilids to be less vulnerable than dytiscids, but the contrast in responses of the two taxa is striking, especially given the fact that the adults, and especially the larvae, are morphologically (and ecologically in the case of larvae), very similar, and similarly vulnerable to fish predation (Merritt et al. 2008); body size, swimming speed, and exoskeleton toughness do not vary systematically between members of the two families represented here (Pintar and Resetarits 2021). However, adult diet does differ and fish are obvious competitors of adult dytiscids as both tend to be generalist predators.

Perhaps most striking is the presence of emergent multi-predator effects, where certain species only respond to predator species combinations, but not to any individual predator, despite matched density and biomass. Whether this synergy derives from differential perception of the combined cues, or differential reaction to the presence of combined cues, is a fascinating question. This range of variation among colonizing species generates variation in communities not only between predator-free and predator patches, but also between patches with different predators and combinations of predators, with the effect being most striking for the dytiscids. Prior work has shown that our assemblage of aquatic insects generally avoid fish patches, and then sort across gradients of patch size (Resetarits et al. 2019); here we see that species also behaviorally assort

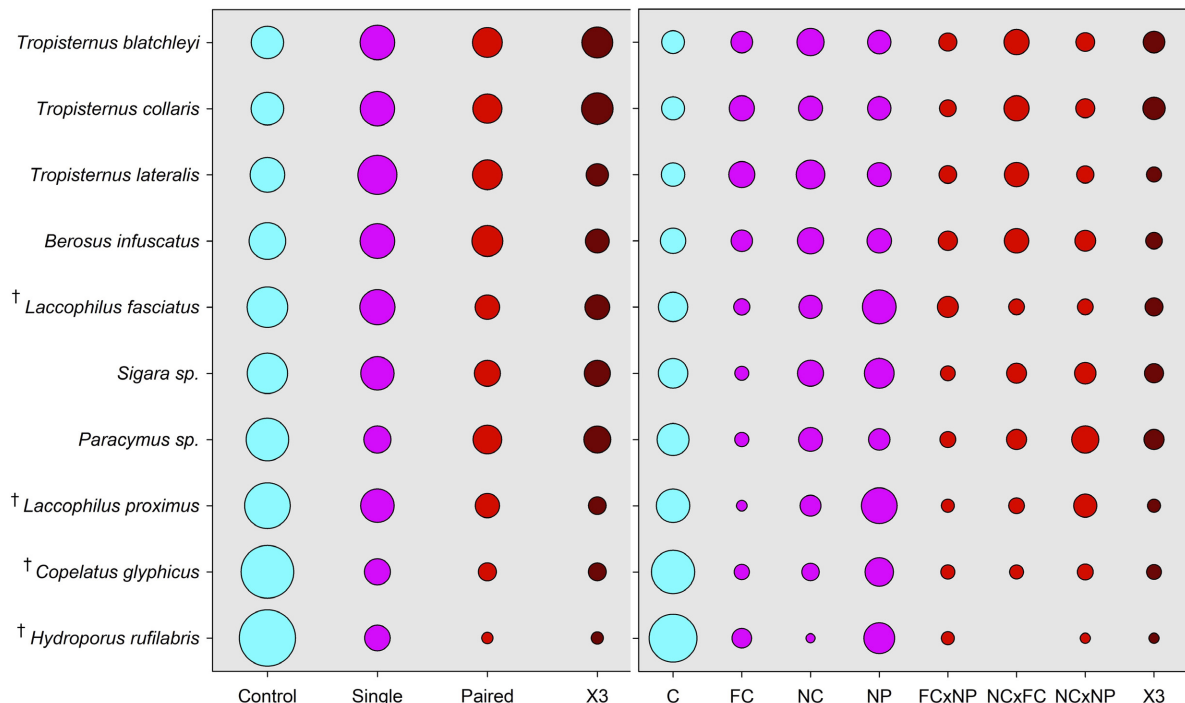


FIG. 12. Bubble plot summarizing the variation among contrast groups (left panel) and treatments for the 10 most abundant taxa, showing how species sort into different assemblages across variation in predator richness and predator identity. Bubble area represents the mean proportional abundance for contrast groups and treatments. *Sigara* sp. is a hemipteran, † indicates dytiscids, others are hydrophilids. See Figs. 2, 7 for relative abundance of higher taxa.

across variation in predator identity and predator richness. The result is that species are not only in competition for enemy-free space (Jeffries and Lawton 1984), but also partition and sort across predator space as a result of habitat preferences (Fig. 12). Thus, patches with different predators and combinations of predators have distinctly different average species composition and both absolute and relative abundance among colonists. Last, our results show a consistent pattern of stronger effects in multi-predator vs. single-predator treatments, despite the strength of the responses to individual predators in many cases. This is seen in the responses of individual species and also in the aggregate measures, such as relative and absolute abundance at higher taxonomic levels, species richness, and multivariate community structure.

Our experiment clearly demonstrates that the effects of predators on the colonist pool of aquatic insects, holding predator density and biomass constant, depends strongly on predator identity, predator richness, and the specific combination of predators present in aquatic patches, as well as the species composition of the potential colonist pool. The complexity of the responses makes prediction of multi-predator effects problematic, and even higher levels of predator species richness, which are common in freshwater habitats, likely further limits our ability to predict responses, but there are some lessons we can learn. Aggregate measures, such as abundance at higher taxonomic levels, and species richness, both of which integrate across these levels of complexity, do have some qualitative predictability, in that increasing predator richness affects aggregate abundance and richness in a fairly consistent manner. This is a result of these measures buffering the idiosyncrasies of individual species responses. More predator species generate more individual avoidance responses, which appear in these aggregate measures and is contrary to expectations based on a generalized fish kairomone. It is important to recognize that, while colonization sets the stage for post-colonization processes, there are also potential feedbacks from consumptive effects back to colonization behavior, as additional species may respond to predator dietary cues that do not respond to predator cues alone, or species may respond more strongly to the presence of both types of cue (Ferrari et al. 2010, Wisenden 2015). Thus, our observed effects may underestimate the total impacts of predators and predator combinations on colonization behavior. So, while post-colonization effects on communities are an integration of multiple sources of mortality in a given patch, including consumptive predator effects, colonization effects are an integration of multiple sources of species responses to aspects of patch quality, including, but not limited to temperature, resources, competitors, patch size, patch context, and, of course, perceived predation risk. A critical, but difficult, task going forward is to gain an understanding of the interaction between demographic habitat selection and post-colonization processes and the relative importance

of the two processes for generating community structure in a variety of systems (Åbjörnsson et al. 2002, Preisser et al. 2005, Vonesh et al. 2009).

It is also important to remember that demographic habitat selection links multiple patches in a landscape and affects multiple spatial scales (Resetarits 2005, Resetarits et al. 2005). So, in Fig. 12, individuals that colonize a predator-free (control) patch are not colonizing any predator patches, and individuals not colonizing predator patches are ending up aggregated in predator-free patches. Prey consumption within a patch has little effect outside of that patch, whereas predator avoidance at the colonization stage affects multiple patches, both those that are avoided and those that are colonized at a higher rate because of the shifting of colonization driven by the presence of avoided (low quality) habitats (spatial compression; Resetarits et al. 2005, Orrock et al. 2010, Resetarits and Silberbush 2016). Thus, demographic habitat selection effects range from the individual to the metacommunity, and the dynamics of habitat selection in response to predators is a complex function of predator identity, predator density, predator richness, predator species composition, and the types of habitats available (patch context).

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SUPPORTING INFORMATION

Additional supporting information may be found online at: <http://onlinelibrary.wiley.com/doi/10.1002/ecm.1474/full>

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Data (Resetarits et al. 2021b) are available in the Dryad digital repository: <https://doi.org/10.5061/dryad.76hdr7swx>