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Hard coral (*Porites lobata*) extracts and homarine on cytochrome P450 expression in Hawaiian butterflyfishes with different feeding strategies

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ABSTRACT

Dietary specialists tend to be less susceptible to the effects of chemical defenses produced by their prey compared to generalist predators that feed upon a broader range of prey species. While many researchers have investigated the ability of insects to detoxify dietary allelochemicals, little research has been conducted in marine ecosystems. We investigated metabolic detoxification pathways in three species of butterflyfishes: the hard coral specialist feeder, *Chaetodon multicinctus*, and two generalist feeders, *Chaetodon auriga* and *Chaetodon kleinii*. Each species was fed tissue homogenate of the hard coral *Porites lobata* or the feeding deterrent compound homarine (found in the coral extract), and the expression and catalytic activity of cytochrome P450 (CYP) 3A-like and CYP2-like enzymes were examined after one-week of treatment. The *P. lobata* homogenate significantly induced content and catalytic activity of CYP2-like and CYP3A-like forms, by 2–3 fold and by 3–9 fold, respectively, in *C. multicinctus*. Homarine caused a significant decrease of CYP2-like and CYP3A-like proteins at the high dose in *C. kleinii* and 60–80% mortality in that species. Homarine also induced CYP3A-like content by 3-fold and catalytic activity by 2-fold in *C. auriga*, while causing non-monotonic increases in CYP2-like and CYP3A-like catalytic activity in *C. multicinctus*. Our results indicate that dietary exposure to coral homogenates and the feeding deterrent constituent within these homogenates caused species-specific modulation of detoxification enzymes consistent with the prey selection strategies of generalist and specialist butterflyfishes.

1. Introduction

In ecological settings, consumers are generally categorized as either generalists or specialists with regard to dietary preference. Generalists tend to consume many different species, even when one dietary species is abundant, whereas specialists rely on one or a few prey species for most of their food (Krebs, 2009). However, the diets of generalists are often limited to species that have few chemical defenses, while specialists take advantage of their detoxifying enzymes to consume more chemically-defended prey than generalists (Marsh et al., 2006). Numerous examples of insects that are resistant to their prey's defenses have been documented (e.g., Strong et al., 1984). Similarly, many marine consumers are also not deterred by chemical defenses produced or stored by the prey on which they feed, even though those compounds have strong deterrent effects on generalists (Pawlik, 2012). Nonetheless, the mechanistic basis for consumption of chemically-defended marine prey species has been rarely examined (e.g., Sotka and Whalen, 2008).

The butterflyfish *Chaetodon multicinctus* is an obligate corallivore endemic to Hawaii that feeds primarily upon *Porites lobata*, as well as *Porites compressa* and *Pocillopora meandrina* (Tricas, 1986; Motta, 1988; Gochfeld, 1997). The hard (scleractinian) corals, *Porites* spp., have been hypothesized to produce biologically active metabolites that impair butterflyfish predation (Gochfeld, 1997, 2004). One such putative allelochemical, homarine (1-Methylpyridin-1-ium-2-carboxylate), has been isolated from several marine invertebrates (e.g., Affeld et al., 2006), including *Porites* spp. and other species of hard corals (Gochfeld, unpublished). However, the biological significance of homarine in these animals has not been well characterized. For example, homarine has been reported to act as an organic osmolyte in osmoregulation (Beers, 1967), an antimicrobial compound (Slattery et al., 1997; Shapo et al., 2007), and a regulator of colony morphology and metamorphosis (Berking, 1987). Homarine has been shown to exhibit feeding deterrent properties, specifically against a generalist Antarctic sea star *Odontaster validus* (McClintock et al., 1994), and against the generalist pufferfish *Canthigaster rostrata* (Gochfeld, unpublished). Although anti-feedant activity of homarine has not yet been tested against either generalist or specialist coral-feeding butterflyfishes,

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C. rostrata does include hard corals in its omnivorous diet, suggesting that homarine likely provides some feeding deterrent activity for corals. The present study seeks to address toxicity implications of homarine in generalist and specialist butterflyfishes.

Chaetodon auriga is primarily a benthic omnivore feeding on non-coralline and coralline invertebrates, including hard corals and soft (alcyonarians) corals, polychaete worms, and algae (Hobson, 1974; Motta, 1980; Harmelin-Vivien and Bouchon-Navaro, 1983). In Hawaii, *Chaetodon kleinii* is a planktivore, feeding largely on copepods and other planktonic invertebrates (Hobson, 1974), while in other regions throughout its range this butterflyfish includes soft corals in its diet (e.g., Anderson et al., 1981; Pratchett, 2007). These two generalists are hypothesized to have liver enzymes that can act on a broad range of substrates, including toxins, to facilitate biotransformation. However, limitations in the capacity of any specific biotransformation enzyme are thought to prevent generalists from metabolizing large quantities of a single toxin, or of similar toxins (Torregrossa et al., 2012).

The expression of novel cytochrome P450 monooxygenase (CYP) enzymes has been associated with the ability of some insects to biotransform and detoxify allelochemicals, which allows those insects to feed on plants that produce defensive allelochemicals (Berenbaum and Feeny, 1981, Berenbaum and Zangerl, 1999). For example, Berenbaum and Zangerl (1998) showed that parsnip webworms exhibited both increased concentrations and activities of CYP in response to the levels of inducible furanocoumarins in host parsnip plant species. Since there have been relatively few studies that have examined biotransformation enzymes as potential mechanisms of allelochemical resistance in marine organisms, the purpose of this study was to investigate the detoxification enzymes of generalist (*C. auriga* and *C. kleinii*) and specialist (*C. multicinctus*) butterflyfishes consuming a chemically-defended hard coral, *P. lobata*. We hypothesized that specialists with higher basal levels of CYP should be better at detoxifying their preferred prey and allelochemicals than generalists. Since CYP is one of the most characterized detoxification systems of dietary allelochemicals, and it is found in higher concentration in butterflyfishes that preferentially feed on allelochemically-rich corals (Vrolijk et al., 1994), we compared the expression profiles and catalytic activities of CYP2-like and CYP3A-like enzymes in species with different dietary preferences.

2. Materials and methods

2.1. Reagents

Analytical grade methanol, ethanol, and acetonitrile along with glycerol, TRIS, and potassium chloride were purchased from Fisher (Pittsburg, PA). ^{14}C-Testosterone (150 μCi/μmol; 97.6% purity) was purchased from Perkin-Elmer (Waltham, MA). MS-222, EDTA, gelatin, and NAPDH were purchased from Sigma-Aldrich (St. Louis, MO). Tween-20 was purchased from EMD Millipore (Billerica, MA). All other materials were obtained from Aldrich Chemical Company, St. Louis, MO and were used as received.

2.2. Homarine synthesis

Homarine (1-Methylpyridin-1-ium-2-carboxylate) was synthesized following methods in McClintock et al. (1994). To a sealed tube equipped with a stir bar was added picolinic acid (2.01 g, 16.34 mmol), ethanol (10 mL), and iodomethane (2.0 mL, 32.13 mmol). The reaction was stirred at 55 °C for 16 h, cooled to 0 °C, filtered and washed with ice-cold ethanol. The crude solid was washed with acetone and recrystallized from water and acetone to yield a light yellow solid (552 mg, 25%). ^{1}H and ^{13}C spectra were recorded on a Varian Inova 400 spectrometer. Proton (^{1}H) chemical shifts are reported in parts per million (δ) with respect to tetramethylsilane (TMS, δ = 0), and referenced internally with respect to the protio solvent impurity. Carbon (^{13}C) chemical shifts were referenced internally with methanol. Deuterated NMR solvents were obtained from Cambridge Isotope Laboratories, Inc., Andover, MA, and used without further purification. Mass spectra were recorded on an LCQ Deca XP Plus mass spectrometer (Thermo Fisher Scientific, San Jose, CA) using electrospray ionization and processed with Thermos Xcalibur 2.0 software.

^{1}H NMR (400 MHz, D_2O) δ 8.72 (d, J = 6.2 Hz, 1H); 8.54 (t, J = 87.9 Hz, 1H); 8.07 (d, J = 7.9 Hz, 1H); 7.98 (t, J = 7.0 Hz, 1H); 4.37 (s, 3H); ^{13}C NMR (100 MHz, D_2O) δ 166.1; 146.9; 146.2; 127.8; 126.8; 126.8; 47.3; HRMS (ESI) *m/z* calculated $C_7H_8NO_2$ $(M + H)^+$ 138.0550; and found 138.0549.

2.3. Collections

Butterflyfishes were collected from Kaneohe Bay, Yokahama Bay, and Mokuleia Bay reef systems surrounding the island of Oahu, Hawaii, during June 2012 and 2013. *C. multicinctus* (11 g ± 3; 8.0 cm ± 0.2), *C. kleinii* (23 g ± 7; 9 cm ± 1), and *C. auriga* (43 g ± 8; 10 cm ± 1) were acclimated in the laboratory for two weeks before treatments, and fed with fish brine shrimp. Fish were held at the Waikiki Aquarium in a large (3 m diameter and 1 m depth) flow-through seawater tank, with floating cages for separation of treatment groups and species.

P. lobata was collected from Kaneohe Bay and immediately transplanted to the laboratory for processing. A Water-Pik was used to remove soft tissue from the coral, which was collected in Tris–HCl buffer (pH 8.2) (Johannes and Wiebe, 1970). The samples were lyophilized, weighed and stored at −20 °C until used in treatments.

2.4. Coral extraction

Homarine concentrations were quantified in aqueous extracts from *P. compressa*, *Porites evermanni* and *P. lobata* collected in Hawaii, using high performance liquid chromatography (HPLC). Laboratory-synthesized homarine was used to develop a standard curve. Aqueous extracts were generated by extracting frozen coral samples for 12 h in Millipore water three times. The combined aqueous extract was filtered, lyophilized and weighed. Following extraction, tissue volume was measured using the waxing method (Gochfeld and Aeby, 2008) to calculate the concentration of homarine per tissue volume.

2.5. Coral homogenate and homarine gavage treatments

To examine the effect of *P. lobata* tissue homogenate and homarine on CYP expression, 4–6 individuals of each butterflyfish species were laid flat on a cushioned dissection tray and orally gavaged (1 mL syringe, stainless steel ball point needle) with either a high dose (250 mg/kg), or a low dose (50 mg/kg) of *P. lobata* tissue homogenate (Summer 2012), or a high dose (54 μg/kg), or a low dose (25 μg/kg) of homarine (Summer 2013), or with a control dose of 10 μL of Tris-buffer (pH 8.2). Doses were based on average daily consumption rates by *C. multicinctus*, *C. auriga*, and *C. kleinii* (Tricas, 1989; Aeby, 2002; Gregson et al., 2008; Cole et al., 2011) and concentrations of homarine in crude extract of *P. lobata*, 0.05–0.02%. Treatments occurred on days 1, 3, and 5. On day 7, fish were euthanized by MS222 overdose and dissected livers were removed and stored at −80 °C until microsomes were prepared for immunoblot and catalytic activity assay.

2.6. Microsome preparations

Livers were individually homogenized in 1:5 w/v of cold 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4, containing 100 mM KCl, and 1 mM EDTA (Sigma Aldrich Inc.). Homogenates were centrifuged at 12,000 ×*g* for 30 min. Supernatant was collected and centrifuged at 100,000 ×*g* for 60 min to obtain microsomal fraction. The resulting supernatant was removed and the microsomal fraction was resuspended in 1:0.5 w/v of 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4, containing 1 mM EDTA, and 20% w/v glycerol. Proteins were measured by the