1 **Hard Coral (*Porites lobata*) extracts and homarine on Cytochrome P450 expression**
2 **in Hawaiian butterflyfishes with different feeding strategies.**

3
4 **Aileen Maldonado^{a*}, Amber Johnson^a Deborah Gochfeld^b, Marc Slattery^c, Gary K.**
5 **Ostrander^d, Jon-Paul Bingham^e, Daniel Schlenk^a**

6
7 *^aDepartment of Environmental Science University of California, Riverside, CA*
8 *2258 Geology, 900 University Ave. Riverside, CA 92521 USA*

9 *^bNational Center for Natural Products Research and Department of BioMolecular*
10 *Science University of Mississippi, P.O. Box 1848, University, MS 38677 USA*

11 *^cDepartment of BioMolecular Science and National Center for Natural Products*
12 *Research University of Mississippi, P.O. Box 1848, University, MS 38677 USA*

13 *^dDepartment of Biomedical Sciences, College of Medicine, Florida State University*
14 *Tallahassee, FL 32306-1330 USA*

15 *^eDepartment of Molecular Bioscience and Bioengineering University of Hawaii at*
16 *Manoa, 1955 East-West Road, Ag. Science 218, Honolulu, HI 96822 USA*

17
18 **Running Title:** Cytochrome P450 Expression in Butterflyfish after exposure to *Porites*
19 *lobata* and homarine

20
21 **ms. has 25 pages, 3 figures**

22 *Corresponding author:

23 Aileen Maldonado

24 Department of Environmental Science University of California, Riverside, CA

25 2258 Geology, 900 University Ave.

26 Riverside, CA 92521 USA

27 Tel: 808-259-1945; Fax: 951-827-3993

28 amald008@ucr.edu

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.

Key words-Allelochemical, *Chaetodon*, coral, cytochrome P450, generalist, specialist.

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 & Whalen, 2008).

The butterflyfish *Chaetodon multicinctus* is an obligate corallivore endemic to Hawaii that feeds primarily upon *Porites lobata*, as well as *P. 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, *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.

90 *C. auriga* is primarily a benthic omnivore feeding on noncoralline and coralline
91 invertebrates, including hard corals and soft (alcyonarians) corals, polychaete worms, and
92 algae (Hobson, 1974; Motta, 1980; Harmelin-Vivien & Bouchon-Navaro, 1983). In
93 Hawaii, *C. kleinii* is a planktivore, feeding largely on copepods and other planktonic
94 invertebrates (Hobson, 1974), while in other regions throughout its range this
95 butterflyfish includes soft corals in its diet (e.g., Anderson, 1981; Pratchett, 2007). These
96 two generalists are hypothesized to have liver enzymes that can act on a broad range of
97 substrates, including toxins, to facilitate biotransformation. However, limitations in the
98 capacity of any specific biotransformation enzyme are thought to prevent generalists
99 from metabolizing large quantities of a single toxin, or of similar toxins (Torregrossa,
100 2012).

101 The expression of novel cytochrome P450 monooxygenase (CYP) enzymes has
102 been associated with the ability of some insects to biotransform and detoxify
103 allelochemicals, which allows those insects to feed on plants that produce defensive
104 allelochemicals (Berenbaum et al., 1981, 1999). For example, Berenbaum and Zangerl
105 (1998) showed that parsnip webworms exhibited both increased concentrations and
106 activities of CYP in response to the levels of inducible furanocoumarins in host parsnip
107 plant species. Since there have been relatively few studies that have examined
108 biotransformation enzymes as potential mechanisms of allelochemical resistance in
109 marine organisms, the purpose of this study was to investigate the detoxification enzymes
110 of generalist (*C. auriga* and *C. kleinii*) and specialist (*C. multicinctus*) butterflyfishes
111 consuming a chemically-defended hard coral, *Porites lobata*. We hypothesized that
112 specialists with higher basal levels of CYP should be better at detoxifying their preferred
113 prey and allelochemicals than generalists. Since CYP is one of the most characterized
114 detoxification systems of dietary allelochemicals, and it is found in higher concentration
115 in butterflyfishes that preferentially feed on allelochemically-rich corals (Vrolijk et al.,
116 1994), we compared the expression profiles and catalytic activities of CYP2-like and
117 CYP3A-like enzymes in species with different dietary preferences.

118 119 **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 $\mu\text{Ci}/\mu\text{mol}$; 97.6% purity) was purchased from Perkin-Elmer (Waltham, MA). MS-222, EDTA, gelatin, and NADPH 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 $^{\circ}\text{C}$ for 16 h, cooled to 0 $^{\circ}\text{C}$, 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%). ^1H and ^{13}C spectra were recorded on a Varian Inova 400 spectrometer. Proton (^1H) chemical shifts are reported in parts per million (δ) with respect to tetramethylsilane (TMS, $\delta=0$), 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 Thermo Xcalibur 2.0 software.

^1H 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 $\text{C}_7\text{H}_8\text{NO}_2$ ($\text{M}+\text{H}$) $^+$ 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 $\pm$ 3; 8.0 cm $\pm$ 0.2), *C. kleinii* (23 g $\pm$ 7; 9 cm $\pm$ 1), and *C. auriga* (43 g $\pm$ 8; 10 cm $\pm$ 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.

Porites 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 & 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 *Porites compressa*, *Porites evermanni* and *Porites 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 hours 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 & 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 (250mg/kg), or a low dose (50mg/kg) of *P. lobata* tissue homogenate (Summer 2012), or a high dose (54µg/kg), or a low dose (25µg/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 *Porites 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.

2.5 Microsome preparations

Livers were individually homogenized in 1:5 w/v of cold 100 mM KH₂PO₄/K₂HPO₄ buffer pH 7.4, containing 100 mM KCl, and 1 mM EDTA (Sigma Aldrich Inc.) Homogenates were centrifuged at 12,000 *xg* for 30 min. Supernatant was collected and centrifuged at 100,000 *xg* 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 KH₂PO₄/K₂HPO₄ buffer pH 7.4, containing 1 mM EDTA, and 20% w/v glycerol. Proteins were measured by the Coomassie Blue method using a commercial kit (Pierce Inc., Rockford, IL) with bovine serum albumin as a standard.

2.7 Immunoblotting

CYP3A-like and CYP2-like protein levels were determined by Western blot as described in Lavado et al. (2009). Microsomal samples were boiled for 5 min in SDS-PAGE buffer (50/50) (Laemmli, 1970), and 10 µg of protein was separated by electrophoresis using 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (BioRad). The membrane was probed with either 1:500 dilution (v/v) of primary rabbit anti-rainbow trout polyclonal CYP2M1 or 1:1000 dilution (v/v) of primary rabbit anti-rainbow trout polyclonal CYP3A27 antibodies provided by Dr. D. R. Buhler, Oregon State University. Blots were incubated at room temperature overnight and rinsed twice with Tris-buffered saline containing 0.2% Tween 20 (v/v) (T-TBS) and once with Tris-buffered saline containing 0.2% Tween 20 (v/v) and 0.5% gelatin (w/v). The blot was then incubated for 1 h with alkaline phosphatase-conjugated anti-rabbit IgG (BioRad) and excess secondary antibody was removed with two washes of T-TBS. The immunoreactive bands at approximately 54-57 kD were tinted by incubation with the

substrates p-nitroblue tetrazolium chloride and 5- bromo-4-chloro-3-indolyl phosphate from a commercial alkaline phosphatase conjugation kit (BioRad). Semiquantification by densitometry was done using Image Lab (BioRad) in a Molecular Imager ChemiDoc XRS+ Imaging System (BioRad) image analyzer. The data is presented in optical density units per mg protein.

2.8 ¹⁴C Testosterone hydroxylase activity

¹⁴C Testosterone hydroxylase activity was measured as described by Martin-Skilton et al. (2006). In short, 50 µL of hepatic microsomal protein was incubated with 3.5 µM [¹⁴C]-testosterone (150 µCi/µmol; 97.6% purity) and 7.5 µM NADPH in a final volume of 0.510 mL of 50 mM Tris–HCl pH 7.4. Samples were incubated for 30 min at 25 °C, additional 10 µL of NADPH added, then incubated for another 30 min at 25 °C. Incubations were stopped with 250 µL of acetonitrile followed by centrifugation at 10,000 *xg* for 10 min, 400 µL of supernatant was injected into a reverse-phase HPLC column. HPLC analyses were performed on a SCL-10AVP Shimadzu HPLC system equipped with a 250Å~4.6 mm Atlantis C18 (5 µm) reverse-phase column (Waters, Milford, MA). Separation of testosterone metabolites was attained using mobile phase systems of (A) 75% water and 25% acetonitrile and (B) 45% water and 55% acetonitrile at a rate of 1 ml/min. The run gradient started with 100% A to 100% B in 30- 35 min. Chromatographic peaks were monitored on a radioflow detector β-ram Model 3 (INUS Systems Inc., Tampa, FL) using U-Flow 2:1 (Lablogic Inc.) scintillation liquid (Cat. SG-BXX-05). Metabolites 6β-, 16α- and 16β-hydroxytestosterone were identified using standard compounds (by co-chromatography). The metabolites were quantified by integrating the area under the radioactive peaks (recovery 98.9%-99.7%); the detection limit was 0.2 pmol/min/mg protein.

2.9 Statistical analyses

For enzyme measurement comparisons, statistical analyses were conducted using Prism5 v5.0a software. Prior to statistical analysis, all data were analyzed to meet the normality and variance assumptions of parametric tests. For normally distributed data, an

initial one-way ANOVA was carried out to evaluate the differences between doses, not between treatments. If a P-value less than 0.05 was observed, it was considered statistically significant, and the Tukey's multiple range test was performed to determine differences between groups. If data did not meet assumptions of the parametric test, a Kruskal–Wallis test and two-tailed multiple comparisons Dunn's test were used.

3. Results

Homarine concentrations in crude aqueous extracts of corals were $0.869 \pm 0.012\%$ in *P. compressa* and $0.725 \pm 0.0075\%$ in *P. evermanni*. In these species, the aqueous extract represents $7.86 \pm 1.22\%$ of the total tissue volume. Thus, the natural concentration of homarine in *Porites* spp. tissue approximates 0.08%. *P. lobata* used in the experiment had a concentration of $0.03\% \pm 0.02\%$ homarine from total tissue volume.

Basal content (2-fold) and catalytic activity (4-11 fold) of CYP3A-like and content of CYP2-like (2-fold) enzymes were significantly higher ($P \leq 0.05$) in *C. auriga* when compared to *C. multicinctus* (i.e., control fish in Figure 1) but not in the homarine exposure, which had significantly more variability (Figure 2). *C. kleinii* basal CYP2-like content (78.1 AU/mg protein) was 3-fold higher and 16 β -hydroxytestosterone (11.4 pmol/min/mg protein) was 1.5-fold higher than in *C. multicinctus* (24.4 AU/mg protein and 7.82 pmol/min/mg protein) ($P \leq 0.05$) in response to the homarine exposure (Figure 2). *C. auriga* basal CYP3A-like content was 1-fold greater than in *C. kleinii* but not following homarine exposure with significantly more variability (Figure 1)

Treatment of fish with homogenates of *P. lobata* did not cause mortality in any of the species. Tissue homogenate of *P. lobata* had no statistically significant effect on CYP3A-like and CYP2K1-like content in liver microsomes of *C. auriga* or *C. kleinii* (Figure 1). In contrast, CYP3A-like proteins exhibited a dose dependent induction ($P < 0.01$) in liver microsomes of *C. multicinctus* and an increasing trend for CYP2-like proteins after treatment with the coral homogenate ($P < 0.01$). In *C. multicinctus*, formation of 16 α -hydroxytestosterone, 6 β -hydroxytestosterone and 16 β -hydroxytestosterone were trending towards induction 2-9 fold by *P. lobata* homogenate

treatment. Although not statistically significant, *C. auriga* showed a slight trend toward increased CYP3A-like proteins with dose and *C. kleinii* showed slight trend in increase of 16 β -hydroxytestosterone with dose (Figure 1).

Homarine treatment caused 60% and 80% mortality in *C. kleinii* in the low and high doses, respectively, while 100% survival occurred in the control. In contrast, 100% survival was observed in *C. auriga* and *C. multicinctus* (Figure 3).

Homarine caused a significant 4-fold increase in CYP2-like content and 2-fold increase in 16 α -hydroxytestosterone catalytic activity in *C. multicinctus* (Figure 3). CYP3A-like catalytic activity in *C. multicinctus* displayed a U-shaped dose response curve, with a significant 3-fold decrease at the low dose and a return to the control level after the high dose treatment. Significant effects were not observed in CYP3A-like content in *C. multicinctus* and *C. kleinii*. The high dose of homarine induced a significant 100% and 320% decrease in CYP3A-like enzyme activities (16 β -hydroxytestosterone and 6 β -hydroxytestosterone formation, respectively) and a 50% decrease in the CYP2-like catalytic activity of 16 α -hydroxytestosterone formation in *C. kleinii* (Figure 3). In *C. auriga*, homarine significantly induced 6 β - and 16 β -hydroxytestosterone formation (by 120%) and CYP3A-like content (by 50%).

4. Discussion

The role of biotransformation enzymes in metabolizing dietary allelochemicals in marine organisms is essentially unknown, although there is evidence in terrestrial systems to suggest that CYPs may play an important adaptive role in feeding on chemically defended plants or animal species (Cohen et al., 1989; Wen et al., 2003; Sasabe et al., 2004). In earlier studies, the concentrations of total hepatic CYP in *Chaetodon capistratus* that regularly preys upon allelochemically-rich gorgonian corals was shown to be 2-3 fold higher, particularly CYP2 and CYP3, than in two sympatric, congeneric butterflyfishes that do not feed on gorgonians (Vrolijk et al., 1994). Similarly, when extracts of the soft coral, *Sinularia sp.* were fed to *C. punctatofasciatus*, which feeds on coral polyps in Australia, CYP2N mRNA was induced (DeBusk et al., 2008). High

levels of CYP2B-like proteins were also found in tropical marine fish that consumed algae rich diets, suggesting that natural products in their diet may be involved in the higher-level expression (Stegeman et al., 1997). These studies indicated that CYPs in families 2 and 3 may be involved in dietary allelochemical biotransformation in butterflyfishes. To better understand the biochemical and molecular mechanisms underlying the ability of *Chaetodon* spp. to consume corals, the effects of a dietary extract and an allelochemical constituent of the tissue extract on hepatic CYP concentration and catalytic activity was examined in three species of butterflyfishes with different feeding strategies.

Although treatment of each butterflyfish species with homogenate from *Porites lobata* failed to elicit acute toxicity, dietary treatment caused significant induction of the detoxification enzymes, CYP2-like and CYP3A-like in *C. multinctus*, which feeds specifically on this coral. The upregulation of CYPs after oral treatment in *C. multinctus* may indicate a unique adaptation allowing consumption of *P. lobata* and related corals in their diet. The evolutionary diversification of CYP has been characterized in insects, and is significantly correlated with specific dietary compounds (Feyereisen et al., 1989, 1999; Berenbaum, 1991, et al., 1999; Cohen et al., 1992; Schuler, 1996; Petersen et al., 2001). For example, studies of the swallowtail butterflies (*Papilio* sp.) provided the principal evidence that feeding specialization within a genus was associated with the coevolution and transcriptional regulation of CYP genes capable of detoxifying the toxic allelochemicals produced by their host plant (reviewed in Schuler, 1996). CYP6B1 mRNA and protein expression were induced in larval swallowtail butterfly after ingesting parsley treated topically with xanthotoxin (Cohen et al., 1992).

Since the *P. lobata* tissue homogenate induced CYP3A-like and CYP2-like in coral-feeding specialist, *C. multinctus*, we predicted that induction would also occur following exposure to homarine, one of the constituents with feeding deterrent activity in the coral extract. Homarine is found in a diversity of marine invertebrates that share the necessity to prevent predation, possibly through chemical defense (Suwetja et al., 1989; Slattery et al., 1997; Affeld et al., 2007; Shapo et al., 2007). Previous studies in sea stars showed that homarine was a feeding deterrent (McClintock et al., 1994) and a similar

study showed feeding deterrence in omnivorous pufferfish (Gochfeld, unpublished). While a trend toward CYP3A-like induction and significant induction of CYP2-like proteins were observed, a non-monotonic dose response curve was observed with each catalytic activity in liver microsomes from homarine treated *C. multinctus*, indicating potential adaptation to stressor-imposed perturbations (Stebbing, 2003a, 2003b; Calabrese, 2008). The low doses may have caused sublethal cytotoxic impairment of protein synthesis, resulting in a decrease in CYP3A-like and CYP2-like activity. At higher doses, hepatocytes may overcompensate, leading to induction and potential detoxification (Calabrese, 2007). It has been reported that increasing hepatic concentrations of copper are associated with decreased lipid peroxidation in wild yellow perch (*Perca flavescens*), a response opposite to that predicted by basic principles, and it has been proposed that this effect might be caused by an increase in the intracellular metallothionein concentrations that might afford protection against reactive oxygen species (Giguere et al., 2005).

Consistent with a potential response to cytotoxicity, acute lethality was observed in *C. kleinii* in a dose dependent manner. In addition, the toxicity was related with decreased activity of CYP3A-like and CYP2-like in *C. kleinii*. Acute toxicity of allelochemicals has been observed in herbivorous insects (Berenbaum, 1978, 1985), although the more common effect is feeding deterrence (Berenbaum et al., 1989). The mechanism for homarine toxicity and its relationship to CYP regulation is unknown. Although it is not known as a substrate for CYP, homarine does alter CYP regulation in a species-specific manner. Induction without catalytic activity is not uncommon. One of the best examples is the industrial contaminant, 2,3,7,8 dibenzodioxin (TCDD), which directly induces CYP1 orthologs by binding to the aryl hydrocarbon receptor (AhR), but does not undergo extensive metabolism by CYP1 (Whitlock, 1990; Olson et al., 1993). Whether toxicity in *C. kleinii* is dependent upon CYP is unclear, but the finding warrants further study.

It is also unclear how homarine alters catalytic activity of CYP3A-like and CYP2-like, but not enzyme content in *C. multinctus*. In vertebrates, CYP3A has a relatively large active site that is prone to cooperative stimulation, particularly in mammals

(reviewed in Sevrioukova & Poulos, 2013). Thus, homarine may have cooperatively stimulated testosterone hydroxylation by CYP3A. Further studies examining potential cooperative interactions may help resolve this issue.

In contrast to coral homogenate treatments, CYP3A-like catalytic activity and protein expression in *C. auriga* were significantly induced by homarine at the high doses. As discussed above, generalists are predicted to have limitations in the capacity of biotransformation enzymes to metabolize large quantities of a single or a similar toxin (Torregrossa et al., 2012). Thus, exceeding basal CYP detoxification capacities may have required induction of the enzyme to avoid toxicity. Given the observation that exposures to *P. lobata* tissue homogenate and homarine caused significantly different toxicities, as well as unique CYP catalytic activity and content in the three species. It appears that the different responses to the coral tissue homogenate and homarine may have resulted from other unknown compounds within the homogenate that potentially served to diminish homarine toxicity relative to *C. kleinii* and CYP induction in *C. auriga* and *C. multinctus*. Why induction occurred in the generalist *C. auriga*, but not *C. kleinii*, is unclear and indicates that additional factors beyond general feeding strategy may be contributing to the differences between the species. For example, *C. auriga* are benthic omnivores and *C. kleinii* prey largely on plankton (Hobson, 1974; Motta, 1980; Harmelin-Vivien & Bouchon-Navaro, 1983). Both species are considered generalists but have different dietary histories and feeding strategies, possibly influencing responses to coral homogenate and homarine.

Based on the insect studies described and preliminary studies in other species of butterflyfish (Vrolijk et al., 1994; Stegeman et al., 1997; DeBusk et al., 2008), specialists with higher basal levels of CYP should be better at detoxifying allelochemicals than generalists. However, *C. auriga* and *C. kleinii* performed as well as *C. multinctus* with regard to toxicity of the coral homogenate, although each species had unique profiles of enzyme expression and activity following treatments. *C. auriga* and *C. kleinii* had elevated basal levels of CYP3A-like and CYP2-like without significant induction, whereas *C. multinctus* had rapidly inducible CYP3A-like and CYP2-like but limited basal expression. As with insects, we also expected the specialists that consistently feed

on *P. lobata*, to constitutively express CYP with somewhat minimal adjustability, and the basal transcript expression of CYP in generalists to be barely detectable but highly inducible upon exposure to a new food source (Sotka & Whalen, 2008). The elevated levels of CYP in generalists may provide an ability to consume toxic uncommon food sources with a limited cost for detoxification. In the generalist marine gastropod *Cyphoma gibbosum*, a controlled feeding assay showed that digestive gland tissues constitutively expressed high activities of the detoxification enzyme glutathione S-transferase (GST) regardless of the gorgonian diet (Whalen et al., 2010b).

Other possible explanations for the differences between the three species may be that each responded differently to the 2 week depuration period during which all fish were fed brine shrimp prior to gavage with coral homogenates. Diet has been shown to significantly alter differences in Phase I and Phase II metabolizing gene expression, particularly in the liver (Parkinson & Ogilvie, 2007). The generalists, *C. auriga* and *C. kleinii*, may have maintained higher CYP levels during the switch to brine shrimp since they consume multiple dietary items. For example, Polar cod, *Boreogadus saida*, were exposed weekly to two doses of dietary crude oil for 4 weeks, and following a 2-week depuration period, mRNA expression of CYP1A and GST returned to basal levels (Nahrgang et al., 2010). The lower basal CYP levels in *C. multinctus* may be due to accelerated reduction of CYP when coral prey (and its associated toxins) was no longer consumed. During exposures in 2012 and 2013, basal CYP contents showed similar trends in each species. However, catalytic activity was significantly higher in 2013 relative to 2012. Although fish were collected from the same locations each year and treated identically, it is unclear why catalytic activity, but not CYP content was different. The dietary contents of each species prior to collection is unknown, so dietary standardization (i.e. brine shrimp feeding) during acclimation is necessary for controlled laboratory experiments to provide normalizing conditions for comparisons. Whether this was responsible for the temporal differences and pre-collection diets requires further study.

In summary, evidence that diet can modulate CYP activities is consistent with previous findings in vertebrates and invertebrates (Vrolijk & Targett, 1991; Schuler,

1996; Berenbaum and Zangerl, 1998; DeBusk et al., 2008; Whalen et al., 2010a). These results display the usefulness of incorporating biochemical methods into ecological studies to improve the understanding of mechanisms that allow marine consumers to tolerate allelochemically defended prey. Furthermore, identifying the molecular foundations of physiological responses has comprehensive implications for understanding the role of the environment in determining gene function in a coevolutionary context.

Acknowledgments. We thank Dr. Andrew Rossiter and the staff of the Waikiki Aquarium for providing laboratory facilities and technical expertise. We would also like to thank Dr. Malin Celander (University of Gothenburg) and Dr. Donald R. Buhler (Oregon State University) for providing the anti-CYP antibodies. We are grateful for the support of undergraduates Bronte Crook, Jenna Harlacher, Danielle Garcia, and Chelsea Wolke in care of the fish.

Compliance with Ethical Standards. The author and co-authors of this paper have acted ethically in conducting the described research, having undertaken careful analysis of data and the submitted manuscript to avoid errors. Animals in this study were treated humanely, with protocols overseen and approved under the University of Hawaii at Manoa, Institutional Animal Care & Use Committee (IACUC), protocol # 13-1701 Ref. 1701.

Funding. Resource Allocation Program of the Agricultural Research Station for UCR and NSF for University of Mississippi.

449

450 **References**

- 451 Aeby, G. S., 2002. Trade-offs for the butterflyfish, *Chaetodon multicinctus*, when
452 feeding on coral prey infected with trematode metacercaria. Behav. Ecol.
453 Sociobiol. 52, 158-165. doi: 10.1007/s00265-002-0490-2.
- 454 Affeld, S., Wagele H., Avila C., Kehraus S., Konig G. M., 2006. Distribution of
455 homarine in some Opisthobranchia (Gastropoda: Mollusca). Bonner Zoologische
456 Beitrage. 55, 181-190.
- 457 Anderson, G.R.V., Ehrlich, A. H., Ehrlich, P. R., Roughgarden, J. D., Russell, B. C.,
458 Talbot, F. H., 1981. The community structure of coral reef fishes. Am. Nat. 117,
459 476–495.
- 460 Beers, J. R., 1967. The species distribution of some naturally-occurring quaternary
461 ammonium compounds. Comp. Biochem. Physiol. 21, 11-21.
- 462 Berenbaum, M. C., 1978. Toxicity of a furanocoumarin to armyworms: a case of
463 biosynthetic escape from insect herbivores. Science. 201, 532-534. doi:
464 10.1126/science.201.4355.532
- 465 Berenbaum, M. C., 1985. The expected effect of a combination of agents: the general
466 solution. J. Theoretical. Bio. 114, 413-431. doi: 10.1016/S0022-5193(85)80176-4
- 467 Berenbaum, M. C., 1995. The chemistry of defense: theory and practice. Proc. Natl.
468 Acad. Sci. 9, 2-8.
- 469 Berenbaum, M. R., 1991. Coumarins. In: Rosenthal, G.A., Berenbaum, M.R. (Eds.),
470 Herbivores: Their Interactions with Secondary Plant Metabolites, vol. 1. Academic Press,
471 California, pp. 221-249.
- 472 Berenbaum, M.R., Feeny, P., 1981. Toxicity of angular furanocoumarins to
473 Swallowtail butterflies: escalation in a coevolutionary arms race? Science. 212, 927-
474 929. doi: 10.1126/science.212.4497.927
- 475 Berenbaum, M.R., Zangerl, A.R., Lee, K., 1989. Chemical barriers to adaptation by a
476 specialist herbivore. Oecologia. 80, 501-506. doi: 10.1007/BF00380073
- 477 Berenbaum, M.R., Zangerl, A.R., 1998. Chemical phenotype matching between a
478 plant and its insect herbivore. Proc. Natl. Acad. Sci. 95, 13743-13748. doi:

479 10.1073/pnas.95.23.13743
 480 Berenbaum, M.R., Zangerl, A.R., 1999. Genetic variation in cytochrome P450-based
 481 resistance to plant allelochemicals and insecticides. 38th Symposium of the
 482 British Ecological Society. 55-83.
 483 Berking, S., 1987. Homarine methylpicolinic acid and trigonelline (Nmethylnicotinic
 484 acid) appear to be involved in pattern control in a marine hydroid. Development. 99, 211-
 485 220.
 486 Calabrese, E. J., 2008. Hormesis: why it is important to toxicology and toxicologists. Env.
 487 Toxicol. Chem. 27, 1451-1474.
 488
 489 Calabrese, E.J., Bachmann, K. A., Bailer, A. J., Bolger, P. M., Borak, J., Cai, L., et al.,
 490 2007. Biological stress response terminology: Integrating the concepts of adaptive
 491 response and preconditioning stress within a hormetic dose-response framework.
 492 Toxicol. Appl. Pharmacol. 222, 122-128.
 493 Cohen, M. B., Berenbaum, M. R., Schuler, M. A., 1989. Induction of cytochrome
 494 P450-mediated detoxification of xanthotoxin in the black swallowtail. J. Chem.
 495 Ecol. 15, 2347-2355.
 496 Cohen, M. B., Schuler, M. A., Berenbaum, M. R., 1992. A host-inducible cytochrome
 497 P-450 from a host-specific caterpillar: Molecular cloning and evolution. Proc.
 498 Natl. Acad. Sci. 89, 10920-10924.
 499 Cole, A. J., Lawton, R. J., Prachett, M. S., Wilson, S. K., 2011. Chronic coral
 500 consumption by butterflyfish. Coral Reefs. 30, 85-93.
 501 DeBusk, B.C., Slattery, M., Ki, J., Lee, J., Aparicio-Fabre, R., Schlenk, D., 2008. Species
 502 differences and effects of soft coral extracts from *Sinnularia maximus* on the
 503 expression of cytochrome P4501A and 2N in butterflyfishes (*Chaetodon spp.*).
 504 Fish. Physiol. Biochem. 34, 483-492.
 505 Feyereisen, R., 1999. Insect P450 enzymes. Annu. Rev. Entomol. 44, 507-533.
 506 Feyereisen, R., Koener, J. F., Farnsworth, D. E., Nebert, D. W., 1989. Isolation and
 507 sequence of cDNA encoding a cytochrome P-450 from an insecticide-resistant
 508 strain of the house fly, *Musca domestica*. Proc. Natl. Acad. Sci. 86, 1465-1469.

509 Giguere A., Campbell, P. G. C., Hare, L., Cossu-Leguille, C. 2005 Metal
510 bioaccumulation and oxidative stress in yellow perch (*Perca flavescens*) collected
511 from eight lakes along a metal contamination gradient (Cd, Cu, Zn, Ni). Can J
512 Fish Aquat Sci. 62, 563–77.
513

514 Gochfeld, D. J., 1997. Mechanisms of coexistence between corals and coral-feeding
515 butterflyfishes: territoriality, foraging behavior, and prey defense. Ph.D.
516 Dissertation, University of Hawaii, Manoa.

517 Gochfeld, D. J., 2004. Predation-induced morphological and behavioral defenses in a
518 hard coral: implications for foraging behavior of coral-feeding butterflyfishes.
519 Mar. Ecol. Prog. Ser. 267, 145-158.
520

521 Gochfeld, D. J., Aeby, G. S., 2008. Antimicrobial chemical defenses in Hawaiian corals:
522 possible protection from disease? Mar. Ecol. Prog. Ser. 362, 119-128.

523 Gregson, M. A., Prachett, M. S., Berumen, M. L., Goodman, B. A., 2008. Relationships
524 between butterflyfish (Chaetodontidae) feeding rates and coral consumption on
525 the Great Barrier Reef. Coral Reefs. 27, 583-591.

526 Harmelin-Vivien, M. L., Bouchon-Navaro, Y., 1983. Feeding diets and significance of
527 coral feeding among chaetodontid fishes in Moorea (French Polynesia). Coral
528 Reefs. 2, 119-127.

529 Hobson, E. S., 1974. Feeding relationships of teleostean fishes on coral reefs in Kona.
530 Hawaii. U.S. Fish Bull. 72, 915-1031.

531 Johannes, R. E., Wiebe, W. J., 1970. Method for determination of coral tissue biomass
532 and composition. Limnol. Oceanogr. 15, 822–824.

533 Krebs, C. J., 2009. Ecology, Sixth Edition. Person Education Inc, San Francisco.

534 Kozl, C.D., Nomikos, A. P., Hampton, T. H., Warnke, L. A., Gosse, J. A., Davey, J. et al.,
535 2008. Laboratory diet profoundly alters gene expression and confounds genomic
536 analysis in mouse liver and lung. Chemico-biological Interactions. 2, 129-140.

537 Laemmli, U. K., 1970. Cleavage of structural proteins during assembly of head of
538 bacteriophage T4. Nature. 227, 680-685.

539 Lavado, R., Rimoldi, J. M., Schlenk, D., 2009. Mechanisms of fenthion activation in
 540 rainbow trout (*Oncorhynchus mykiss*) acclimated to hypersaline environments.
 541 Toxicol. Appl. Pharm. 235, 143-152.

542 Marsh, K.J., Wallis, I. R., Mclean, S., Sorensen, J. S., Foley, W. J., 2006. Conflicting
 543 demands on detoxification pathways influence how brushtail possums choose
 544 their diets. Ecology. 87, 2103-2112.

545 Martin-Skilton, R., Lavado, R., et al. 2006. Evidence of endocrine alteration in the red
 546 mullet, *Mullus barbatus* from the NW Mediterranean. Environ. Pollut. 141, 60–68.

547 McClintock, J.B., Baker, B. J., Hamann, M. T., Yoshida, W., Slattery, M., Heine, J. N.,
 548 Bryan, P. J., Jayatilake, G. S., Moon, B. H., 1994. Homarine as a feeding
 549 deterrent in common shallow-water Antarctic lamellarian gastropod *Marseniopsis*
 550 *mollis*: a rare example of chemical defense in a marine prosobranch. J. Chem.
 551 Ecol. 20, 2539-2549.

552 Motta, P. J., 1980. Functional anatomy of the jaw apparatus and related feeding behavior
 553 of the butterflyfishes (Chaetodontidae) including a review of jaw protrusion in
 554 fishes. Ph.D. Dissertation, University of Hawaii.

555 Motta, P. J., 1988. Functional morphology of the feeding apparatus of ten species of
 556 Pacific butterflyfishes (Perciformes, Chaetodontidae): an ecomorphological
 557 approach. Env. Biol. Fish. 22, 39-67.

558

559 Nahrgang, J., Camus, L., Gonzalez, P., Jönsson, M., Christiansen, J. S., Hop, H., 2010.
 560 Biomarker responses in polar cod (*Boreogadus saida*) exposed to dietary crude oil.
 561 Aquat. Toxicol. 96, 77-83.

562 Olson, J. R., McGarrige, B. P., Giguotti, P. J., Kumar, S., McReynolds, J. H., 1993.
 563 Hepatic uptake and metabolism of 2,3,7,8-Tetrachlorodibenzo-p-dioxin and
 564 2,3,7,8 Tetrachlorodibenzofuran. Fund. Appl. Toxicol. 22, 631-640.

565 Parkinson, A., Ogilvie B. W., 2008. Biotransformation of Xenobiotics. In: Klaassen, C.D.,
 566 (Eds.), Casarett and Doull's Toxicology The Basic Science of Poisons, Seventh
 567 Edition, McGraw-Hill.

568 Pawlik, J. R., 2012. Antipredatory Defensive Roles of Natural Products from Marine

569 Invertebrates. In: Fattorusso, E., Gerwick, W.H., Taglialatela-Scafati, O., (Eds.),
570 Handbook of Marine Natural Products, Springer Science. doi: 10.1007/978-90-
571 481-3834-0_12

572 Petersen, R.A., Zangerl, A. R., Berenbaum, M. R., Schuler, M. A., 2001. Expression of
573 CYP6B1 and CYP6B3 cytochrome P450 monooxygenases and furanocoumarin
574 metabolism in different tissues of *Papilio polyxenes* (Lepidoptera: Papilionidae).
575 Insect Biochem. Mol. Biol. 31, 679– 690.

576 Pratchett, M. S., 2007. Dietary selection by coral-feeding butterflyfishes
577 (Chaetodontidae) on the Great Barrier Reef, Australia. Raffles Bull. Zool. 14,
578 155-160.

579 Sasabe, M., Wen, Z., Berenbaum, M. R., Schuler, M. A., 2004. Molecular analysis of
580 CYP321A1, a novel cytochrome P450 involved in metabolism of plant
581 allelochemicals (furanocoumarins) and insecticides (cypermethrin) in *Helicoverpa*
582 *zea*. Gene. 338, 163-175.

583 Schuler, M. A., 1996. The role of cytochrome P450 monooxygenases in plant–insect
584 interactions. Plant Physiol. 112, 1411–1419.

585 Sevrioukova, I. F., Poulos, T. L., 2013. Understanding the mechanism of cytochrome
586 P450 3A4: recent advances and remaining problems. Dalton Transactions. 42,
587 3116-3126.

588 Shapo, J. L., Moeller, P. D., Galloway, S. B., 2007. Antimicrobial activity in the common
589 seawhip, *Leptogorgia virgulata* (Cnidaria: Gorgonaceae). Comp. Biochem. and
590 Phys. 148, 65-73. DOI: 10.1016/j.cbpb.2007.04.019

591 Slattery, M., Hamann, M. T., McClintock, J. B., Perry, T. P., Puglisi, M. P., Yoshida, W.,
592 1997. Ecological roles for waterborne metabolites from Antarctic soft corals. Mar.
593 Ecol. Prog. Ser. 161, 133-144.

594 Sotka, E. E., Whalen, K. E., 2008. Herbivore offense in the sea: the detoxification and
595 transport of secondary metabolites. In: Amsler, C.D., (Eds.), Algal Chemical
596 Ecology. Springer Verlag, Berlin, pp. 203-228.

597 Stebbing, A. R. D., 2003a. A mechanism for hormesis-a problem in the wrong discipline.
598 CRC Critical Reviews in Toxicol. 33, 463-467.

599 Stebbing, A. R. D., 2003b. Adaptive responses account for the β -Curve--hormesis is
600 linked to acquired tolerance. *Nonlinearity Biol. Toxicol. Med.* 1, 493-511.

601 Stegeman, J.J., Woodin, B. R., Singh, H., Oleksiak, M. F., Celander, M., 1997.
602 Cytochromes P450 (CYP) in tropical fishes: catalytic activities, expression of
603 multiple CYP proteins and high levels of microsomal P450 in liver of fishes from
604 Bermuda. *Comp. Biochem. Physiol. C: Pharmacol Toxicol. Endocrinol.* 116, 61-
605 75.

606 Strong, D. R., Lawton, J. H., Southwood, T. R. E., 1984. *Insects on plants: community*
607 *patterns and mechanisms.* Harvard University Press, Cambridge.

608 Suwetja, I. K., Hori, K., Miyazawa, K., Ito, K., 1989. Changes in content of ATP-related
609 compounds, homarine, and trigonelline in marine invertebrates during ice storage.
610 *Bull Japanese Soc. Sci. Fisheries.* 55.

611 Torregrossa, A.M., Azzara, A. V., Dearing, M. D., 2012. Testing the diet-breadth trade-
612 off hypothesis: differential regulation of novel plant secondary compounds by a
613 specialist and a generalist herbivore. *Oecologia.* 168, 711–718.

614 Tricas, T. C., 1986. Life history, foraging ecology, and territorial behavior of the
615 Hawaiian butterflyfish, *Chaetodon multicinctus*. Ph.D. Dissertation, University of
616 Hawaii at Manoa.

617 Tricas, T. C., 1989. Prey selection by coral-feeding butterflyfishes: strategies to
618 maximize the profit. *Env. Biol. Fish.* 25, 171-185.

619 Vrolijk, N. H., Targett, N. M., 1991. Comparison of in vivo detoxification enzyme
620 activities among three members of the genus *Chaetodon* (butterflyfish). *Proc.*
621 *Second Intl. Mar. Biotechnol. Conf. IMBC'91.*

622 Vrolijk, N.H., Targett, N. M., Woodin, B. R., Stegeman, J. J., 1994. Toxicological and
623 ecological implications of biotransformation enzymes in the tropical teleost
624 *Chaetodon capistratus*. *Mar. Bio.* 119, 151-158.

625 Wen, A., Pan, L., Berenbaum, M. R., Schuler, M. A., 2003. Metabolism of linear and
626 angular furanocoumarins by *Papilio polyxenes* CYP6B1 co-expressed with
627 NADPH cytochrome P450 reductase. *Insect Biochem. Molec. Bio.* 33, 937-947.

628 Whalen, K.E., Starczak, V. R., Nelson, D. R., Goldstone, J. V., Hahn, M. E., 2010a.

Cytochrome P450 diversity and induction by gorgonian allelochemicals in the marine gastropod *Cyphoma gibbosum*. BMC Ecology. 10, 24.

Whalen, K.E., Lane, A. L., Kubanek, J., Hahn, M. E., 2010b. Biochemical warfare on the reef: the role of glutathione transferases in consumer tolerance of dietary prostaglandins. PloS One. 5, e8537. doi: 10.1371/journal.pone.0008537

Whitlock, J. P. Jr., 1990. Genetic and molecular aspects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin action. Annu. Rev. Pharmacol. Toxicol. 30, 251-277.

Legends

Figure 1. Effects of dietary exposure to *P. lobata* tissue homogenate on the hepatic expression (left) and catalytic activity (right) of CYP2K1 and CYP3A27 in three species of butterflyfish. Different letters indicate significant ($P \leq 0.05$) differences between untreated and treated fish.

Figure 2. Effects of dietary exposure to homarine on the hepatic expression (left) and catalytic activity (right) of CYP2K1 and CYP3A27 in three species of butterflyfish. Different letters indicate significant ($P \leq 0.05$) differences between untreated and treated species.

Figure 3. The percentage survival of butterflyfishes after a 7 day exposure to homarine at a low and high dose (N=6-8).

Figure 1

Figure 2

Figure 3