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The Role of Detoxification Enzymes in Coral-Consuming Butterflyfish of Different Feeding Strategies From Hawaii and the Indo Pacific

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Dedication

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ABSTRACT OF THE DISSERTATION

The Role of Detoxification Enzymes in Coral-Consuming Butterflyfish of Different Feeding Strategies From Hawaii and the Indo Pacific

by

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Doctor of Philosophy, Graduate Program in Environmental Sciences
University of California, Riverside, August 2015
Dr. Daniel Schlenk, Chairperson

A primary mechanism reef predators use to cope with dietary toxins is through detoxification. Detoxification enzymes cytochrome P450 (CYP) (specifically CYP3 and CYP2) were found in higher concentration in butterflyfish that preferentially feed on allelochemically rich corals. Butterflyfish (*Chaetodon sp*.) have unique feeding strategies with some species being generalist feeders and others feeding on several species of chemically-defended coral. This research elucidates the coevolutionary relationship between butterflyfish and corals by understanding the role detoxification enzymes play in allowing butterflyfish of differing feeding strategies to consume toxic allelochemicals produced by corals. To examine this issue detoxification enzymes CYP1, 2, 3, epoxide hydrolase, glutathione S-transferase (GST) and UDP-glucuronosyltransferase (UGT) were analyzed in butterflyfish of species *C. kleinii, C. lunulatus C. auriga* and *C.*

unimaculatus from Hawaii and Australia. These results have indicated that species consuming soft coral have significantly increased CYP2 and 3A expression and catalytic activity, and GST activity. Also hard coral feeding preference on *Porites spp.* over *Acorapora spp.* had significant CYP2 and 3A expression and catalytic activity, and GST activity. Further investigation into hard coral *Porites lobata* and the feeding deterrent compound homarine (found in the coral extract), found that oral exposure to *P. lobata* homogenate significantly induced content and catalytic activity of CYP2 and CYP3A, by 2-3 fold and by 3-9 fold, respectively, in preferred specialist *C. multicinctus*, but not in *C. auriga* or *C. kleinii*. Homarine caused a significant decrease of CYP3A and CYP2 at the high dose in *C. kleinii* and 60-80% mortality in that species. Soft coral *Sinularia maxima* and isolated compound 5-Episinuleptolide (5ESL) were deterrent to butterflyfish generalist feeders, but not specialists. Oral exposure to 5-ESL caused 100% mortality on the hard coral specialist *C. multicinctus* and the least toxicity on soft coral specialist *C. unimaculatus*, correlated with the incredible induction of CYP3A catalytic activities and concentrations, NADPH dependent metabolism, and CYP3A identified metabolite. My 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.

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Chapter 1: Introduction

Coral reefs are one of the most biologically diverse and economically important ecosystems on the planet. Coral reefs provide essential habitats and resources for thousands of species. Being one of the most species-rich habitats of the world, coral reefs are important in maintaining a genetic library for future generations (Moberg, 1999). Almost a third of the world's marine fish species are found on coral reefs (McAllister et al., 1991). Specifically corallivorus fish are dependant on coral reefs for food, shelter and living space. However, due to the increasing threat of climate change and anthropogenic pollution, coral reefs are experiencing significant declines (Bellwood et al., 2004; Gardner et al., 2003; Hughes et al., 2003; McClanahan et al., 2001). Based on the world's most extensive time series data on reef condition (2,258 surveys of 214 reefs over 1985–2012), they show a major decline in coral cover from 28.0% to 13.8% (0.53% y−1), a loss of 50.7% of initial coral cover (De'ath et al., 2012).

Corallivorous fish are particularly sensitive to the declining abundance of certain coral prey and are typically among the first and most affected fish during extensive coral loss (Cole et al., 2008; Graham, 2007; Mora et al., 2011; Pratchett et al., 2006). While the relationship between fish and coral reefs is significant, progress in the evaluation of the ecological significance of corallivory has been hindered by limited information (Jones et al. 1991). Given the number of fish species that feed on corals, it is likely that the coral-corallivore link in the coral reef food web may be more important than traditionally thought. The largest family of corallivorus fish species is Chaetodontidae (butterflyfish), making up 61% of coralivorous fish (Bellwood et al., 2010). In an intensely dynamic

environment butterflyfish often compete for food resources that are mechanically and chemically defended. Feeding upon chemically defended coral species may give them some distinct advantages within their ecosystem (Hay & Steinberg, 1992). A primary mechanism for coping with dietary allelochemicals is to biochemically alter the diet-derived compound to a less toxic metabolite.

The overall goal of this project is to further knowledge of dietary detoxification and the role of the biotransformation enzyme in corallivorous fish species of the genus *Chaetodon*. This study will enhance our knowledge of the biochemical and molecular mechanisms underlying the ability of *Chaetodon* spp. to consume corals and how these mechanisms relate to feeding strategies and diet choice. The coevolutionary relationship between corals and butterflyfish will provide critical insight into the susceptibility of butterflyfish to coral reef declines. Since chemical defense is generally considered to be a major selective force driving the evolution of CYP gene families, this research will increase our understanding of the role of allelochemical (secondary metabolites) biotransformation and detoxification in marine organisms, which holds significance for understanding patterns of predation and herbivory in marine environments. The role of biotransformation enzymes in the metabolism of dietary allelochemicals in marine organisms is essentially unknown, though there is evidence in terrestrial systems to suggest that CYP may play an important adaptive role in feeding on chemically defended plants or prey species (Wylie et al., 1989, Cohen et al., 1989; Sasabe et al., 2004; Wen et al., 2003).

Chemical Arms Race

Coevolution fascinates biologists because it suggests a view of nature in which close associations between species have shaped their life histories and ecologies in a way that fundamentally alters how they interact. The evolution between insects and plants is an extensively cited example of coevolution (Cohen et al., 1989; Sasabe et al., 2004; Wen et al., 2003; Becerra et al., 2009; Berenbaum et al., 1986; Berenbaum et al., 1998). Coevolution has been described as the process by which plants (prey) and insects (predator) radiate and speciate in association with one another (Ehrlich & Raven, 1964). The process described entails the evolution of chemical defense by plants directed against herbivores, which then frees the plant from herbivore pressure. Interacting herbivores that evolve a counter-adaptation or tolerance to this defense will be able to exploit the new competitor-free adaptive zone, with a consequential likelihood of radiation and speciation.

The pairwise reciprocal coevolutionary scenario of Jazen (1980), following Ehrlich and Raven (1964), predicts the development of a chemical "arms race" (Dawkins and Krebs, 1979), in which reciprocity between plant chemical defenses and insect counter-adaptation takes place. Berenbaum (et al., 1986; et al., 1998; et al., 1999) and Feeny (1981) tested this hypothesis and found that insect-plant specialization corresponds to host plant chemistry, and that this correspondence was maintained through lineages of insects and plants in a system comprising Apiaceae, *Papilio*, and secophoris/furanocoumarins. Insect-host plant affiliates were seen to reflect single chemical changes through evolution of detoxification systems, consistent with a

reciprocal arms-race scenario. Berenbaum and Zangerl (et al., 1986; 1998; 1999) in almost 20 years of work have pieced together one of the most compelling examples of coevolution for plant-herbivore systems. This "reciprocal stepwise evolution" can be of principal importance in the generation of biological diversity and association between insects and their host. Despite the fact that a chemical arms race may be paramount for biological diversification, few studies have looked at this scenario in marine systems, specifically coral chemical defense and butterflyfish counter-adaptive detoxification.

Biotransformation

The detoxification and excretion of xenobiotics (i.e., foreign compounds, including diet-derived allelochemicals) involve complex processes that allow an organism to respond to its internal and external chemical environments. Such metabolic resistance "involves the biochemical transformation of a substance, ultimately reducing its capacity to interact with a target molecule" (Li et al., 2007). Biotransformation is a process conducted primarily through enzymatic reactions that often lead to detoxification and elimination of the parent compounds by radically altering the chemistry of nonpolar lipophilic chemicals to polar water-soluble metabolites. Detoxification pathways can often be divided in to three phases. Phase I enzymatic reactions either add or expose polar atoms within a xenobiotic or endobiotic compound. When polarity has been enhanced through phase I reactions, phase II reactions generally attempt to further enhance polarity through conjugation of the phase I products with a bulky polar endogenous molecule. Alternatively, phase II reactions may protect against bioactivation by masking functional

groups prone to reactive intermediate formation with groups that likely provide steric hindrance rather than augmented polarity. Finally, unmodified allelochemicals and their phase I and II metabolites can be excreted from the cell to the extracellular space or compartmentalized into subcellular organelles (i.e., peroxisomes or vacuoles) (Oude Elferink et al., 1993; Van Luyn et al., 1998), by phase III transmembrane ATP-dependent efflux pumps (Bard, 2000; Flugge & van Meer, 2006; Sorensen & Dearing, 2006).

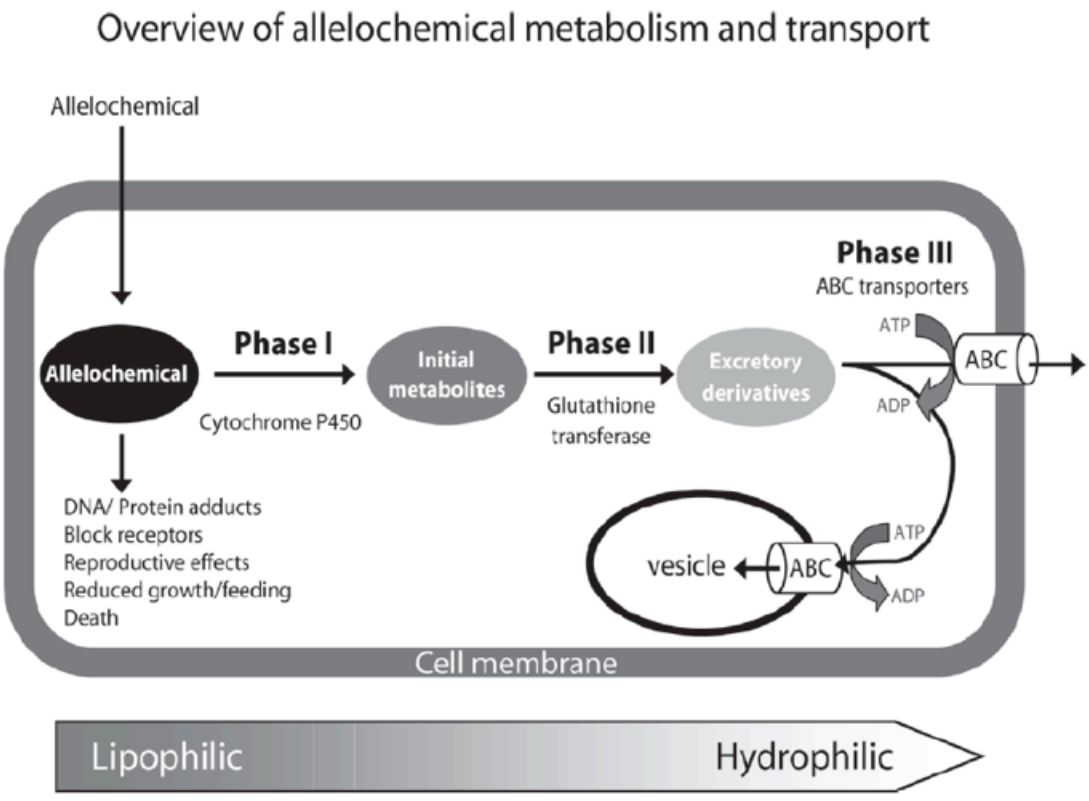

Figure 1.1. An overview of the three phases of allelochemical metabolism and transport (Sotka, 2008)

Three primary reactions occur in Phase I biotransformation: hydrolysis, reduction, and oxidation. Various enzymes are involved in the oxidation of xenobiotic and endobiotic compounds. Biochemical detoxification is believed to be the most important mechanism by which many organisms handle anthropogenic or allelochemical compounds (Kennedy et al., 2013).

Cytochrome P450 monooxygenases

The most dominant enzyme system responsible for oxidation processes in phase I biotransformation and detoxification of dietary allelochemicals is the cytochrome P450 monooxygenase (CYP) family. CYP enzymes can be found in virtually all organisms including bacteria, fungi, yeast, plants, insects, fish and mammals. All CYP genes have probably evolved from one single ancestral gene, which existed before the time of prokaryote/eukaryote divergence (Nelson 1996). CYPs constitute a superfamily of heme containing proteins that have broad substrate specificity. CYP is prevalent in the liver in association with the endoplasmic reticulum or mitochondria (Porter and Coon, 1991). Analysis of sequences of several teleost genomes indicate that fish species contain a complement of CYP gene families similar to those found in mammals; however, tissue distribution, mechanisms of gene regulation, and the catalytic function of many of these enzymes remain unknown (Schlenk et al., 2008).

A complete cytochrome P450 system consisting of CYP and NADPH-P450 reductase (alternatively cytochrome b_5) in membrane-bound form is required for catalytic activity (Lu et al., 1968; Lu et al., 1969). In eukaryotic microsomal CYPs, electrons for

the reduction of molecular oxygen are supplied by NADPH in two consecutive steps via the flavoprotein, NADPH-cytochrome P450 reductase (NPR). NPR contains two redox cofactors: flavin adenine dinucloetide (FAD) and flavin mononucleotide (FMN).

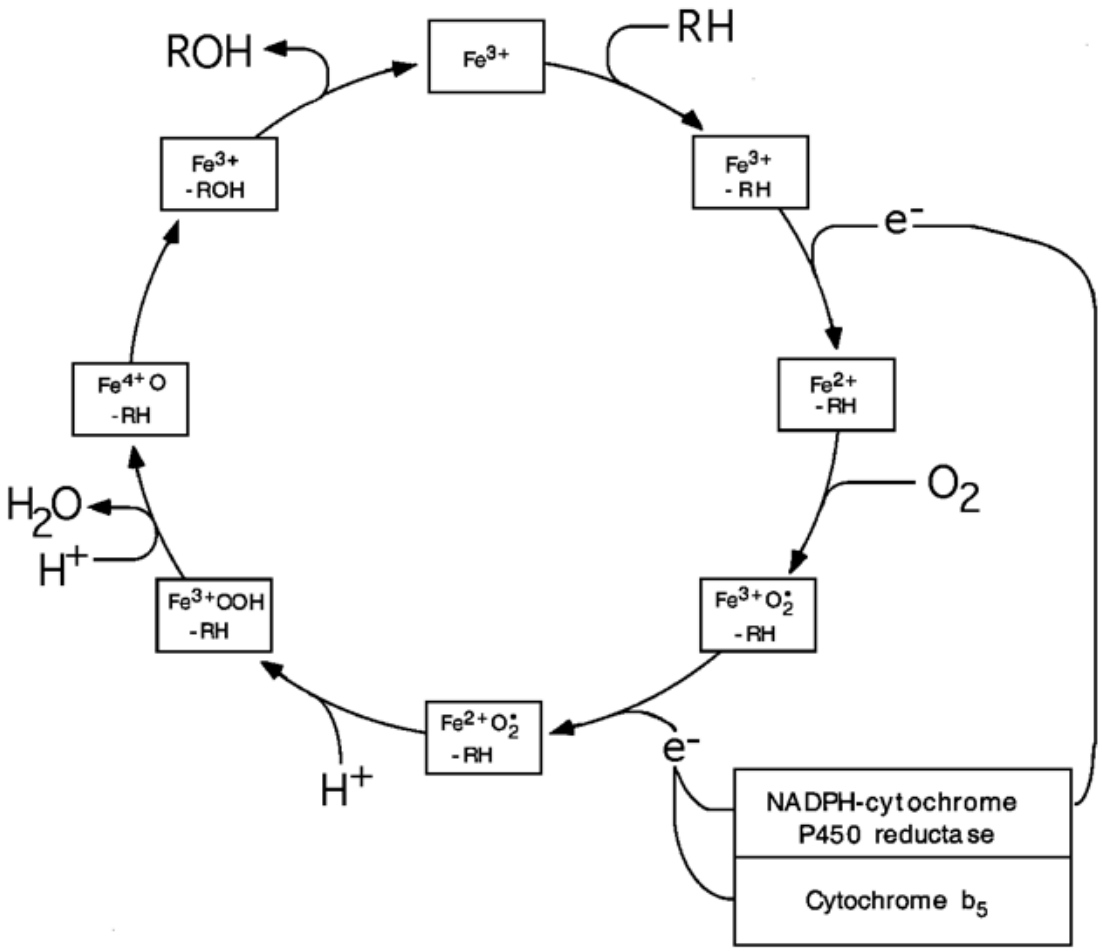

Figure 1.2. Catalytic mechanism of microsmal P450 mediated monooxygenation (Schlichting et al., 2000; Guengerich, 1991).

Therefore, electron flow in the CYP system ends with oxygen. CYP thus uses the chemical potential of oxygen for organic reactions by controlling, via the heme group, the transfer of electrons, creating reactive oxygen intermediates (Hayaishi et al., 1955).

The catalytic cycle begins with the substrate binding to the ferric form: the ground state in which the sixth coordination site is occupied by molecular water. The enzyme-substrate complex is then reduced by an electron transferred from NADPH via NADPH cytochrome P450 reductase. Molecular oxygen binds to the reduced enzyme-substrate complex to form an enzyme-O_2-substrate complex followed by the introduction of a second electron transferred from NADPH via NADPH-cytochrome P450 reductase or from cytochrome b5. The next step involves addition of a proton leading to the formation of an enzyme-OOH-substrate complex. The addition of a second proton results in homolytic cleavage of the oxygen-oxygen bond with one atom of oxygen being released as water. The retained oxygen atom is then inserted into the substrate, the oxidized product is released, and the ferric form of the enzyme is regenerated.

Regulation of CYP may be affected by various factors including age, sex, reproductive status, species, and environmental conditions. Basal levels of individual CYP mRNAs and proteins are regulated via transcriptional and post-transcriptional processes, including mRNA and protein stabilization or degradation. A process of particular interest is the regulation of CYP expression, especially up-regulation or induction by substrates. In many cases inducers of CYPs are also substrates for the CYPs that they induce and therefore stimulate their own metabolism or detoxification. For

example polycyclic aromatic hydrocarbons induce and are metabolized by CYP1A (Billiard et al., 2002).

CYP nomenclature has been standardized so that an Arabic numeral represents the gene family, a capital letter indicates the subfamily, and an Arabic numeral identifies the specific subfamily member. Enzymes within a family must share > 40% sequence homology, while members of a subfamily share > 55% homology. The emergence of new CYP genes results from several evolutionary events, including speciation, gene duplication, divergence, and drift as a function of mutation and fixation all withstanding evolutionary pressures (Nebert et al., 1989; Nelsen, 1999). Taken together, the broad substrate specificity, catalytic versatility, and diversity of forms make the CYP gene superfamily an effective means of protection against a variety of dietary compounds (Scott & Wen, 2001).

CYP2 is the most diverse CYP gene family, with 14 known CYP2 subfamilies in fish: 2J, 2K, 2M, 2N, 2P, 2R, 2U, 2V, 2X, 2Y, 2Z, 2AA, 2AD, and 2AE (Buhler et al., 1994; Yang et al., 1998; Mosadeghi et al., 2007; Schlenk et al., 2002; Oleksiak et al. 2003; Wang et al., 2007). Members of the CYP2 gene family in fish are involved in the metabolism of endobiotics, such as arachidonic acid, lauric acid, and sex steroid hormones, as well as xenobiotics such as aflatoxin, alkoxyresorufins, and benzphetamine (Buhler and Wang-Buhler, 1998; Oleksiak et al., 2003; Yang et al., 1998, 2000). The reason for the observed differences in CYP2 like expression among species is not known. However it has been suggested that natural dietary compounds may be the cause of these differences, since higher protein levels of CYP2B-like protein was observed in

butterflyfish that consumed corals containing high levels of allelochemicals when compared to fish that avoided those corals (Vrolijk et al., 1994). In the Bermuda, herbivorous fish had higher CYP2 levels compared to carnivorous fish (Stegeman et al., 1997).

In the CYP3 gene family four subfamilies have been identified, including CYP3A to CYP3D (Celander & Stegeman, 1997; Hegelund & Celander, 2003; Kullman & Hinton, 2001; Lee & Buhler, 2003; Lee et al., 1998). Currently 13 teleost CYP3A genes have been identified by sequence homologies. CYP3A enzymes are functionally among the most versatile forms of CYP's. Mutation and docking studies have demonstrated that CYP3A proteins have a large substrate-binding pocket in comparison to other members of the CYP superfamily (Khan & Halpert, 2000). This large pocket is thought to enable CYP3A enzymes to bind multiple substrate molecules at any given time. CYP3A have broad substrate specificities for both endogenous and exogenous substrates, including steroids, bile acids, retinoids, xenobiotics such as pharmaceuticals, and procarcinogens (Aoyama et al., 1990; Gillam et al., 1993; Li et al., 1995; Smith et al., 1996; Waxman et al., 1998). CYP3A proteins were initially purified from several teleost species, including scup, rainbow trout, and Atlantic cod (Celander et al., 1989; Klotz et al., 1986; Miranda et al., 1989). Identification of these proteins as CYP3A was initially based on cross-reactivity with CYP3A specific antibodies and steroid hydroxylase activity. Subsequent cloning and sequencing studies confirmed their identity (Celander et al., 1996; Miranda et al., 1991). Functional characterization of recombinant CYP3A enzymes has been determined for CYP3A27, CYP3A45, CYP3A38, and CYP3A40 (Lee et al., 2002; Lee et

al., 2003; Kashiwada et al., 2005;) by heterologous expression in baculovirus systems. CYP3A expression is prominent in the digestive tract (intestinal mucosa and liver), suggesting a role for these enzymes in first-pass metabolism of xenobiotics. The fact that these genes are expressed in tissues that act as barriers to the environment, such as digestive and respiratory tracts, together with the broad substrate specificities of CYP3A enzymes suggest that they evolved as a biochemical defense to prevent toxicity of xenobiotics. Modulation of teleost CYP3A expression following xenobiotic exposure is highly variable and species dependent (Gonzales 1989; Nelson et al. 1996).

Expression of novel CYP isoforms has been associated with the ability of some insects to metabolize toxic allelochemicals and consequently allow them to feed on plants that produce those chemicals (Berenbaum et al., 1981, 1995). Plant secondary compounds have influenced the diversification of the cytochrome P450 gene superfamily in insects (Cohen et al., 1992). Berenbaum and Zangerl (1998) showed that parsnip webworms exhibited both increased amounts and activities of CYP in response to the levels of inducible furanocoumarins in host parsnip plants. In a previous study black swallowtail (*Papilio polyxenes*) where exposed to xanthotoxin, which is a dietary allelochemical found in many of the host plants. When added to the diet xanthotoxin induced the CYP activity in a dose-dependent manner, increasing sevenfold (Cohen, 1989). This result may reflect a physiological adaptation to the levels of xanthotoxin encountered by the black swallowtail in its diet in nature (Cohen, 1989). It has been argued that the evolution of plant chemical defenses and herbivore counter defenses has allowed plants and herbivores to colonize new adaptive zones, within which further evolutionary divergence

could occur in the relative absence of previous competition and predation. While the biochemical interactions between some insects and their host plants have been fairly well characterized, little is known about biotransformation and detoxification of allelochemicals derived from dietary products in marine organisms.

Other Detoxification Enzymes

Xenobiotic epoxides and arene oxides are usually formed by cytochrome P450-dependent oxygenation of a double bond or an aromatic ring, that readily react with lipids, proteins, and DNA bases. The function of the detoxification enzymes epoxide hydrolase (EH) is to catalyze the addition of water to an epoxide or arene oxide (Schlenk et al., 2008). Microsomal epoxide hydrolase is of particular importance for arene oxides produced by the action of CYP on polycyclic aromatic hydrocarbons (PAH). The importance of epoxide hydrolase activity in the biotransformation of xenobiotic compounds was highlighted in a study of the in vitro metabolism of benzo(a)pyrene in scup hepatic microsomes (Stegeman and James, 1985). In fish, however, induction of epoxide hydrolase activity following administration of these agents has not been demonstrated (James and Little, 1981; James et al., 1997). Indeed, in stingrays treated with 3-methylcholanthrene at a dose that induced AHH activity tenfold, epoxide hydrolase activity was significantly lower in hepatic microsomes from treated fish relative to controls (James and Bend, 1980).

Primary phase II enzymes include glutathione S-transferase (GST) and UDP-glucuronosyltransferase (UGT) (Acosta, 2010). GSTs are primarily soluble enzymes

identified in prokaryotic and eukaryotic organisms that detoxify electrophillic and genotoxic compounds through conjugation with GSH. To date GSTs have been divided into six classes, namely α, μ, π, θ, σ, and κ, and members of several classes have been identified in fish. 1-Chloro, 2,4-dinitrobenzene (CDNB) is utilized as a model substrate for characterization of GST activity (Habig and Jakoby, 1981). Activities ranging from 100 to 4000 nmol/min/mg protein have been reported in teleosts and may reflect an increased need for protection from reactive intermediates produced during periods of oxidative stress or as a protective buffer from compounds found in their diets (Schlenk et al., 2008). A number of plant phenols have been shown to be potent inhibitors of GST (Lee, 1991), and chronic exposure to similar compounds or evolutionary adaptation to exposure may result in the increased levels observed in corallivorous butterflyfish (Vrolijk et al., 1994). GST has been implicated in the biotransformation of natural products in aquatic and marine environments similar to responses observed in terrestrial plants by insects (Vrolijk et al., 1992; 1994; Debusk et al., 2000). The induction of GSTs is much less pronounced than that observed in CYPs with exposure to xenobiotics, with only two to five fold increases at the most (Acosta, 2010). Some GSTs are induced through oxidative stress pathways, and thus induction often accompanies increases in CYP1A, but the inter-individual variations often mask changes in GST (Schlenk et al., 2008). Additionally, changes in GST often differ in direction and magnitude depending on treatment schedule and differential effects on various isoforms (Schlenk et al., 2008). Purification and subsequent characterization of GST proteins has proceeded rapidly in the last few years, but molecular characterization, particularly in fish, has been much less

successful (Dominey et al., 1991; Lee et al., 2006). Currently 20 plus gene sequences for fish GSTs have been identified to date, but several groups are currently pursuing further efforts in this area (http://www.ncbi.nlm.nih.gov/biosystems).

The UDP-glucuronosyltransferases (UGTs) are responsible for the process of glucuronidation, a major part of phase II metabolism. Glucuronidation is the major pathway for the conversion (and inactivation) of both endogenous and exogenous compounds to polar, water-soluble compounds that are then excreted in the bile or urine (Schlenk et al., 2008). The UGTs are located in the endoplasmic reticulum (ER), with the active site facing inward into the lumen of the ERUGT has also been known to play an important role in the biotransformation of natural toxins and anthropogenic toxicants that are absorbed into organisms (Acosta, 2010). In vertebrates, membrane-bound UGTs are regarded as major members of the phase II drug metabolizing enzymes, conjugating a large number of xenobiotics as well as endobiotics, such as bilirubin and steroid hormones with UDP-glucuronic acid (Bock, 2003). Insect UGTs play an important role in detoxication of plant allelochemicals encountered by many insects in their diets (Ahmad et al., 1993). Consequently, UGT-catalyzed biotransformation of xenobiotics has been implicated in some cases of insecticide resistance (Bull et al., 1972). In addition, insect UGTs play important roles in several processes, including cuticle formation, pigmentation, and olfaction (Kramer et al., 1987; Hopkins et al., 1992; Wang et al., 1999). Enzyme activities of the insect UGTs are detected in the fat body, midgut and other tissues (Ahmad and Hopkins, 1993b), and are directed towards a variety of plant allelochemicals (Ahmad and Hopkins, 1993a; Luque et al., 2002; Sasai et al., 2009).

Furthermore, insect phenol UDP-glucosyltransferase has been characterized in the silkworm (*Bombyx mori*), which is likely involved in detoxification of plant phytoalexins (Luque et al., 2002). However, only limited molecular information on insect UGTs is available.

A study took a pharmacological approach to determine how a specialist (*Neotoma stephensi*) of juniper foliage (*Juniperus monosperma*) and a generalist (*N. albigula*) may process the same dietary plant secondary compounds (Haley et al., 2007). They investigated the xenobiotic metabolizing enzymes of the specialist and generalist on a juniper diet. The results showed overall the generalist demonstrated induction of UGT and GST conjugation enzymes and constitutively higher levels of UGT enzyme, the specialist did exhibit a greater level of induction of UGT compared to the generalist. A previous study (Lamb et al., 2004) measuring levels of similar xenobiotic metabolizing enzymes in recently caught *N. stephensi* found levels of UGT activity higher than those measured in the specialist on a high juniper diet. The assumption was that the recently caught specialist was consuming, in the wild, a diet containing high levels of juniper. Activities should, therefore, be similar to specialists consuming the high juniper diet in this study. This difference in UGT activities between these studies requires further work for clarification.

Early observations suggested co-regulation of UGTs and CYPs by prototypical phenobarbital- and 3-methylcholanthrene-type inducers (Inscoe et al., 1960; Bock et al., 1973; Owens 1977; Watkins et al., 1982). Recently, these observations were established in the case of CYP2B/3A and UGT1A genes controlled by the nuclear pregnane X

receptor and the constitutive androstane receptor (Xie et al., 2003). In addition, co-regulation of both rat and human CYP1A1 and UGT1A6 (and other UGT1 genes, Fig. 3) by the AhR was demonstrated (Emi et al., 1996; Munzel et al., 1998). Co-regulation facilitates detoxication of plant phytoalexins and dietary PAH contaminants, such as benzo(a)pyrene. For example, phenols generated by induced CYP1A1 can be more efficiently detoxified by co-induced phenol UGTs. In fact, utilizing the Ames test it was shown that co-regulation of CYPs and UGTs attenuates the mutagenicity of benzo(a)pyrene (Bock et al., 1990).

Butterflyfish

Of the 5000 or more fish species recorded on coral reefs only 128 eat corals (Cole et al., 2008; Rotjan & Lewis, 2008). This highlights the exceptional abilities of the few corallivores that have managed to subsist on corals, and the extraordinary status of butterflyfish (Bellwood et al., 2010). The oldest estimated record of Chaetodontidae coevolution with corals is 15.7 Ma (Bellwood et al., 2010). The family contains over 130 species with representatives in all coral reef regions (Allen et al., 1998; Kuiter, 2002). Butterflyfish have been regularly used as a bioindicator species of reef health around the Indo Pacific (Reese et al., 1975; Roberts et al., 1988; Kulbicki et al., 2005). Just over 50% of Chaetodontidae feed at least in part on coral and are an important link in the coral reef food web on eastern Pacific reefs with over 287 important food web links between coral prey, invertebrate and fish corallivores, and top level carnivores (Glynn, 2004). Their colorful patterns, and ease of identification and observation has ensured that the

behavioral, ecological, morphological and biogeographic characteristics of butterflyfishes have been extensively studied (e.g. Motta, 1988; Ferry-Graham et al., 2001; Findley & Findley, 2001; Pratchett, 2005). However, there have been very few studies attempting to understand the ability of butterflyfish to detoxify allelochemicals that are typically present in corals. Based on both observations of feeding and analysis of stomach contents, Chaetodontidae can be divided into three broad feeding categories: coral feeders (facultative and obligative), omnivores (generalist), and plankton feeders (Reese, 1977).

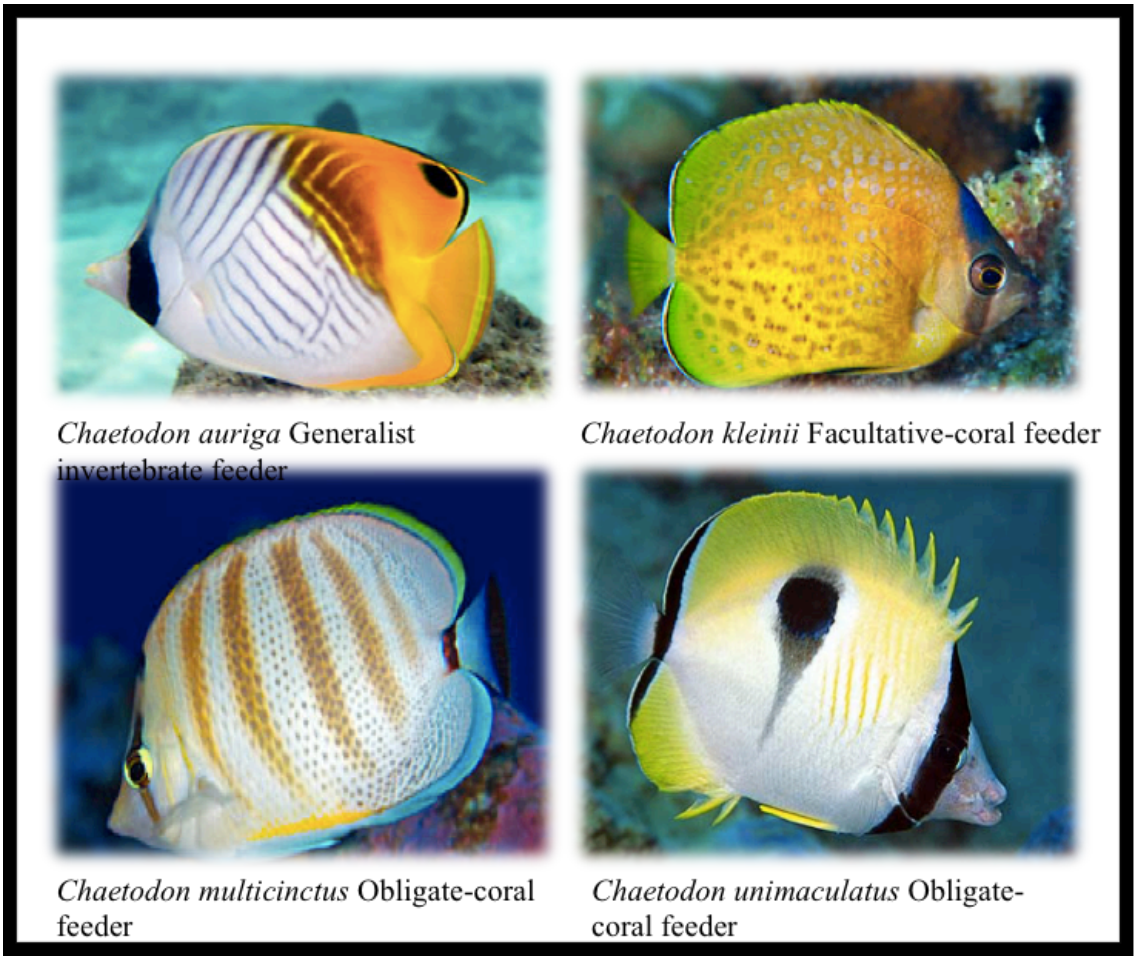

Figure 1.3. Four species of butterflyfish, feeding habits described based on species found in Hawaii

Four species that readily occur within coral reefs throughout the world and have varied feeding strategies (Fig 3.) include: *C. multicinctus* (obligate hard coral feeder), *C. kleinii* (facultative coral feeder), *C. auriga* (omnivore), and *C. unimaculatus* (predominantly obligate soft coral feeder but also hard coral). *C. multicinctus* are hard coral feeding butterflyfish endemic to Hawaii, specializing in *Pocillipora meandrina* and *Porites lobata* (Hoover et al., 2007). *C. kleinii* in Hawaii feed primarily on algae and corals off of reef faces. However, in Australia they are known to subsist largely on soft coral *Lobophytum spp.* (Pratchett et al., 2005). *C. kleinii* feed on hard-corals, soft-corals, gorgonians and hydroids in Australia (Pratchett, 2005).*C. auriga* are generalists, feeding on a variety of corals and sand dwelling invertebrates both in Hawaii and Australia. *C. auriga* were observed feeding intensively on recently damaged colonies of soft coral *Lobophyllia hemprichii* in Australia (Pratchett, 2005). *C. lunulatus* consumes the hard coral *P. lobata* in Hawaii (Cox, 1994) and the hard coral *Acropora spp.* in Australia (Pratchett, 2005). *C. unimaculatus* are specialists primarily feeding on the hard coral *Montipora sp.* in Hawaii (Cox, 1994) and soft corals *Lobophytum spp.* and *Sinularia spp.* in Australia (Pratchett, 2005). *C. unimaculatus* feeds mainly (72.9%) on soft corals in Australia (Pratchett, 2005). However, in Guam they are specialized to feed on the soft coral *Sinularia maximus* (Wylie & Paul, 1989). *C. unimaculatus* has an unusual tolerance for the secondary metabolites of *Sinularia*, enabling this fish to exploit a food source unavailable to most other marine organisms. Certain species of obligate coral feeding butterflyfish, like *C. unimaculatus*, are selective and have evolved to feed exclusively on chemically defended species of corals (Wylie & Paul, 1989). Despite being one of the

most intensively studied families of reef fish, the evolution of specialist feeding strategies remains poorly understood, especially in regards to mechanisms of detoxification.

Figure 1.4. Butterflyfish *Chaetdond lunulatus*

The studies conducted on the role of detoxification in butterflyfish are few and inconclusive. A study conducted by Vorlijk and colleagues (1994) looked at the hepatic CYP and GST in *Chaetodon capistratus*. *C. capistratus* is the only teleost that regularly preys upon allelochemically-rich gorgonian corals. *Chaetodon striatus* and *C. ocellatus*, two sympatric, congeneric butterflyfish species, do not feed on gorgonians and possess 2- to 3-fold less total CYP. The latter two species also have significantly less CYP2B-like and CYP3A-like proteins, suggesting that CYPs in families 2 and 3 may be involved in dietary allelochemical biotransformation (Vorlijk, 1994). Vorlijk (1994) also analyzed GST in *C. capistratus* and found it to be among the highest ever reported in fish. These results suggest that GST may be involved in detoxification of cnidarian dietary allelochemicals in *C. capistratus*. Another study conducted by DeBusk et al. (2008) in

Australia looked at the expression of CYP1A and CYP2N mRNAs in several butterflyfish species, which can feed on numerous chemically defended soft and hard corals. Phylogenetic analyses of the CYP1A cDNA from 12 species of butterflyfish (DNA, amino acid) indicate well-separated groupings according to their feeding strategies (Debusk et al., 2008). In addition, they looked at the effect of soft coral extracts from *Sinularia maxima* on expression of hepatic CYP1A and CYP2 mRNAs. The non-coralline feeding generalist *Chaetodon xanthurus* exhibited a 7-fold higher basal expression of CYP2N8 relative to the other specialist butterflyfish species studied. Although induction of CYP2N8 expression was observed in *C. punctatofasciatus*, these results indicated groupings of feeding strategy with CYP1A phylogeny in *Chaetodon*, but generally unaltered expression of CYP1A and CYP2N following dietary treatment with an extract from a chemically defended soft coral. This study was inconclusive in determining the role of these isoforms in the detoxification of chemicals in these extracts. As mentioned above, herbivorous fish from Bermuda coral reef systems had higher CYP levels (including CYP2B-like proteins) compared to carnivorous fish (Stegeman et al., 1997). Bainy et al. (1999) observed increased levels of CYP2- like proteins in tilapia that were potentially attributable to consumption of chemically-defended algae. It remains to be shown if natural dietary chemicals may act as inducers of CYP2 forms in fish or if other mechanisms are involved. Additional studies examining other CYP orthologs, particularly in obligate coral feeding species, are necessary to better understand the biochemical mechanisms that allow fish to feed on chemically defended organisms (Debusk, 2008).

Impacts of Feeding Strategies on Detoxification and Fitness

Distinct feeding strategies lead to different capacities for detoxification of allelochemicals. Specialist and generalist feeding strategies incur trade-offs; however, specialists seem to be particularly vulnerable to coral reef declines. Generalists are predicted 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 particular type of biotransformation enzyme are thought to prevent the generalist from metabolizing large quantities of a single or a similar toxin (Torregrossa, 2012). In contrast, specialists are thought to have evolved liver enzymes with greater specificity and capacity to metabolize specific toxins, often in high concentrations. The trade-off is that specialists will have reduced abilities to process novel toxins (Sorensen et al., 2005a,b; Torregrossa, 2012). Together, these two hypotheses predict that the generalist will perform (e.g. detoxify) better than the specialist when exposed to novel toxins (Torregrossa, 2012). For example, when the specialist woodrat, *Neotoma stephensi*, and generalist woodrat, *Neotoma albigula*, were exposed to a novel dietary toxin from *Larrea tridentate* bushes, fitness metrics of the specialists were more negatively impacted than the generalists (Sorensen et al, 2005a). Specialist woodrats more effectively neutralize dietary toxins found in the natural diet of both woodrat species, when compared to its more generalist counterpart (Sorensen et al. 2004).

Studies of swallowtail butterflies (genus *Papilio*) provided the first evidence that herbivore feeding specialists are associated with the evolution and transcriptional

regulation of CYP genes capable of detoxifying the toxic allelochemical furanocoumarins produced by wild parsnips (reviewed in Schuler 1996). *Papilio polyxenes* is a specialist species that feeds solely on furanocoumarin-containing plants. Two generalist papilionids are *P. glaucus* that feeds occasionally on furanocourmarin-containing plants and *P. canadensis* that rarely encounters furanocoumarins. All three *Papilio spp*. expressed CYP6B genes with specific reactivities reflecting the degree to which furanocourmarins are present in their diets (reviewed in Schuler 1996; Li et al. 2001, 2003). In addition, four times the number of furanocoumarin-metabolizing CYP6B genes have been isolated from generalist *Papilio* species than from the specialist *P. polyxenes*, suggesting that the wider the dietary breadth of the consumer, the greater the requirement for a diversity of detoxification genes to cope with the range of dietary allelochemicals (Li et al. 2007). Because specialists consistently feed on host plants containing furanocoumarins, it is not surprising that their CYP6B genes would be constitutively expressed with somewhat minimal adjustability, while in generalists transcript expression of CYP6B is barely detectable but highly inducible upon exposure to furanocoumarins (Li et al. 2007).

Molecular modeling studies comparing CYP6B1 from the specialist *P. polyxenes* and CYP6B8 from the generalist *Helicoverpa zea*, revealed that CYP6B8 from the generalist had an overall greater flexibility, a more elastic catalytic pocket, and an additional substrate access channel than did CYP6B1 (Li et al. 2004). Subsequent recombinant expression of both enzymes confirmed that the generalist CYP6B8 metabolized a greater range of structurally diverse plant allelochemicals, however at a lower catalytic efficiency, than can the specialist CYP6B1. To the generalist, the cost of

increased flexibility, resulting in a less efficient metabolism of furanocoumarins, was balanced by the acceptance of a wider variety of allelochemicals at the catalytic site and the ability to better contend with the unpredictability of plant defenses (Li et al. 2004).

Berumen (2008) compared the growth rates of two coral feeding butterflyfishes, *Chaetodon trifascialis* (specialized) and *Chaetodon plebeius* (generalist), maintained on exclusive diets of preferred vs. non-preferred prey in Australia. Both butterflyfish selected coral species that maximized juvenile growth; but contrary to expectations, the more specialized species (*C. trifascialis*) did not outperform the generalist species (*C. plebeius*) when both consumed their preferred prey. Increased dietary specialization therefore appears to be a questionable strategy, as there was no evidence of any increased benefits to offset increases in susceptibility to disturbance (Berumen et al., 2008).

Corals and Chemical Defensive Allelochemicals

Encircled by the waters of the North American Countercurrent, the Hawaiian archipelago is among the most isolated subtropical regions in the Indo-Pacific (Simon, 1987). Due to its limited exchange with other central Pacific reef systems, Hawaii has the highest number of endemic corals in the Pacific (Hughes et al., 2003) and may potentially be a place of isolated coevolutionary adaptations (LaJeunesse et al., 2004). Corals are fundamental in providing essential habitats and resources for numerous organisms including butterflyfish.

The most commonly found coral species on the Hawaiian Islands is *Porities lobata*. *P. lobata* is a hermatypic or reef-building hard coral and forms part of the reef

biome. It is important for sheltering many reef species including pairs of the butterflyfish. *C. multicinctus* pairs will often defend feeding territories on *P. lobata.* Grazing of *Porities spp.* by *C. multicinctus* induced changes in polyp behavior (prolonged withdrawal of coral polyps) in the short term, and increases in nematocyst (coral stinging cells) density over the longer term, and these changes were associated with reductions in palatability and subsequent predation rates on the damaged corals (Gochfeld, 2004).

The genus Acropora (Scleractinia, *Acroporidae*) is one of the most widespread genera of corals, spanning the Indian and Pacific Oceans and the Caribbean Sea. In the Great Barrier Reef in Australia, the coral genus *Acropora* has some 73 species (Veron and Wallace, 1984) and in Hawaii and Northwestern Hawaiian Islands it has 4 species (Kenyon, 1992; Asher et al., 2012). *Acropora* is a genus of small polyp stony coral in the phylum Cnidaria. Some of its species are known as table coral, elkhorn coral, and staghorn coral. *Acropora* species are one of the major reef corals responsible for building the immense calcium carbonate substructure that supports the thin living skin of a reef.. Since *Acropora* is abundant on many reefs, the species that it shelters are clearly common, albeit inconspicuous, components of the reef community. The most generalist hard-coral feeder*, C. lunulatus*, consumed a total of 51 coral taxa in broadly similar proportions throughout the Indo-Pacific. However, in Lizard Island Australia Berumen et al. (2005) showed that *C. lunulatus* prefers to feed on *Acropora hyacinthus* and in some locations around the Island feed almost exclusively on this coral prey. Moreover, the physiological condition of *C. lunulatus* was strongly and positively correlated with proportional consumption of *A. hyacinthus* (Berumen et al. 2005, 2008). *C. lunulatus* can,

nonetheless, persist in habitats around Lizard Island with very limited access to *A. hyacinthus* (Pratchett et al. 2004). It may be competitive dominance, therefore, that enables *Chaetodon baronessa* and *Chaetodon trifascialis* to feed predominantly on *A. hyacinthus*, and given equal access to all corals, other butterflyfishes, including *C. lunulatus*, may also feed more extensively on this coral in Lizard Island.

Sinularia maxima is a gorgonian soft coral species and is one the most widely distributed genus of soft coral in the indo Pacific. However, it is not found in Hawaii. *Sinularia sp.* produce secondary compounds which are thought to function in predator defense, competition for space, antifouling and reproduction (Wylie & Paul, 1989). *Sinularia* extracts and secondary metabolites are very effective at deterring natural populations of carnivorous fishes in field assays in Guam (Wylie & Paul, 1989). These soft corals appear to be avoided by most fish predators; however, the butterflyfish *Chaetodon unimaculatus* feed almost exclusively on the soft corals *Sinularia sp*., *Sinularia polydactyla* (Ehrenberg) and *Sinularia maxima Verseveldt* on a Cocos Lagoon patch reef on Guam. *C. unimaculatus* generally feed from the tips of the soft corals where secondary metabolite concentrations are highest, likely because these are the most accessible portions of the colonies (Motta, 1985).

In contrast with fast, mobile organisms plants and corals with a limited range of movement that cannot run away from potential predators are well represented among the chemically defended. Sessile marine invertebrates are particularly accomplished chemists, including sponges, crinoid echinoderms, polychaetes, bryozoans, brachiopods, and particularly anthozoan corals (Teeyanpant & Proksch, 1993; Stachowicz et al., 1997;

Thornton et al., 2002; Carte & Faulkner, 1983; Pohnert, 2004). However, there are fewer reports of secondary metabolites from morphologically defended scleractinian corals (Faulkner, 1993). This observed distribution of secondary metabolites suggests that hard corals produce beautifully sculptured aragonitic homes for their polyps, allowing their polyps to withdraw completely into these exoskeletons to avoid predation. Hard corals may have less chemical defenses than soft corals, but hard coral families *Acroporidae, Poritidae, Merulinidae* and *Pocilloporidae* have been found to be toxic (Gunthorpe & Cameron 1990). Chemically defended taxa tend to be more speciated than those lacking chemical defenses. Defensive chemicals used by organisms for protection against potential consumers are generally products of secondary metabolism (Pohnert, 2004).

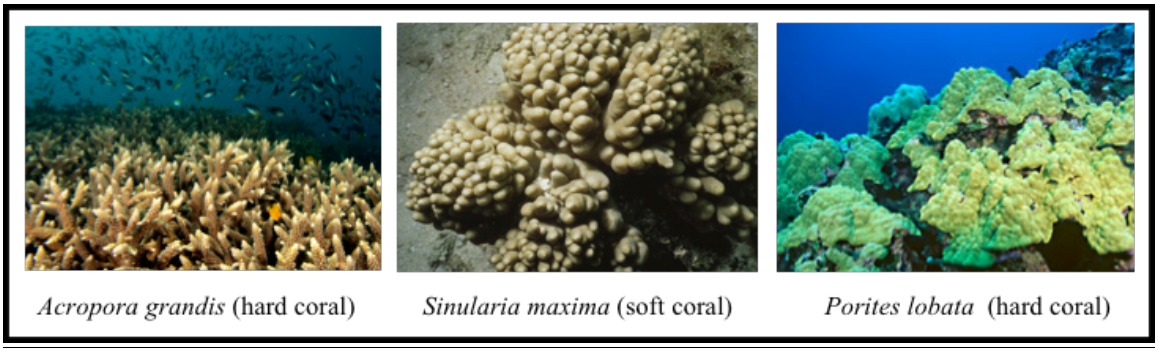

Figure 1.5. Three Indo Pacific species of corals.

Secondary Metabolites and Chemical Defense

Thousands of secondary metabolites have been isolated from marine organisms (Hay et al., 1987; Pohnert, 2004; Faulkner, 1984, 1996, 1999, 2000, 2001). Examples

include phlorotannins, terpenes, sterols, nitrogenous compounds, acetogenins, and compounds of mixed biogenesis (Blunt, 2004). Often, secondary metabolites have been shown to exhibit one or more of a wide range of activities that can be broadly categorized as either attractants (Faulkner, 1999; Pawlik, 1992; Maier, 1993; Maier, 1995; Forward et al., 1994; Boland et al., 1995; Coll et al., 1994; Coll et al., 1995) deterrents (Hay, 1996; Coll, 1992; Hay et al., 1992; Paul, 1992; Lindquist, 1992; Proksch et al., 1994; Pawlik et al., 1995), or protectants (Nolen et al, 1995; Pavia et al., 1997). Much of the work on secondary metabolites has defined organismal responses to compounds through behavioral assays. More recent studies have begun to address the physiological and biochemical mechanisms underlying these responses (Adler, 1994). Not surprisingly, the mechanistic underpinnings of the behavioral responses are complex and multivariate. Given the number and diversity of secondary metabolites and their broad range of bioactivities, it is interesting to speculate on the effect of these compounds (which can be present in plants and prey at concentrations that constitute approximately 20% of the dry tissue mass) once they are consumed.

During the last decade evidence has accumulated that chemical defense can play an important role influencing the composition of pelagic communities (Pohnert et al., 2007). Secondary chemistry differs from primary chemistry principally in its distributional variability and it is this variability that has intrigued ecologists for the past 30 years (Berenbaum, 1995). Their presence in an organism is generally characterized by specialized synthesis, transport, or storage (Adler, 1994).

Two coral compounds that occur in butterflyfish diets are 5-episinuleptolide (5ESL) (isolated from *S. maxima*) (Bowden, 1978) and Homarine (isolated from *P. lobata*) (Gochfeld, unpublished). Both compounds deter fish predation, which may indicate possible toxicity by learned avoidance (Slattery, unpublished; McClintock, 1994). 5ESL is a norcembranolide diterpene (a cembrane lacking the C-18 methyl group at C-4) first isolated from *Sinularia leptoclados* (Bowden, 1978) and later from several other species including S. maxima (Tsai et al, 2013; Sheu et al, 2002; Ahmen et al., 2003; Li & Pattenden, 2011). 5ESL showed moderate cytotoxicity in assays against four human cancer cell lines and had an inhibitory effect on LPS-induced TNF-a production (Takaki, 2003). Control and treated pellets were offered to 4 species of butterflyfishes in the field (n=15 replicates of each), representing generalist (*C. auriga* and *C. vagabundus*) and specialist (*C. melannotus* and *C. unimaculatus*) feeding strategies. The results, in Figure 4, from this study indicated the extract and 5ESL were significantly deterrent to generalists, but not to specialists.

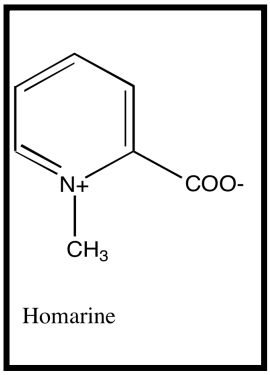

Figure 1.6. Molecular structure of Homarine

Homarine (N-methyl piolinic acid betaine) (Fig. 6) is soluble in water and alcohol and characterized by an absorption minimum at 240 nm and maximum at 272 nm (Berking, 1987). Although it has been found in many invertebrate marine organisms it appears to be absent in fresh water invertebrates. Homarine has been found at high concentrations in the tissues of sea anemones in the Portuguese man-of-war, Physalia (Mathias et al., 1960) and in the hard coral *P. lobata* making up 0.6-1% of the crude extract (Gochfeld et al., unpublished). There is a tendency for more complex marine invertebrates to have a higher concentration of homarine than lower invertebrates (McClintock et al., 1994). UV irradiation destroys the material but heat does not appear to have any significant effect (Berking, 1987). Homarine does not appear to have significant neurohumoral effects (Gasteiger et al, 1955) and the biological significance in these animals has not been well characterized. In *Hydractinia,* homarine was applied to whole animals at several larval stages. It prevented metamorphosis from larval to adult stages and altered the patterns of adult structures in the organism (Berking, 1987). Homarine is present during the entire life cycle of *Hydractinia* indicating a possible role in organism development. Homarine control and treated pellets were offered to omnivorous pufferfish *C. rostrata* (methods found in Gochefeld et al., 2012), treated pellets were significantly deterrent (McNemar**'**s test, $P<0.05$) (Gochfeld, unpublished).

Structurally 5ESL compound appears to have the ability to modify CYP. Preliminary computer docking studies with 5ESL (Fig. 5) indicate the double bond between carbons 16-17 may allow CYP3 to add oxygen to form an epoxide (Goldstone, unpublished). Several low energy (high affinity) positions were found, well within the

oxidizable distance to a computed Fe-O position, suggesting a high likelihood that 5-episinuleptolide will be oxidized by CYP3 orthologs (Goldstone, unpublished).

Overview

This project was designed to further knowledge of the tight coevolutionary relationship between butterflyfish and coral reefs by understanding the role of detoxification enzymes in butterflyfish of differing feeding strategies that consume corals, which produce toxic allelochemicals. The work that follows characterizes these effects in butterflyfish, generalists and specialists, and attempts to address the issue in terms of whole food chemical defense, as well as single compound effects. Several reports state (Whalen et al., 2010; Debusk et al., 2000; Debusk et al., 2008; Kuhajek & Schlenk, 2003) a need for more research on the contribution of CYP to detoxification of natural toxins in marine organisms because the theories for expression differentiation and the role of biotransformation enzymes are based exclusively on studies of terrestrial plant-herbivore interaction (Berenbaum et al., 1995). Since the biochemical interactions between some insects and their host plants have been fairly well characterized, this study seeks to understand mechanisms of biochemical detoxification in a marine organism, butterflyfish, that has the ability to consume toxin laden prey, corals. The following hypotheses were addressed:

a. Butterflyfish basal CYP levels will be higher in generalist feeding species of butterflyfish relative to specialists.
b. When consuming preferred prey a dose dependent induction of CYP2 and/or CYP3 isoforms will occur within specialist butterflyfish

c. Generalists will have greater survival than specialist when exposed to lethal concentrations of novel toxins.
d. 5-Episinuleptolide will be metabolized by liver micosomes from specialists that feed on *Sinularia sp.* (*C. unimaculatus*)
e. Butterflyfish that consistently consume soft corals will have higher CYP and conjugation enzymes than hard coral feeding counterpart

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Chapter 2: The Role of Biotransformation Enzymes in Diet of Coral-consuming Fish in Hawaii versus Australia.

Abstract

Soft corals have been described as chemist of the ocean, using allelochemicals as their major defense against predation, whereas hard corals have been known to use their hard exoskeleton to prevent predation however few deterrent chemicals have been isolated. Many predatory fishes feed on chemically-defended hard and soft corals; however, little is known about the molecular mechanisms that underpin allelochemical biotransformation and detoxification. This study examines the detoxification enzymes CYP1, 2, 3, epoxide hydrolase, GST, and UGT in livers of four coral-feeding butterflyfish species (family Chaetodontidae) in Australia and Hawaii with similar niches but significantly different feeding strategies. *Chaetodon kleinii, Chaetodon auriga,* and *Chaetodon unimaculatus* feed on soft corals in Australia but facultatively feed on hard corals, and sessile invertebrates in Hawaii. By contrast, *Chaetodon lunulatus* primarily consumes hard coral *Montipora captata* in Australia and hard coral *Porites lobata* in Hawaii. Hawaiian *C. auriga* in the current study had significantly higher CYP1A mRNA, CYP1A activity, and GST activity than Australian and Hawaiian species suggesting exposure to plainar aromatic hydrocabons. *C. lunulatus* from Hawaii had higher CYP2, CYP3, and GST levels than *C. lunulatus* from Australia. Higher levels of CYP2 and CYP3A isozymes and of GST activity in Australian species that feed on allelochemically-rich prey suggest that these biotransformation enzymes may be involved in detoxification of coral dietary allelochemicals in butterflyfish.

Introduction

Numerous marine organisms are known to produce toxins that prevent predation, particularly sessile cnidarians such as sea anemones, soft corals and hard (scleractinia) corals (Pawlik, 2012). Soft corals and gorgonians are sessile invertebrates that usually attach to the reefs or seabeds and lack hard exoskeletons for defense (Brusca et al. 2003). When encountering their predators, they cannot escape freely and quickly (Brusca et al. 2003). However, they can survive in the competitive coral reef environment against numerous predators, mainly depending on their many toxic and antifeeding secondary metabolites (allelochemicals) (Faulkner, 2001). Diterpenoids, sesquiterpenoids, terpenoids and sterols are the main compound classes accounting for the anti-feeding activity and icthyotoxicity of soft corals and gogonians (Ne'eman et al., 1974; Weinheimer et al., 1977, Faulkner, 2001). In contrast, there are fewer reports of secondary metabolites from morphologically defended scleractinian corals (Gunthorpe et al.1990). This observed distribution of secondary metabolites suggests that hard corals produce beautifully sculptured aragonitic homes for their polyps, allowing their polyps to withdraw completely into exoskeletons to avoid predation. Although hard corals may have less chemical defenses than soft corals, families *Acroporidae, Poritidae, Merulinidae* and *Pocilloporidae* have been found to be ichthyotoxic (Gunthorpe et al.1990).

A primary mechanism reef predators use to cope with dietary toxins is to biochemically alter the diet-derived compound to a less toxic metabolite through

detoxification. In marine systems, however, knowledge of enzymes conferring consumer tolerance to dietary allelochemicals is in its infancy. Detoxification pathways can often be divided in to three phases (Acosta, 2010). Phase I enzymatic reactions either add or expose polar atoms within a xenobiotic or endobiotic compounds. After polarity has been enhanced through phase I reactions, phase II reactions generally attempt to further enhance polarity through conjugation of the phase I products with a bulky polar endogenous molecule that may mask functional groups prone to reactive intermediates. Finally, unmodified allelochemicals and their phase I and II metabolites can be excreted from the cell to the extracellular space or compartmentalized into subcellular organelles by phase III transmembrane ATP-dependent efflux pumps (Bard, 2000; Flugge & van Meer, 2006; Sorensen & Dearing, 2006). Biochemical detoxification is believed to be the most important mechanism by which many organisms handle allelochemical compounds.

Principle phase I detoxification enzymes are cytochrome P450 (CYP) and epoxide hydrolase (EH) (Acosta, 2010). CYP's have been studied extensively for over 30 years as the predominant enzyme system responsible for the detoxification of dietary chemicals and other xenobiotics (Gonzales 1989; Nelson et al. 1996). CYP is perhaps the most important xenobiotic metabolizing enzyme system in vertebrates, invertebrates and plants. It has been shown to confer resistance in numerous terrestrial insects that consume toxin-laden plants (Frank and Fogelman 1992; Hung et al. 1995). For example, *Papilio polyxenes, P. glaucus P.* and *canadensis* all feed on furanocoumarin (FC)-containing plants. These species of butterflies have different feeding strategies and express CYP6B genes with specific catalytic activity reflecting the degree to which FC were present in

their diets (reviewed in Schuler 1996; Li et al. 2001, 2003).

A study conducted by Vrolijk and his colleagues (1994) compared the CYPs of butterflyfish, *Chaetodon capistratus* that preferentially feeds on allelochemically-rich gorgonian corals and *Chaetodon striatus* and *C. ocellatus,* which do not feed on gorgonians. The study concluded that the latter species possessed 2- to 3-fold less total CYP and significantly less CYP2B-like and CYP3A-like proteins. These results suggest that CYP2 and 3 may be involved in dietary allelochemical biotransformation. Another study conducted by DeBusk et al. (2008) in Australia looked at the expression of CYP1A and CYP2N mRNAs in several butterflyfish species. A CYP1A cDNA phylogenetic tree from 12 species of butterflyfish (DNA, amino acid), indicated well separated groupings according to feeding strategy.

Microsomal epoxide hydrolase is of particular importance for arene oxides produced by the action of CYP on polycyclic aromatic hydrocarbons (PAH). The importance of epoxide hydrolase activity in the biotransformation of xenobiotic compounds was highlighted in a study of the in vitro metabolism of benzo(a)pyrene (BaP) in scup (*Stenotomus chrysops*) hepatic microsomes (Stegeman, 1985). BaP can be activated to electrophilic epoxide metabolites, which can be hydrolyzed with water by epoxide hydrolase or conjugated by other Phase II enzymes. Primary phase II enzymes are glutathione transferase (GST) and UDP-glucuronosyltransferase (UGT) (Acosta, 2010). Electrophilic metabolites can react with cellular nucleophiles such as nucleic acids and proteins, potentially initiating cytotoxicity (James 1986). Vrolijk (1994) also analyzed GST in *C. capistratus* and found it to be among the highest ever reported in

fish. These results suggest that GST may be involved in detoxification of cnidarian dietary allelochemicals in *C. capistratus*. UGT has also been known to play an important role in the biotransformation of natural toxins and anthropogenic toxicants that are absorbed into organisms. An insect phenol UGT has been characterized in the silkworm (*Bombyx mori*), which is likely involved in detoxification of plant toxin phytoalexins (Luque et al., 2002).

The butterflyfish (*Chaetodon spp*.) family makes up the majority of fish that consume chemically defended corals. This study examines the detoxification enzymes in butterflyfish in Hawaii compared to those of the same species living in Australia with a variety of diets. Hawaii has approximately 50 species of corals compared to the hundreds of species found in Indonesia and Australia (LaJeunesse et al., 2000). Based on both feeding observations and analysis of stomach contents, butterflyfish can be divided into three broad feeding categories: coral feeders (facultative and obligative), omnivores (generalist) and plankton feeders (Reese, 1977). *C. kleinii* in Hawaii feed primarily on algae and hard corals. However, in Australia they subsist largely on soft coral *Lobophytum spp.* (Pratchett et al., 2005). *C. auriga* are generalists, feeding on a variety of corals and sand dwelling invertebrates both in Hawaii and Australia. *C. unimaculatus* are specialists, primarily feeding on the hard coral *Montipora sp.* in Hawaii (Cox, 1994) and soft corals *Lobophytum spp.* and *Sinularia spp.* in Australia (Pratchett, 2005). *C. lunulatus* consumes the hard coral *P. lobata* in Hawaii (Cox, 1994) and the hard coral *Acropora spp.* in Australia (Pratchett, 2005). This study allows for the direct comparison of the same species with different or the same feeding strategies between locations to

determine if diet may influence expression and activity of detoxification enzymes. Elucidating the molecular mechanisms governing allelochemical resistance is crucial for understanding the genetic basis of adaptation in consumers like butterflyfish that regularly feed on chemically defended prey.

Material And Methods

Chemicals

Methanol, ethanol, and acetonitrile were analytical grade, glycerol, tris, and potassium chloride, purchased from Fisher (Pittsburg, PA). ^{14}C-Testosterone (150 μCi/μmol; 97.6% purity) was purchased from Perkin-Elmer (Waltham, MA). MS22, EDTA, gelatin, NAPDH, 4-nitrophenol (PNP), 4-nitrophenyl-β-D-glucuronide (PNP-G) was purchased from Sigma-Aldrich (St. Louis, MO). Tween was purchased from EMD Millipore (Billerica, MA).

Animal Collections

Butterflyfish from Hawaii were collected from Kaneohe Bay (*C. auriga, C. lunulatus* and *C. unimaculatus*) and Yokahama Bay (*C. kleinii*) reef systems, surrounding the island of Oahu during summer 2014 (n=7-11). *C. lunulatus* (45.5 ± 9 g; 11.9 ± 0.7 cm), *C. kleinii* (23 ± 7 g; 9 ± 1 cm), *C. auriga* (43 ± 8 g; 10 ± 1 cm) and *C. unimaculatus* (10.4 ±6 g; 7.2 ±1.7 cm) were dissected immediately after being caught. Butterflyfish from Australia were collected from Lizard Island reef system during summer 2014. *C. lunulatus* (50 g ± 11; 13.3 cm ± 0.4), *C. kleinii* (21 g ± 3; 8 cm ± 0.7), *C. auriga* (49 g ±

10; 14 cm ± 2) and *C. unimaculatus* (14.2 g ±5; 9.1 cm ±2.4) were dissected immediately after being caught.

Measurement of CYP gene expression

RNA isolation, reverse transcription and first strand cDNA synthesis

Expression level of CYP1, 2, 3, and β-Actin genes, for which primers have been designed, was determined using quantitative real-time polymerase chain reaction (qPCR). Total RNA was isolated from organs using the SV Total RNA Isolation System (PROMEGA, Madison, WI) as recommended by the manufacturer's instruction. The concentration and purity of the total RNA was analyzed spectrophotometrically at 260 and 280 nm (NanoDrop, Wilmington, DE). One μg of total RNA was reverse transcribed with 1 U of Reverse Transcription System (PROMEGA, Madison, WI) in the presence of random primers according to the manufacturer's instructions. Realtime PCR amplifications used the following primers:

For CYP1A
5’ -TG CCA CTG RTT GAT GAA GAC RCA KGT GTC YTT GG-3’
5’-ATC TGY GGN ATG TGC TTY GGC CGR CGC TA-3’

For CYP2N
5’-AAG GAK CCT CCW GGW CC-3’
5’-GAA GAA SAG RAA SAG CTCCA-3’

For CYP3A
5’-GTG TYC TCT CTC CYT CCT TC-3’
5’-TCT GGG AAT CAA TCA TCA GC-3’

For β-Actin (normalization, house-keeping gene)
5′-ACT CAC ATC TGC TGG AAG GT-3′
5′-TCA CCA ACT GGG CTG ACA TG-3′

The CYP genes were quantified with the Power SYBR Green PCR Master Mix (Applied Biosystems, Grand Island, NY) based Realtime PCR method using an iCycler iQ apparatus (Biorad, Hercules, CA) according to the manufacturer's instructions.
The PCR cycling conditions used were as follows: an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min, primer annealing at 55 °C for 2 min, extension at 72 °C for 2 min and an additional step at 72 °C for 10 min to measure the SyBr Green I fluorescence for avoiding primer–dimer signal. Finally melting curve analysis was performed by heating from 50 °C to 90 °C (0.5 °C for 10 s per cycle for 80 cycles) with simultaneous detection of the SyBr Green I fluorescence signal. All PCR's were performed in technical triplicate for measuring CYP gene expressions. Expression relatively was quantified by densitometric methods previously described using b –actin to normalize samples between treatments (Schlenk et al. 1997). For CYP3A resulting, cDNAs were amplified by PCR in the presence of 10 pmol each primer and PCR Master Mix (PROMEGA, Madison, WI) on Peltier thermocycler. PCR product was purified using Zymoclean Gel DNA Recovery Kit (Zymo Research Co, Orange, CA) and sequenced to verify fragment identity.

Preparation Of Microsomal Fractions

The 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 g for 30 min. Supernatant was collected and centrifuged at 100,000 g for 60 min to obtain microsomal fraction. Supernatant was

removed and the microsomal fraction was re-suspended 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 Croomassie Blue method using a commercial kit (Pierce Inc., Rockford, IL) with bovine serum albumin as a standard.

Western immunoblot

CYP3 and CYP2 protein levels were determined by Western blot as described in (Maldonado et al. 2015b). Microsomal samples were boiled for 5 min in SDS-PAGE buffer (50/50) [44] and 10 μg of protein were 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 CYP2K antibodies provided by Dr. Buhler, Oregon State University or 1:1000 dilution (v/v) of primary rabbit anti-rainbow trout polyclonal CYP3A27 antibodies provided by Dr. Malin Celander University of Gothenburg. 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). Then the blot was incubated for 1 h with alkaline phosphatase-conjugated anti-rabbit IgG (BioRad). Then excess secondary antibody was removed with two washes of T-TBS. The immunoreactive bands 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 were presented in optical density units per mg protein.

^{14}C Testosterone hydroxylase activity

^{14}C Testosterone hydroxylase activity was measured as described by Martin-Skilton et al. (2006). 100 µg of hepatic microsomal protein was incubated with 3.5 µM [^{14}C]-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. An additional 10 µl of NADPH was added, and then incubated for another 30 min at 25 °C. Incubations were stopped with 250 µl of acetonitrile, followed by centrifugation at 10,000 g for 10 min. Then 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 with a radioflow detector β-ram Model 3 (INUS Systems Inc., Tampa, FL) using U-Flow 2:1 (Lablogic Inc.) scintillation liquid. Metabolites 6β-, 16α- and 16β-hydroxytestosterone were identified using authentic standard compounds (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.

EROD and PROD activities

EROD and PROD activities were measured kinetically using a fluorimetric microplate method modified from Lavado et al. (2006). Excitation and emission wavelengths for measuring resorufin formation were 537 and 583 nm, respectively, on a 1420 Multilabel Counter (PerkinElmer, Richmond, CA). Resorufin formation was measured over 10 min and the rate of product formation in samples was obtained from the linear portion of the delta-fluorescence measurements over time. Based on the slope obtained by the linear regression of standards, EROD and PROD activities were normalized to the protein concentration under initial rate conditions and expressed as nmol of resorufin/min/mg protein. EROD/PROD detection limit is 0.05 nmols/min/mg protein of resorufin.

Epoxide hydrolase activities

Microsomes (approximately 1–3 mg protein) from livers were incubated for 20 min in an incubation mixture containing 1 mM styrene oxide in 0.5 M Tris buffer (pH 9.0 unless otherwise indicated) with a total volume of 0.42 ml. Incubations were carried out at 37°C in 25-ml vials with caps having rubber/Teflon septa (Pierce, Rockford, IL) in a Eppendorf heating block. The reaction was terminated by the addition of 2 ml of cold ethyl ether. After vortexing to extract the styrene glycol, samples were placed in a dry ice/ethanol bath for 30 seconds to freeze the aqueous layer. The ether layer was removed and placed in separate tubes. The extraction procedure was repeated twice, yielding 6 ml

of ether layer per sample. The ether layer was evaporated under nitrogen at room temperature. The samples were re-dissolved in heptane/isopropanol (9:1).
The samples were analyzed using a Luna C18 (Phenomenex, Torrance, CA) analytical column (4.6 Å~ 250 mm) on a Shimadzu high-performance liquid chromatograph (HPLC). The mobile phase was heptane/isopropanol (95:5) plus 0.1% trifluoroacetic acid at a rate of 1 ml/min. Ultraviolet (UV) detection was at 209 nm. Standards were prepared from styrene glycol in heptane/isopropanol (9:1). The detection limit was 0.5 pmol/min/mg protein.

GST activities

Cytosolic GST activities toward 1-chloro-2,4-dinitrobenzene (CDNB) was determined spectrophotometrically using the assays of Habig and Jakoby (1981). CDNB was initially dissolved in ethanol prepared so that the final concentration of alcohol was less than 0.01% in the reaction, 50 µl of 20 mM. Substrate and GSH concentrations were 1 mM and 1 mM for CDNB assays. Assay buffers were 100 mM phosphate buffer for CDNB (pH 7.4) in total volume of 1ml. The reaction was started and after 5-min, pre-incubation of GSH and protein in buffer was completed by adding the appropriate substrate. The linear portion of change of absorbance, as determined through preliminary studies, was monitored over 5-min using a Shimadzu 1601 UV/Visible spectrophotometer (Shimadzu Corporation, Torrance, CA**).** GST catalytic activity assays were run in triplicate and corrected for nonenzymatic activity**.** Activity was determined from the linear portion of the curve and expressed as pmol of CDNB-GSH conjugate

produced per minute per mg protein using the extinction coefficient 9.6 mM: cm. Detection limit 50 ng/mg protein.

UGT activity

Microsomal 4-nitrophenol glucuronidation activities were measured using a method described by Krishnaswamy et al. (2003a) with slight modifications. Incubation mixtures contained 25 mM phosphate buffer (pH 7.5), 2.5 mM MgCl2 and 5 mM UDPGA in a final volume of 100 µl. Protein concentrations of 0.05 mg/ml of microsomes were used. Incubations were performed in a heat block for 20 min at 30°C and terminated by addition of 100 µl of acetonitrile containing acetaminophen as an internal standard. After centrifugation at 16,000g for 10 min, the supernatants were dried down in a vacuum oven at 45°C and reconstituted with 150 µl of water and used for HPLC. Standard curves were generated using purified 4-nitrophenol-UGT (10 to 100 nmol). The lower limit of sensitivity of the assay under these conditions was 3 pmol/min/mg of protein.
HPLC analyses were used in a similar method to Almasi et al. 2006, with modifications as follows: Shimadzu 1601 UV/Visible spectrophotometer was used for analysis (Shimadzu Corporation, Torrance, CA**)**. A Luna C18 reversed phase column (150 mmÅ~4.6 mm I.D., 5 µm particle size) was employed for all separations. The mobile phase used consisted of methanol–water (50:50, v/v) containing 0.01 M TBAB. The sample volume was 200 µL, the flow rate was 0.7 mL/min and detection was effected at 290 nm. The wavelength was then determined for optimal expression 4-nonylphenol and 4-nonylphenol-Glucuronidation. The analyses were performed at ambient temperature

allowing a 15 minute equilibration after the start of pumping mobile phase through the column. Detection limits was 0.04 pmol/min/mg protein.

Statistical analyses

Statistical analyses were conducted using Prism5 v5.0a software. Data were expressed as a ratio of CYP2 gene copy to β-actin gene copy per ng of total RNA and log normalized for equality of variance testing. Differences in average expression results were calculated using Tukey's oneway ANOVA at 95% confidence interval with Prism Statistical Software.

Prior to statistical analysis, all data was analyzed to meet the normality and variance assumptions of the parametric tests. For normally distributed data, an initial one-way ANOVA was performed to evaluate the differences between locations and between species from the same location. If a P-value less that 0.05 was observed, it was considered statistically significant, and if there was significance, the Tukey's multiple range test was preformed 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 was used.

Results

CYP1A fragments of 607 bp were used to measure expression following treatment with four species of butterflyfish from Hawaii and Australia. CYP1A mRNA expression was significantly greater (11-37 fold) in *C. auriga* than other species in

Hawaii and significantly higher than *C. auriga* from Australia ($P \leq 0.05$). The levels of CYP1A mRNA expression in Austrailian *C. unimacuatus* were significantly lower (5-13 fold) than levels seen in the other three species from Australia ($P \leq 0.05$).

Sequence fragments of 207 bp were used to measure expression of CYP2N mRNA from livers of Australian and Hawaiian fish (Fig). The levels of CYP2N mRNA expression in Hawaiian species were significantly different between each other ($P \leq 0.05$). CYP2N mRNA expression levels were significantly higher (2-28 fold) in the Australian *C. kleinii* than seen in the other three species from Australia and from the Hawaiian species of *C. kleinii.* CYP2N mRNA expression levels were significantly higher (15-fold) in Hawaiian *C. lunulatus* than from the Australian species of *C. lunulatus*. CYP2N mRNA expression levels were significantly higher (14-fold) in Australian *C. unimaculatus* than from the Hawaiian species of *C. unimaculatus.*

Sequence fragments of 379 bp were used to measure expression of CYP3A mRNA from livers of Australian and Hawaiian fish. CYP3A mRNA expression in both *C. lunulatus*'s from Hawaii and Australia were significantly greater (1-100 fold) than other species from their locations. The CYP3A mRNA levels in *C. lunulatus* from Hawaii were significantly higher (1-fold) than *C. lunulatus* from Australia. The CYP3A mRNA levels were greater in Hawaiian *C. auriga* and *C. lunulatus* than Hawaiian *C. kleinii* and *C. unimaculatus*. The CYP3A mRNA levels were greater in Australian *C. auriga, C. kleinii* and *C. unimaculatus,* than Hawaiian *C. auriga, C. kleinii* and

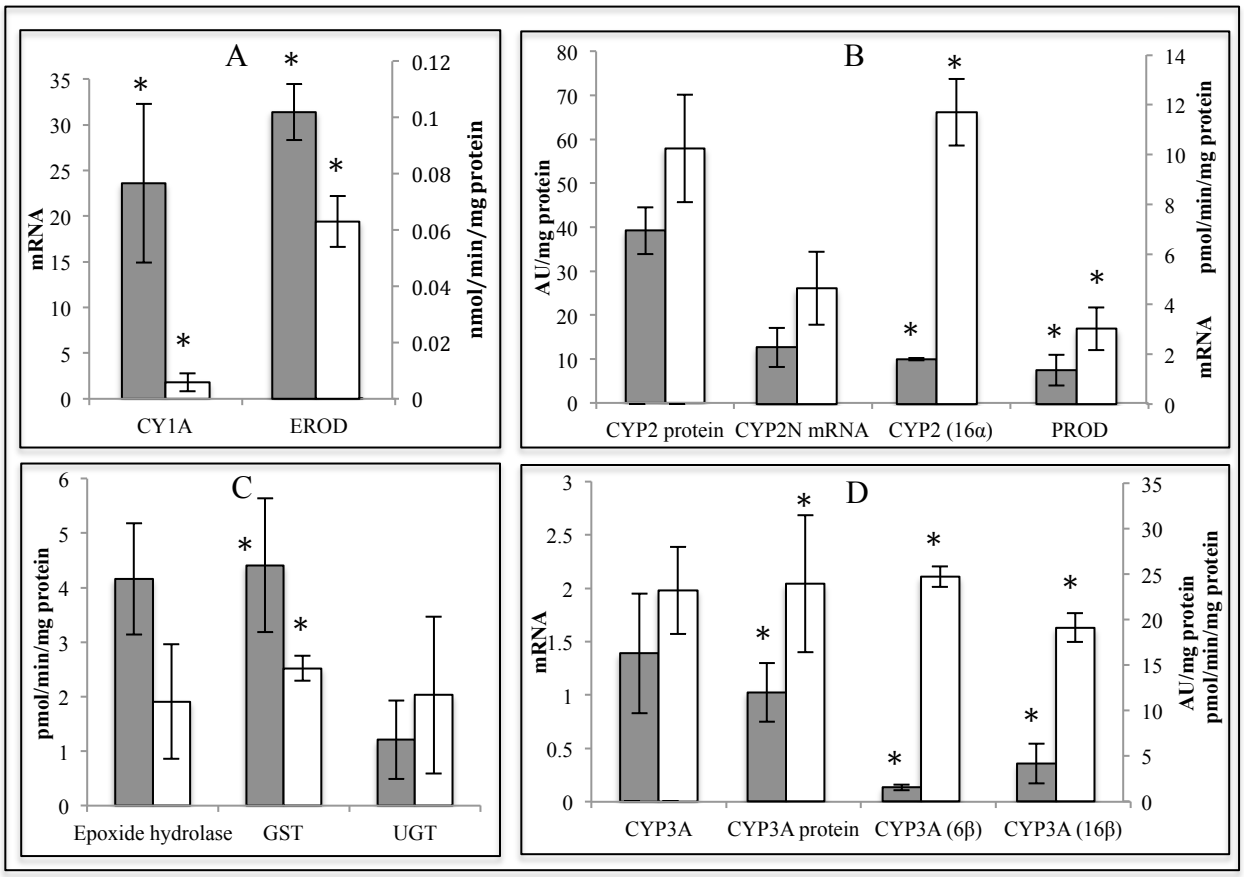

Figure 2.1. Hepatic mRNA, content, catalytic activity in *C. auriga* from Hawaii (grey) and Australia (white). (A) CYP1A mRNA expression (left axis) and CYP1 activity EROD expressed in nmol per minute per mg of microsomal protein (right axis). (B) CYP2 microsomal protein content (left axis), CYP2N mRNA expression (right axis), CYP2 (16α) catalytic activity and PROD both expressed in pmol per minute per mg of microsomal protein (right axis). (C) Epoxide hydrolase microsomal activity, GST cytosolic activity, and UGT microsomal activity all expressed in in pmol per minute per mg of microsomal or cytosolic protein. (D) CYP3A mRNA expression (left axis), CYP3A protein content expressed in AU per mg protein (right axis), CYP3A (6, 16β) microsomal catalytic activity expressed in pmol per minute per mg of microsomal protein (right axis). Significant difference between locations indicated by (*). Values are means of measurements from 6-10 individuals.

C. unimaculatus. Anti-rainbow trout CYP2K1 also recognized several protein bands in all four species of butterflyfish that indicate several CYP2 homologues. The regression of CYP2N mRNA and CYP2 content is R^2=0.853 with a molecular weight of 5.3 kDa. CYP2 total content was significantly lower (4-6 fold) in Hawaiian *C. unimaculatus* than the other three species in Hawaii and *C. unimaculatus* from Australia. Australian *C. kleinii* had significantly more CYP2 content than the other three species from Australia and *C. kleinii* from Hawaii.

Immunoblot analysis with a polyclonal antibody to rainbow trout cytochrome CYP3A27 detected a single band, suggesting that all four species of butterflyfish also possess a CYP3A homologue. There were significant differences observed between Hawaiian *C. auriga* and *C. kleinii* and the Hawaiian *C. lunulatus* and *C. unimaculatus*. CYP3A content was significantly higher (2-4 fold) in *C. kleinii* and *C. unimaculatus* from Australia than Hawaii.

The formation of 16α-hydroxytestosterone from testosterone was significantly greater (5-fold) in *C. lunulatus* than other three Hawaiian species. The formation of 16α-hydroxytestosterone from testosterone was significantly greater (11-fold) in Australian *C. auriga, C. kleinii* and *C. unimaculatus* than Hawaiian *C. auriga, C. kleinii* and *C. unimaculatus.* The formation of 16α-hydroxytestosterone from testosterone was

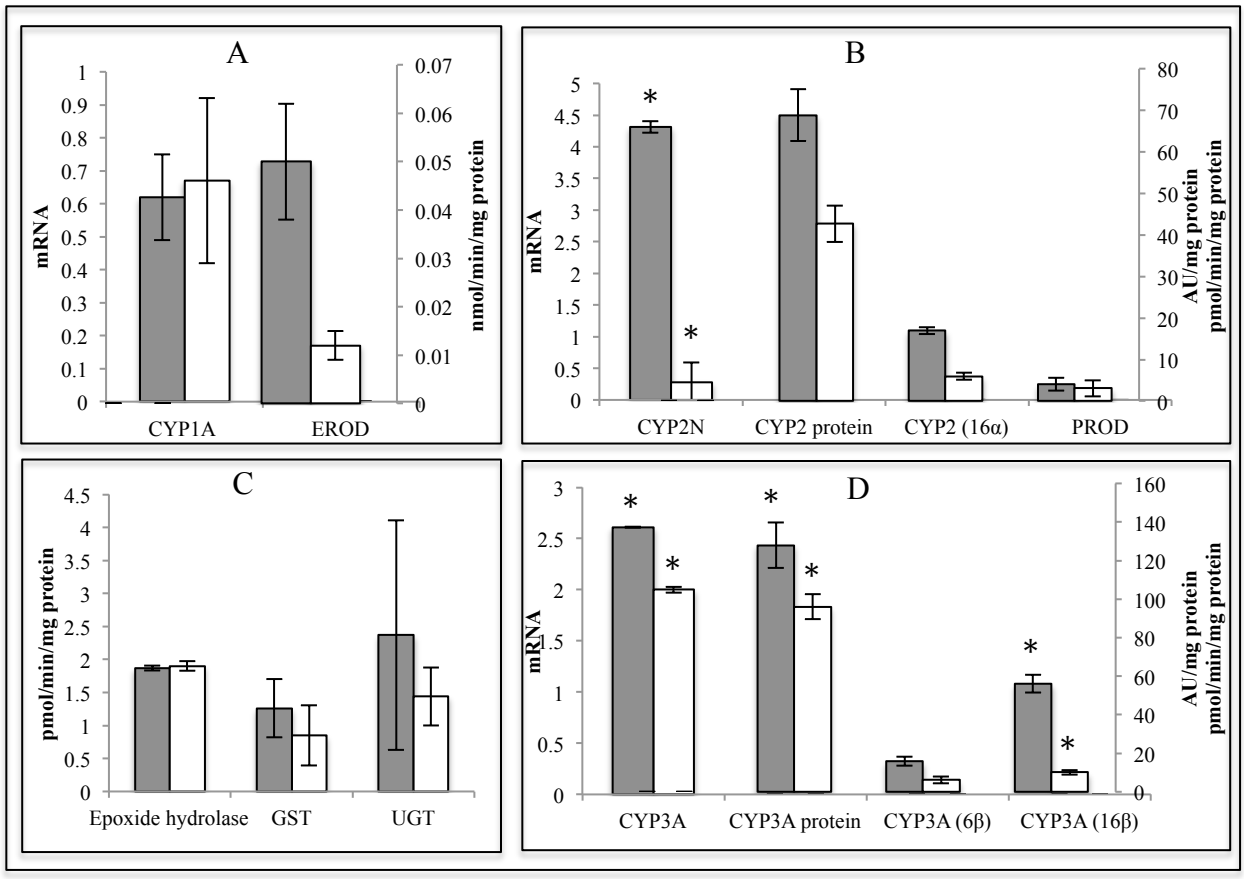

Figure 2.2. Hepatic mRNA, content, catalytic activity in *C. lunulatus* from Hawaii (grey) and Australia (white). (A) CYP1A mRNA expression (left axis) and CYP1 activity EROD expressed in nmol per minute per mg of microsomal protein (right axis). (B) CYP2 microsomal protein content (left axis), CYP2N mRNA expression (right axis), CYP2 (16α) catalytic activity and PROD both expressed in pmol per minute per mg of microsomal protein (right axis). (C) Epoxide hydrolase microsomal activity, GST cytosolic activity, and UGT microsomal activity all expressed in in pmol per minute per mg of microsomal or cytosolic protein. (D) CYP3A mRNA expression (left axis), CYP3A protein content expressed in AU per mg protein (right axis), CYP3A (6, 16β) microsomal catalytic activity expressed in pmol per minute per mg of microsomal protein (right axis). Significant difference between locations indicated by (*). Values are means of measurements from 6-10 individuals.

significantly different between Australian butterflyfish species. The regression of CYP2 total content and CYP2 catalytic activity was R^2=0.892.

The formation of 6β-hydroxytestosterone and 16β-hydroxytestosterone from testosterone was greater (up to 15-fold and up to 20-fold, respectively) in liver microsomes from Hawaiian *C. lunulatus* compared to other Hawaiian species and the Australian *C. lunulatus* (Fig 2.2). The formation of 6β-hydroxytestosterone was significantly greater (5-20 fold) in Australia *C. auriga* and *C. unimaculatus* than the other two Australian species and than the Hawaiian *C. auriga* and *C. unimaculatus*. The formation of 16β-hydroxytestosterone from testosterone was significantly greater (3-28 fold) in Australian *C. kleinii* and *C. unimaculatus* than the other two Australian species and that the Hawaiian *C. kleinii* and *C. unimaculatus*. The regression CYP3A mRNA, CYP3A content, and CYP3A catalytic activity is R^2=0.842-0.987.

EROD rates were greater (2-fold) in Hawaiian *C. auriga* than in other Hawaiian species and the Australian *C. auriga*. EROD rates were lower (3-5 fold) in Australian *C. lunulatus* than the other Australian species. PROD rates were not significantly different within each location. PROD rates were significantly greater (3-fold) in Australian *C. auriga* than Hawaiian *C. auriga.*

Epoxide (styrene oxide) hydrolase activities were similar in all Australian butterflyfish species. Epoxide hydrolase activities were significantly higher (1-4 fold) in Hawaiian *C. auriga* than the three other Hawaiian species and than the Australian *C. auriga.* Epoxide hydrolase activities were similar in Hawaiian *C. lunulatus* and *C. unimaculatus* and significantly different from Hawaiian *C. auriga* and *C. kleinii.*

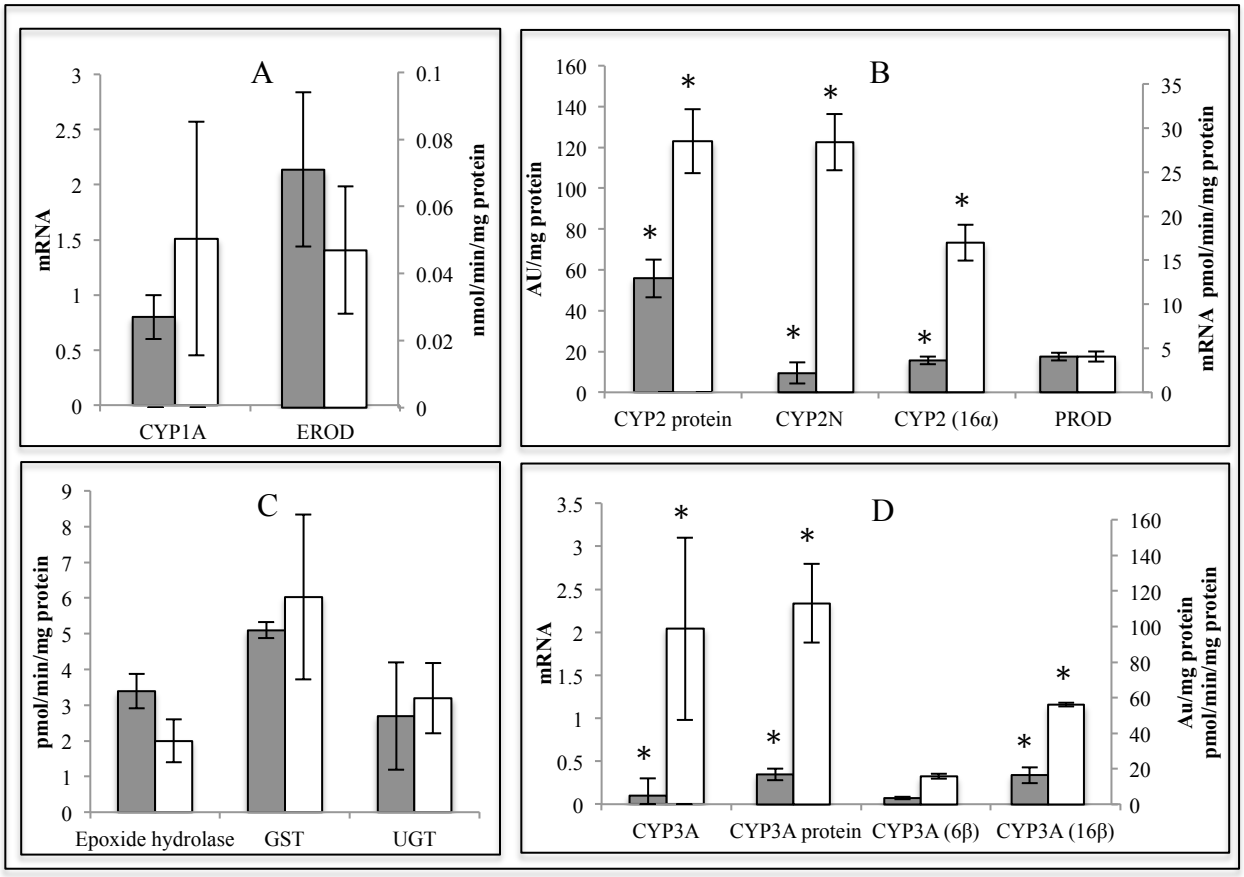

Figure 2.3. Hepatic mRNA, content, catalytic activity in *C. kleinii* from Hawaii (grey) and Australia (white). (A) CYP1A mRNA expression (left axis) and CYP1 activity EROD expressed in nmol per minute per mg of microsomal protein (right axis). (B) CYP2 microsomal protein content (left axis), CYP2N mRNA expression (right axis), CYP2 (16α) catalytic activity and PROD both expressed in pmol per minute per mg of microsomal protein (right axis). (C) Epoxide hydrolase microsomal activity, GST cytosolic activity, and UGT microsomal activity all expressed in in pmol per minute per mg of microsomal or cytosolic protein. (D) CYP3A mRNA expression (left axis), CYP3A protein content expressed in AU per mg protein (right axis), CYP3A (6, 16β) microsomal catalytic activity expressed in pmol per minute per mg of microsomal protein (right axis). Significant difference between locations indicated by (*). Values are means of measurements from 6-10 individuals.

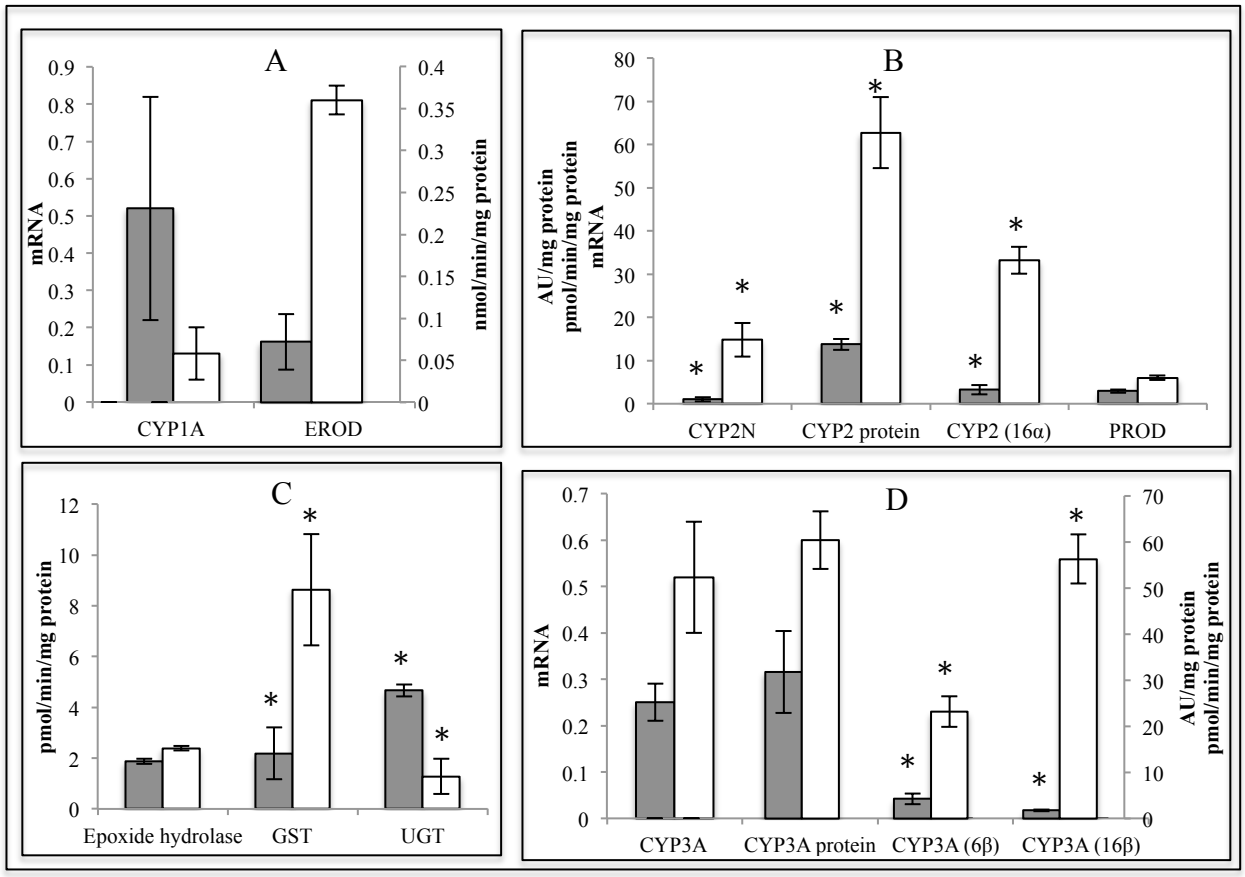

Figure 2.4. Hepatic mRNA, content, catalytic activity in *C. unimaculatus* from Hawaii (grey) and Australia (white). (A) CYP1A mRNA expression (left axis) and CYP1 activity EROD expressed in nmol per minute per mg of microsomal protein (right axis). (B) CYP2 microsomal protein content (left axis), CYP2N mRNA expression (right axis), CYP2 (16α) catalytic activity and PROD both expressed in pmol per minute per mg of microsomal protein (right axis). (C) Epoxide hydrolase microsomal activity, GST cytosolic activity, and UGT microsomal activity all expressed in in pmol per minute per mg of microsomal or cytosolic protein. (D) CYP3A mRNA expression (left axis), CYP3A protein content expressed in AU per mg protein (right axis), CYP3A (6, 16β) microsomal catalytic activity expressed in pmol per minute per mg of microsomal protein (right axis). Significant difference between locations indicated by (*). Values are means of measurements from 6-10 individuals.

Activity of glutathione S -transferase toward CDNB was significantly higher in Hawaiian *C. auriga* and Australian *C. unimaculatus* than the Australian and Hawaiian counter part (Fig 2.1, 2.4). GST activity was significantly greater (1-5 fold) in Hawaiian *C. kleinii* than the other three species from the same location. GST activity was significantly (3-9 fold) lower in Australian *C. lunulatus* than the three other species from Australia.

UDP-glucuronosyl transferase activity appeared to be higher (1-2 fold) in Australian *C. kleinii* than other Australian species (Fig 2.3). UGT activity was significantly greater (1-3 fold) in Hawaiian *C. unimaculatus* than the other three Hawaiian species and Australian *C. unimaculatus*. UGT activity was similar between Hawaiian *C. auriga* and *C. kleinii*, but both were significantly different from both Hawaiian *C. lunulatus* and *C. unimaculatus*.

Discussion

CYP1 Analysis

Regulation of CYP in teleosts may be affected by various factors including age, sex, reproductive status, species, environmental conditions and diet (Schlenk et al., 2008). CYP1A mRNA expression and EROD rates were significantly greater in Hawaiian *C. auriga* than other species in the study. Fish CYP1A induction has been found to be an extremely useful biomarker of environmental exposure to polycyclic aromatic hydrocarbons (PAHs), petroleum hydrocarbons, co-planar polychlorinated biphenyls (PCBs) and dioxin-like chemicals (Safe, 1995). In a review by Bucheli and Fent (1995), they found that 93% of the field studies (68/76) showed that CYP1A

induction in fish was related to contaminant levels in the environment. The Hawaiian *C. auriga* in the current study had significantly higher CYP1A mRNA and EROD rates, which may suggest exposure to plainar aromatic hydrocarbons. Phylogenetic analyses of the CYP1A cDNA from 12 species of butterflyfish (DNA, amino acid) indicate well-separated groupings according to their feeding strategies (Debusk et al., 2008). However, CYP1A mRNA was largely unaffected or diminished by soft coral *S. maxima* extract treatment in *C. xanthurus, C. punctato, C. kleinii*, and *C. auriga* species of butterflyfish (Debusk et al., 2008). We are not inclined to think it is due to diet as a CYP1A gene has been cloned and sequenced from butterflyfish *C. capistratus*, but did not respond to treatment with curcuhydroquinone, which is a gorgonian coral sesquiterpene naturally found in the diet of *C. capistratus* (Vrolijk and Chen 1995).

CYP2 Analysis

CYP2N8 mRNA, CYP2K-like total protein, 16α-hydroxytestosterone (CYP2B, C activity), and PROD rates (CYP2B activity) were all significantly or trending higher in Australia *C. auriga, C. kleinii,* and *C. unimaculatus* than the same species in Hawaii. The reason for the observed differences in CYP2 like expression among species is not known. However it has been suggested that natural dietary compounds may be causing these differences, as higher CYP2B-like protein levels were observed in *C. capistratus* that consumed gorgonians (containing high levels of allelochemicals) compared to butterfly fish that avoided gorgonians (Vrolijk et al., 1994). The major components of the organic soluble extracts of many gorgonians are terpenoids (Tursch et al. 1978; Faulkner 1984).

Terpenoids such as monoterpene, sesquiterpene, diterpene and triterpene classes are known inducers of the CYP2B and CYP3 gene families in mammals (Nebert et al. 1989; Nelson, 2013). CYP is also induced in herbivorous insects, which feed on terpenoid containing plants (Ahmad et al. 1986).

Besides terpenoid natural products, other constituents may be responsible for the higher CYP2 concentrations in the Australia species. Bainy et al. (1999) observed increased levels of CYP2- like proteins in tilapia that were potentially attributable to consumption of chemically-defended algae. In the Bermuda, herbivorous fish had higher CYP2 levels compared to carnivorous fish (Stegeman et al., 1997). *Chaetodon xanthurus, Chaetodon punctato, C. kleinii*, and *C. auriga* CYP2N mRNA expressions were not altered by oral exposures to a coral extract from *Sinularia maxima* (Debusk et al. 2008). However, CYP2-like proteins and 16α-hydroxytestosterone CYP2 activities were significantly induced by oral exposures to the same extracts from *S. maxima* (soft coral) in *C. kleinii* and *C. unimaculatus* (Maldonado et al. 2015a). Australia *C. auriga, C. kleinii,* and *C. unimaculatus* have diets containing soft corals, whereas the same species in Hawaii do not. *C. auriga* were observed feeding intensively on recently damaged colonies of soft coral *Lobophyllia hemprichii* in Australia (Pratchett, 2005). *C. kleinii* feed on hard-corals, soft-corals, gorgonians and hydroids in Australia (Pratchett, 2005). *C. unimaculatus* feeds mainly (72.9%) on soft corals in Australia (Pratchett, 2005). The higher levels of CYP2 in Australian species may suggest the involvement of these enzymes in detoxification of dietary compounds.

CYP3 Analysis

CYP3A mRNA, CYP3A-like protein and CYP3A catalytic activity were all significantly or trending higher in Australian *C. auriga, C. kleinii,* and *C. unimaculatus* than the same species in Hawaii. Although the physiological function of CYP3A is still unknown, the fact that these genes are expressed in tissues that act as barriers to the environment (i.e., digestive, liver and respiratory tract) together with the broad substrate specificities of CYP3A enzymes suggest that they evolved as biochemical defense to prevent bioaccumulation of xenobiotics (Schlenk et al., 2008). Gene families 2 and 3 of CYP are believed to have evolved partially in response to allelochemicals (Gonzalez and Nebert 1990). CYP3A in laboratory rats was induced upon exposure to plant toxins 1,8 cineole and cadinene (Hiroi et al., 1995). Gorgonia feeding *C. capistratus* possess significantly more CYP3A proteins than sympatric congener butterflyfish species that do not feed on gorgonians (Vrolijk et al. 1994). *C. unimaculatus* from Hawaii was orally exposed to the toxin, 5-episinuleptolide, that occurs in *S. maxima,* which *C. unimaculatus* preferentially feeds on in Guam. Exposed fish had 100% survival, a dose dependent induction of CYP3A protein as well as catalytic activity, and an increased ability to metabolize 5-episinuleptolide to epoxide metabolites (Maldonado et al. 2015a). As mentioned above *C. auriga, C. kleinii,* and *C. unimaculatus* all feed on chemically defended soft coral species in Australia, whereas the Hawaiian species of butterflyfish do not. CYP3A is functionally versatile and may be higher in Australia corallivorous butterflyfish species because they require it for the multitude of toxins encountered in

Australia as Hawaii has much fewer species of corals and even less soft corals (LaJeunesse et al., 2000).

Epoxide Hydrolase Analysis

Epoxide hydrolase activity was not significantly higher in the Hawaiian *C. auriga* than other Hawaiian species and the Australian *C. auriga.* Studies with mammalian enzymes have shown that two major epoxide hydrolase enzymes utilize xenobiotic epoxides as substrates (Schlenk et al., 2008). Microsomal epoxide hydrolase is of particular importance for arene oxides produced by the action of CYP on polycyclic aromatic hydrocarbons. For most arene oxides, conversion to the dihydrodiol results in detoxification of the PAHs. In fish, however, induction of epoxide hydrolase activity following administration of these agents has not been demonstrated (James and Little, 1981; James et al., 1997). Flatfish exposed to PAH- and PCB-contaminated Puget Sound sediments showed no increase in epoxide hydrolase activity (Collier and Varanasi, 1991; Collier et al., 1986). Likewise, channel catfish and brown bullhead treated with 10 mg/kg BaP showed no significant induction or species difference in liver microsomal hydrolase activities (Willett et al., 2000). This data supports the absence of high of epoxide hydrolase activity despite the levels of CYP1 mRNA and EROD rates in Hawaiian *C. auriga.*

Although not statistically significant there was a trend of epoxide hydrolase rates higher in Australian *C. auriga, C. kleinii,* and *C. unimaculatus* than Hawaiian. Epoxidation of dietary toxins largely by CYP's can produce epoxides harmful to an

organism. The enzymes EH catalyzed hydration of the epoxide, thereby detoxifying it to a more excretable metabolite. This reaction is advantageous since epoxides have high reactivies, and are common in the herbivore environment. Rats were fed for 2 weeks on a control or 25% Brussels sprouts, in comparison with controls intestinal EH activity was increased 2.4-fold in animals fed either Brussels sprouts (Salbe & Bjeldanes 1985). Hepatic EH activity has been reported to increase 1.4-fold over control (basal diet) levels in mice fed Brussels sprouts, respectively (Hendnch & Bjeldanes, 1983). In previous study with butterflyfish, CYP3A was found to detoxify soft coral toxin 5-episinulaptolide adding an epoxide to the compound. The epoxide metabolite may require EH to further detoxify (Maldonado et al., 2015a). The higher levels of EH in soft coral consuming Australian species *C. auriga, C. kleinii,* and *C. unimaculatus* may correlate to CYP detoxification of toxins.

C. lunulatus Analysis

In contrast to the other three species, CYP2N mRNA, CYP3A mRNA, CYP2K-like protein, CYP3A-like protein, CYP3A catalytic activity, 16α-hydroxytestosterone (CYP2B, C), and PROD rates (CYP2B) were all significantly or trending higher in *C. lunulatus* from Hawaii versus *C. lunulatus* form Australia. Both species are hard coral specialists feeding different species of hard coral. Bioactivity profiles of extracts from species of scleractinian coral in order to determine potential for chemical defense showed that extracts from *Porities spp.* showed significant toxicity to mice, fish and cytotoxicity, whereas *Acroporidae spp.* only showed significant cytotoxicity (Gunthorpe & Cameron

1990). A putative allelochemical found in *Porities spp.*, homarine (1-Methylpyridin-1-ium-2-carboxylate), has been isolated in several marine invertebrates (e.g., Affeld et al. 2006), and was found to be a feeding deterrent to pufferfish *Canthigaster rostrata* (Gochfeld unpublished). Homarine caused a significant decrease of CYP3A and CYP2 at the high dose in *C. kleinii* and 60-80% mortality in that species, however homarine also induced CYP3A content by 3-fold and catalytic activity by 2-fold in *C. auriga* and high dose *C. multicinctus* (Maldonado et al., 2015b). Although more research is necessary to understand the full scope of deterrent chemicals between *Porities spp.* and *Acroporidae spp.* thus far *Porities spp.* is more toxic. Therefore *Porities spp.* may contain more allelochemicals that require *C. lunulatus* from Hawaii to have higher CYP2, CYP3, and GST levels than *C. lunulatus* from Australia.

GST Anlysis

GST activities were significantly or trending higher in Australian *C. klenii*, *C. unimaculatus,* and Hawaiian *C. lunulatus* than the same species in the opposite location. GST's provide cellular protection against the toxic effects of a variety of endogenous and environmental chemicals. The role of GSTs in the resistance to plant chemicals has been studied in numerous crop-feeding lepidopteran species and in insects feeding on deciduous trees (Yu, 1996). In the aphid *Myzus persicae*, the overproduction of GSTs is probably responsible for the adaptation of the insect to glucosinolates and isothiocyanates contained in its Brassicaceae host plants (Francis et al., 2005). Studies in mammalian systems indicate that pi GSTs are more involved than other GST classes in the

detoxification of prostaglandins and other electrophilic α, β-unsaturated carbonyl compounds (Bogaards et al. 1997). The highest concentrations of prostaglandins in nature have been found in Caribbean gorgonians (Weingeimer et al., 1969), where the acetoxy acids, hydroxyl methyl esters and hydroxyl acids of 15(R)-prostaglandin A2 function as feeding deterrents against generalist reef fish (Pawlik et al. 1989; Gerhart, 1984). One study (Vrolijk et al. 1992) examined GST activity in the digestive gland of a generalist gastropod, *Cyphoma gibbosum*, which exclusively feeds on several families of chemically defended gorgonian corals. The authors reported significantly higher GST activity in field-collected *C. gibbosum* feeding on gorgonians *Gorgonia ventalina* and *Briareum asbestinum*, suggesting that GST expression varies in response to different suites of gorgonian allelochemicals. High GST has been measured in *C. capistratus* and may be related to dietary allelochemicals in its preferred prey gorgonians (Vrolijk et al., 1994). As mentioned previously butterflyfish, CYP3A was found to detoxify soft coral toxin 5-episinulaptolide adding an epoxide to the compound. The epoxide may be further detoxified by GST. We hypothesize that Australian *C. klenii*, *C. unimaculatus* GSTs may conjugate gorgonian allelochemicals, like prostaglandins or 5-episinuleptolide, potentially alleviating their toxicity.

GST activity is also significantly higher in Hawaiian *C. auriga* than the Australia *C. auriga*, this is a different trend than CYP2 and CYP3 measurements but similar to CYP1A measurements. In mammals, the AhR controls the transcription of the genes CYP1A1, CYP1A2, CYP1B1, as well as phase II enzymes such as GST (Safe, 1995). Many laboratory studies of GST induction in fish have typically been conducted in

conjunction with studies of CYP1A induction and have therefore involved AhR inducers (George, 1994). Triclosan a personal care product was exposed to yellow catfish (Pelteobagrus fulvidraco), resulting in significantly elevated CYP1A and GST but decreased CYP3A expression (Ku et al. 2014). When rockfish (*Sebastes schlegeli*) were exposed to BaP, northern blots showed that the CYP1A1 and GST transcripts were barely detectable in the control group but were significantly induced in BaP exposed groups (Woo et al., 2007). In Hawaiian *C. auriga* high levels of GST may be correlated with the high levels of CYP1A, suggests that these high levels may be environmental exposure to PAHs, dioxins, polychlorinated biphenyls or other pollutants.

UGT Anlaysis

Similar to the previous detoxification enzymes we see UGT activity was trending higher in Australian *C. auriga*, *C. kleinii and* Hawaiian *C. lunulatus* than the same species in the opposite location. However, significantly higher UGT activity was seen in Hawaiian *C. unimaculatus* than Australian *C. unimaculatus*. Another enzyme family implicated in insect resistance to plant chemicals is the UDP-glycosyltransferases (UGTs) (Macknezie et al., 2005), which act as catalysts for the transfer of a glycosyl group from UDP-glucose to a variety of acceptor molecules. Like CYPs, UGTs likely evolved in response to the challenge of dietary allelochemicals (Bock 2003). Gumboot chiton *Cryptochiton stelleri* readily feeds upon algae *Odonthalia dentate*, which have relatively high amounts of allelochemicals lanosol a known feeding deterrent (Fenical, 1975; Kurata and Taniguchii, 1997; Kuhajek et al. 2003). UGT activity was highly variable but

appeared to increase at the two highest treatments lanosol in *C. stelleri* (Debusk et al. 2000). In the koala (*Phascolarctos cinereus*), which specialist feeder on Eucalyptus, there is negligible conjugation of terpene metabolites, which instead are extensively oxidized to enable their excretion (Boyle et al. 2000, 2001). However, the koala conjugates phenolic metabolites with UGT (McLean et al. 2003), possibly because they are not readily oxidized by CYP enzymes. A similar situation could be occurring in Hawaiian *C. unimaculatus,* where toxins found in the hard coral *Monipora spp.* may not be readily oxidized by CYP enzymes but detoxified by UGT. In Australia, *C. unimaculatus* feeds mainly on soft corals that may have toxins readily metabolized by CYP enzymes. UGT and CYP activities were both higher in Australian *C. auriga*, *C. kleinii* and Hawaiian *C. lunulatus* than the same species in the other location. The coral toxins could be metabolized like certain plant allelochemicals such as hyperforin, β -napthoflavone and indole-3-carbinol inducing UGTs as well as CYPs (Bock & Kohle 2004).

Conclusion

The role of biotransformation enzymes in the metabolism of dietary allelochemicals in marine organisms is essentially unknown, though there is evidence to suggest that they may play an important adaptive role in feeding on chemically defended plants or prey species (e.g. Vrolijk et al. 1994; Stegeman et al., 1997; Whalen et al., 2010). *C. auriga, C. lunulatus, C. kleinii,* and *C. unimaculatus* in Australia and Hawaii represent an ideal model system for studying this concept because we are able to study the same species, in similar environments, with the same reproductive strategy, but

significantly different feeding preferences. Since feeding on allelochemically-rich prey by some of the Australian species is well documented (Pratchett, 2005), quantitatively high levels of CYP2 and CYP3A isozymes and high levels of GST activity suggest that biotransformation enzymes may be involved in detoxification of dietary allelochemicals in butterflyfish. It is quite possible that marine consumers that regularly exploit a range of allelochemically rich prey may have evolved an equally diverse array of detoxification mechanisms. Elucidating the molecular mechanisms governing allelochemical resistance is crucial for understanding the genetic basis of adaptation in consumers like butterflyfish that regularly feed on chemically defended prey.

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Chapter 3: The Role of Cytochrome P450 in Biotransformation of a Hard Coral (*Porites Lobata*) Extract and Homarine in Butterflyfishes

Abstract

Dietary specialists tend to be less susceptible to the effects of chemical defenses produced by their prey than generalist predators that feed upon a broader range of prey species. While many studies 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), with expression and catalytic activity of cytochrome P450 (CYP) 3A and CYP2 examined after one week of treatment. The *P. lobata* homogenate significantly induced content and catalytic activity of CYP2 and CYP3A, by 2-3 fold and by 3-9 fold, respectively, in *C. multicinctus*. Homarine caused a significant decrease of CYP3A and CYP2 at the high dose in *C. kleinii* and 60-80% mortality in that species. Homarine also induced CYP3A content by 3-fold and catalytic activity by 2-fold in *C. auriga*, while causing non-monotonic increases in CYP2 and CYP3A 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.

Introduction

In ecological settings, consumers are generally categorized as either generalist or specialist with regard to dietary preference. Generalists tend to eat 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 host'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, 2004). One such putative allelochemical, homarine (1-Methylpyridin-1-ium-2-carboxylate), has been isolated in 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). While homarine has been shown to exhibit feeding deterrent properties, specifically against a generalist Antarctic sea star *Odontaster validus* (McClintock et al., 1994), also against generalist pufferfish *Canthigaster rostrata* (Gochfeld, unpublished). Research to date has not shown anti-feedant activity of this compound against generalist or specialist butterflyfishes.

C. auriga is primarily a benthic omnivore feeding on noncoralline and coralline invertebrates, including hard corals and soft (alcyonarians) corals, polychaete worms, and algae (Hobson, 1974; Motta, 1980; Harmelin-Vivien & Bouchon-Navaro, 1983). In Hawaii, *C. 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, 1981; Pratchett, 2007). These generalists are predicted 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, 2012).

The expression of novel cytochrome P450 monooxygenase (CYP) isoforms 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 et al., 1981, 1995). 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 which prey on a chemically-defended hard coral, *Porites lobata*. We hypothesize 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 CYP2 and CYP3A expression profiles and catalytic activities and their regulation in species with different dietary preferences.

Materials And Methods

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.

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.

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 mm ± 0.2), *C. kleinii* (23 g ± 7; 9 mm ± 1), and *C. auriga* (43 g ± 8; 10 mm ± 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.

Porites lobata was collected from Kaneohe Bay and immediately brought 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 overnight, weighed and stored at -20°C until used in treatments.

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 12 hours in Millipore water three times. The combined aqueous extract was filtered (0.2µm), lyophilized in freeze dryer and weighed. Following extraction, tissue volume was measured using the waxing method (Gochfeld & Aeby, 2008) to calculate the concentration of homarine per tissue volume.

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* stated bellow. 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.

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 *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 KH_2PO_4/K_2HPO_4 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.

Immunoblotting.

CYP3A and CYP2 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 eletrophoresis 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.

^{14}C Testosterone hydroxylase activity.

^{14}C 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 [^{14}C]-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.

Statistical analyses.

For enzyme measurement comparisons, statistical analyses were conducted using Prism5 v5.0a software. Prior to statistical analysis, all data was 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 that 0.05 was observed, it was considered statistically significant, and if there was significance, 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.

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 and content of CYP2 (2-fold) were significantly ($P \leq 0.05$) higher in *C. auriga* when compared to *C. multicinctus* (i.e., control fish in Fig. I) but not in the following homarine exposure which had significantly more variability. *C. kleinii* basal CYP2 content (78.1 AU/mg protein) was 3-fold higher and 16β-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) ($P \leq$

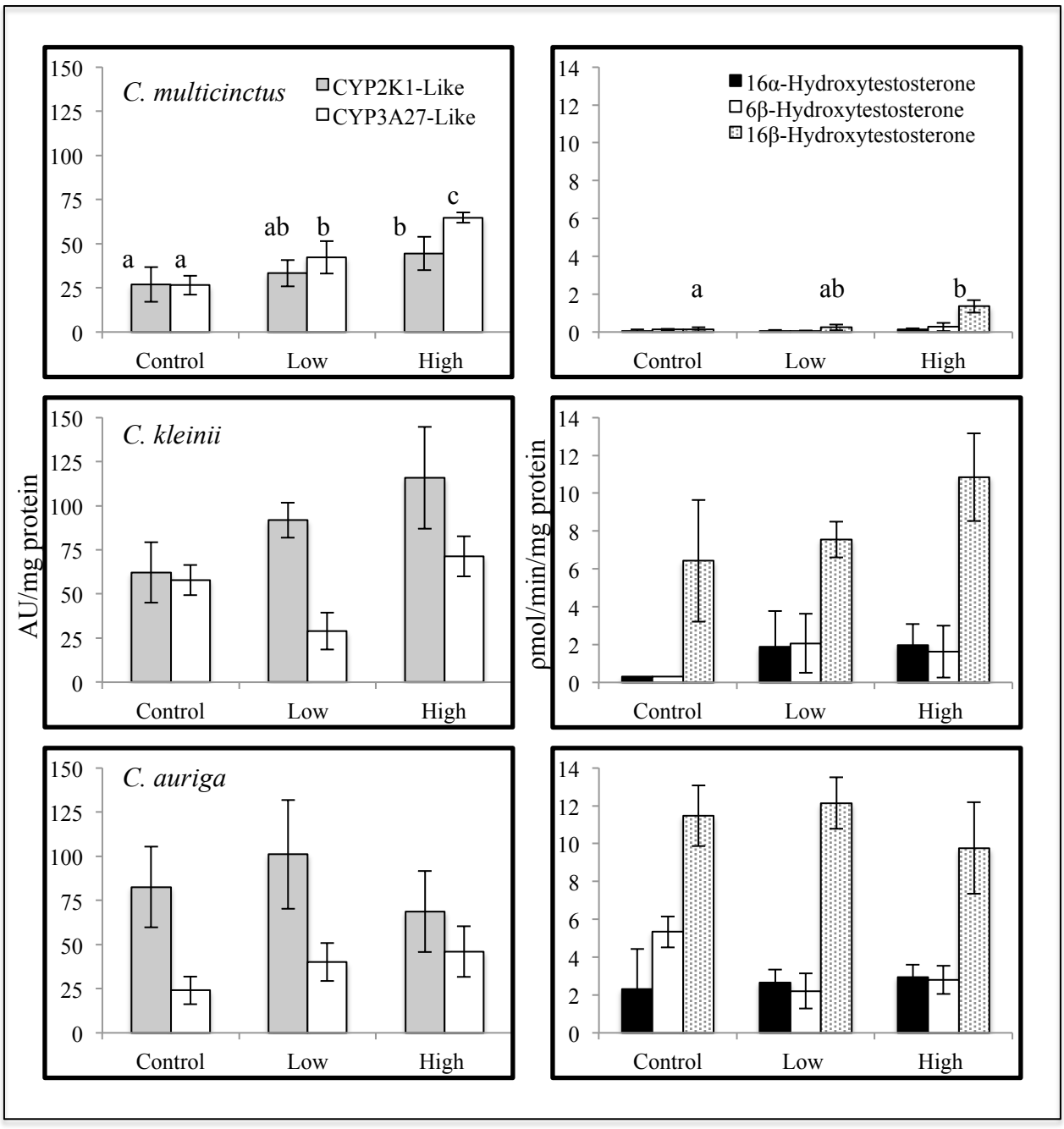

Figure 3.1. Effects of dietary exposure to *P. lobata* tissue homogenate on the hepatic expression (left) and catalytic activity (right) of CYP2K1 and CYP3A27 in three species of butterflyfish. Different letters indicate significant ($P \leq 0.05$) differences between untreated and treated fish

0.05) concurrent with the following exposure (Fig. III). *C. auriga* basal CYP3A content was 2-fold greater than in *C. kleinii* but no the following homarine exposure with significantly more variability.

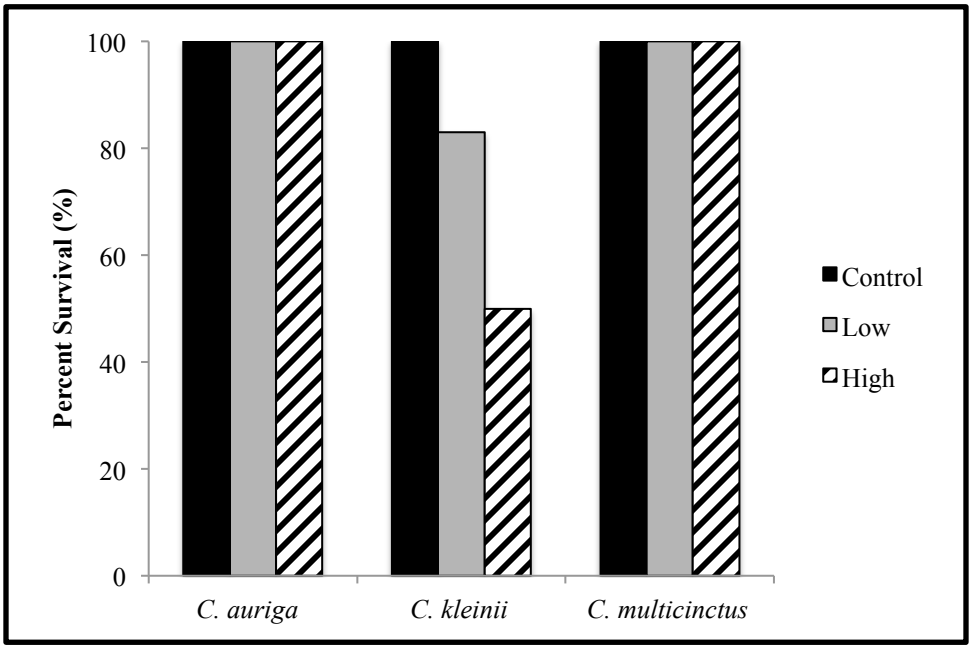

Figure 3.2. The percentage survival of butterflyfishes after a 7 day exposure to homarine at a low and high dose (N=6-8)

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* and *C. kleinii.* 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 proteins after treatment with the homogenate ($P<0.01$). In *C. multicinctus,* formation of 16α-hydroxytestosterone, 6β-hydroxytestosterone and 16β-hydroxytestosterone were induced 2-9 fold by *P. lobata*

homogenate treatment. Not statistically significant however, *C. auriga* had a slight trend on increase CYP3A-like proteins with dose and *C. kleinii* had slight trend in increase of 16β-hydroxytestosterone with dose (Fig. I).

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* (Fig. II).

Homarine caused a significant 4-fold increase in CYP2 content and 27-fold increase in 16α-hydroxytestosterone catalytic activity from a basal level of 0.25 ±0.15 pmol/min/mg protein to 6.75 ±0.83 pmol/min/mg protein in *C. multicinctus* (Fig. III). CYP3A catalytic activity in *C. multicinctus* fit a U-shaped dose response curve, with a significant 3-fold decrease at the low dose and a return to control level at the high dose treatment. Significant effects were not observed in CYP3A content in *C. multicinctus* and *C. kleinii*. The high dose of homarine induced a significant 100% and 320% decrease in CYP3A (16β-hydroxytestosterone and 6β-hydroxytestosterone) and a 50% decrease from 3.61±0.43 pmol/min/mg protein to 2.09±0.68 pmol/min/mg protein in CYP2 catalytic activity of 16α-hydroxytestosterone in *C. kleinii* (Fig. III). In *C. auriga,* homarine significantly induced 6β and 16α-hydroxytestosterone (by 120%) and CYP3A content (by 50%).

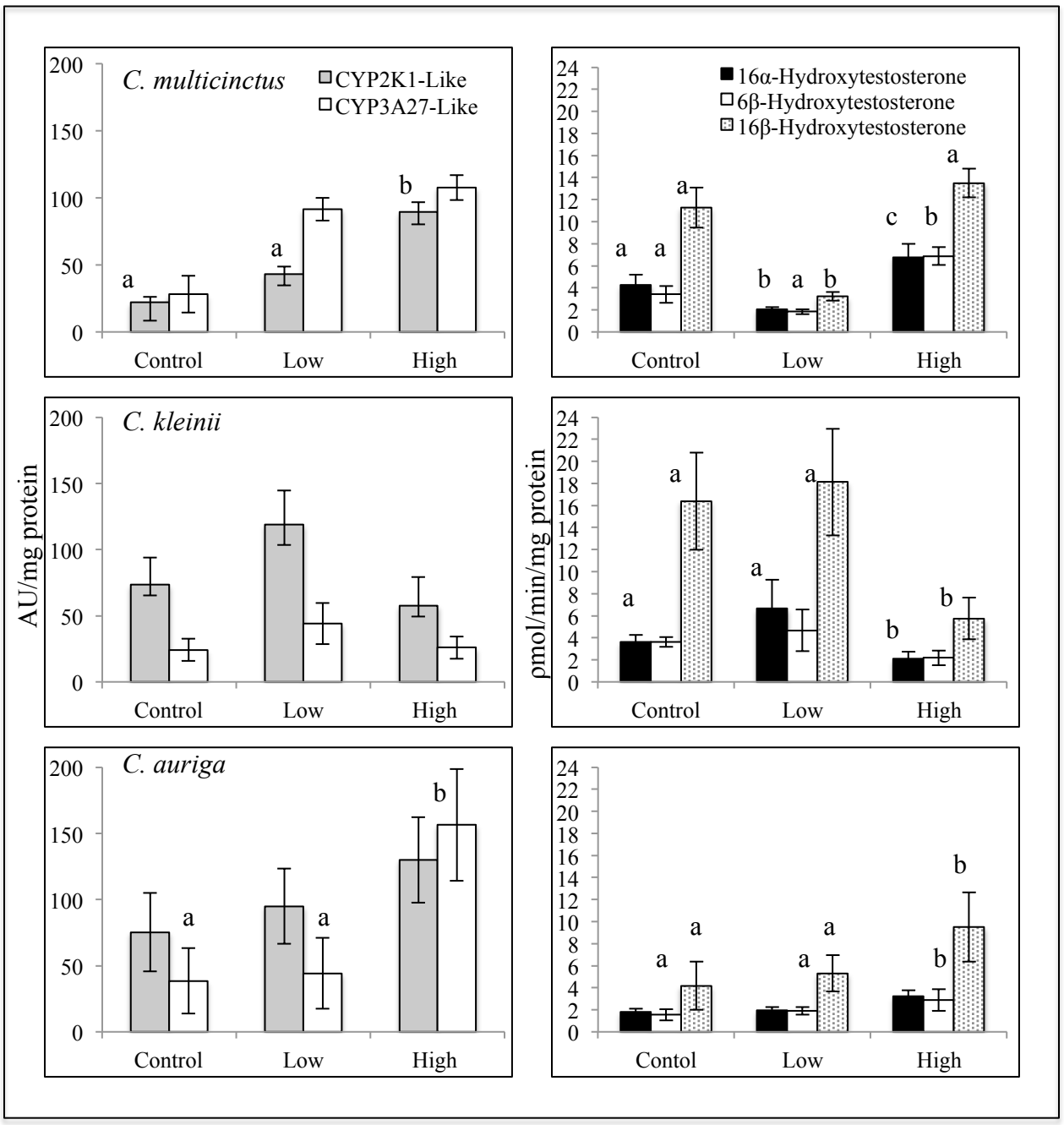

Figure 3.3. Effects of dietary exposure to homarine on the hepatic expression (left) and catalytic activity (right) of CYP2K1 and CYP3A27 in three species of butterflyfish. Different letters indicate significant ($P \leq 0.05$) differences between untreated and treated species

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). The concentrations of total hepatic CYP in *Chaetodon capistratus* that regularly preys upon allelochemically-rich gorgonian corals were 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. In furthering our knowledge of 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 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 and CYP3A in *C. multicinctus,* which feeds specifically

on this coral. The upregulation of CYPs after oral treatment in *C. multicinctus* 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, 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 and CYP2 in *C. multicinctus*, we predicted that induction would also occur following exposure to homarine, one of the constituents responsible for feeding deterrence in the coral extract. While a trend toward CYP3A induction and significant induction of CYP2 were observed, a non-monotonic response curve was detected with each catalytic activity in treated *C. multicinctus,* indicating potential hormesis (Calabrese et al., 2007). Hormesis may occur as a consequence of 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 and CYP2 activity. At higher doses, hepatocytes may overcompensate, leading to induction and potential detoxification (Calabrese, 2007). Stebbing (1981) similarly found increased growth in

colonies of the hydroid *Campanularia flexuosa* in low concentrations of cadmium and copper, while high doses caused decreases in growth (Moore et al., 1993; Correia et al., 2001). It is unclear how homarine alters catalytic activity of CYP3A and CYP2, but not enzyme content in *C. multicinctus*. 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.

Homarine is found in a diversity of marine invertebrates, which 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). Similar unpublished studies (Gochfeld, unpublished) showed deterrence in pufferfish (*Canthigaster rostrata*).

When the three species of butterflyfish were fed (gavaged) with homarine, species-specific effects were observed. Acute lethality was observed in *C. kleinii* in a dose dependent manner. In addition, the toxicity was related with decreased activity of CYP3A and CYP2 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 is unknown. Although it is not known as a substrate for CYP, homarine may alter CYP regulation. Many compounds may alter CYP expression without serving as substrates.

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.

In contrast to coral homogenate treatments in *C. auriga,* CYP3A catalytic activity and protein expression 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 is clear 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. multicinctus*. 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 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. multicinctus* 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 and CYP2 without significant induction, whereas *C. multicinctus* had rapidly inducible CYP3A and CYP2 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 maybe that each responded differently to the 2 week depuration period where 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. multicinctus* may be due to accelerated reduction of CYP when coral prey (and its associated toxins) was no longer consumed. During both exposures basal CYP contents had similar trends, however catalytic activity varied potentially attributed to annual variability. It is difficult to resolve this issue as the dietary contents of each species prior to collection is unknown. Equivalent dietary treatment (i.e. brine shrimp feeding) during acclimation is necessary for controlled laboratory experiments to provide normalizing conditions for comparisons. Also CYP basal levels were not significantly different in homarine exposure most likely due to the high variability in *C. auriga* samples.

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.

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Chapter 4: Biochemical Mechanisms for Geographical Adaptations to Novel Toxin Exposures in Butterflyfish

Abstract

Butterflyfish have coevolved with corals for millions of years, yet the mechanism of this highly specialized coral feeding strategy remains poorly understood. Certain butterflyfish have the ability to feed on allelochemically rich soft coral, e.g. *Sinularia maxima*. A primary mechanism for coping with dietary allelochemicals is through detoxification enzymes. There has been compelling genetic and molecular evidence for an evolutionary participation of insect cytochrome P450's (CYP) in plant allelochemical metabolism. CYP2 and CYP3A content have been associated with butterflyfish that preferentially consumes allelochemically rich soft corals, potentially allowing them to feed on those corals. Feeding deterrence results indicated that *S. maxima* and allelochemical isolated from *S. maxima* 5-episinuleptolide (5ESL) were deterrent to butterflyfish that were generalist feeders (consume a wide range of invertebrates), but not specialist feeders (consume a narrow range of corals). After oral exposure to the coral toxin 5ESL, which is not normally encountered in the Hawaiian butterflyfish diet, an endemic specialist, *Chaetodon multicinctus* experienced 100% mortality compared to a generalist, *Chaetodon auriga,* which had significantly more CYP3A (3-6 fold higher) basal content and catalytic activity. The specialist, *Chaetodon unimaculatus*, which preferentially feed on *S. maxima* in Guam, but not in Hawaii, had 100% survival, a significant induction of 8-12 fold CYP3A, and an increased ability (2-fold) to metabolize 5ESL over other species. In vitro incubations with liver microsomes coupled with computer modeling data of CYP3A4 are consistent with the formation of 5ESL epoxide

metabolite and correlated with CYP3A content, catalytic activity, induction and NADPH dependent oxidase formation. Higher rates in 5ESL hydroxylation were found in control *C. unimaculatus* and *C. auriga* and high *C. unimaculatus* compared to control. Butterflyfish and corals appear to represent a parallel marine model to terrestrial insect plant interactions, in that butterflyfish may have evolved inducible CYP enzymes to detoxify dietary allelochemicals found in corals.

Introduction

Charles Darwin, in *the Origin of Species*, was the first to emphasize the importance of interspecific interactions in driving the diversification of life. In spite of the long-recognized importance of such interactions, little is known about the diverse system that governs interspecific interactions. Unlike adaptation to the physical environment, adaptation to another species can produce reciprocal evolutionary responses that either impede these adaptive changes or, in mutualistic interactions, amplify their effects (Thompson, 2005). The interactive and iterative nature of coevolution is a powerful process in shaping biodiversity (Thompson, 2005). The evolutionary interaction between insects and plants is a widely cited example of what generally is referred to as coevolution.

The pairwise reciprocal coevolutionary scenario can predict the development of a chemical "arms race", in which reciprocity between chemical defenses and counter-adaptive detoxification takes place. It has been argued that the evolution of plant chemical defenses and insect counter detoxification has been one of the most well

documented cases of a chemical arms race (Berenbaum, 1980; Crwford, 1980; Cohen et al., 1989; Li et al., 2004; McDonell et al., 2004). Berenbaum and Zangerl (Berenbaum et al., 1998) showed that parsnip webworms (*Depressaria* pastinacella) exhibited resistance, through increased amounts and activity of CYP, to the levels of furanocoumarins in host parsnip plants. *Papilio plyxenes* (black swallowtail butterfly) also undergo CYP induction when consuming a diet consisting of plants containing xanthotoxin, (Gonzalez et al., 1990; Berenbaum, 1980; Crwford, 1980; Cohen et al., 1989; Li et al., 2004; Cohen et al., 1992; Berenbaum et al., 1998; Li et al., 2003; McDonell et al., 2004). Much of the apparent diversity of CYP isoforms in insects is likely due to reciprocal evolutionary influences between host chemical defenses and consumer detoxification mechanisms. (Gonzalez et al., 1990; Li et al, 2003).

In marine systems, however, knowledge of genes conferring consumer tolerance to dietary allelochemicals is in its infancy, when compared to that of terrestrial systems (Debusk et al., 2008; Sotka et al., 2008; Sotka et al., 2009). Butterflyfish-coral interactions are an ideal model to study consumer tolerance in marine ecosystems because they share many of the same characteristics as plant-insect interactions. Plants and corals are sessile creatures producing allelochemicals to prevent predations (Fritz et al., 1992; Lages et al., 2010). Just as insects have coevolved with plant hosts for 400 million years, butterflyfish have evolved with corals over the past 50 (Labandeira, 2007; Cole et al., 2008). Coral consumption (corallivory) is unique adaptation as only 128 fish eat corals, out of the 5000 or more fish species recorded from coral reefs, and 61% belong to a single-family: butterflyfish (*f. Chaetodontidae*) (Cole et al., 2008; Rotjan et

al., 2008). Despite being one of the most intensively studied families of reef fishes, the evolution of coral feeding remains poorly understood (Bellwood et al., 2010).

Among corallivorous butterflyfish, sympatric species often exhibit highly contrasting levels of dietary specialization (Berumen et al., 2008). Specialist species tend to have a narrow dietary range, while generalist species are able to thrive on a widely varied diet (Krebs et al., 2009). Generalists, such as *Chaetodon auriga*, consume species from polychaete worms to hard corals (Pratchett 2005), are hypothesized to have liver enzymes that can act on a broad range of substrates to facilitate the biotransformation of a wide variety of toxins. By utilizing a generalist feeding strategy, generalists consume small amounts of a variety of toxins that are processed through a diverse set of detoxification pathways without overloading any one pathway. This hypothesis has become firmly entrenched in the ecological literature to the extent that it is accepted as the predominant factor regulating the foraging ecology of generalist herbivores (e.g. Sorensen et al., 2003). However, there have been relatively few empirical tests to confirm this hypothesis (Dearing & Cork, 1999; Sorensen et al., 2003; Sorensen et al., 2005). Specialists, such as *Chaetodon multicinctus*, consume three species of corals (Tricas et al., 1989), limiting their range of available food and potentially increasing their capacity to detoxify specific toxins in high concentrations.

Specialists are thought to have evolved novel liver enzymes with greater specificity (Crwford, 1980; Cohen et al., 1989; Vrolijk et al., 1995; Li et al., 2004; Torrefrossa et al., 2012). The potential negative effect is that specialists are less energetically and/or mechanistically efficient at eliminating novel toxins (Sorensen et al.,

2005). For example *Chaetodon capistratus* preferentially feeds on allelochemically-rich gorgonian corals. *Chaetodon striatus* and *C. ocellatus* do not feed on gorgonians. *C. capistratus* possessed 2- to 3-fold more total CYP and significantly more CYP2B-like and CYP3A4-like proteins when compared to *C. striatus* and *C. ocellatus*. Suggesting that CYPs in families 2 and 3 may be involved in allelochemical biotransformation conferring potential feeding advantages (Vrolijk et al., 1995). Expressions of hepatic CYP1A in Australian butterflyfish were related to feeding strategies (Debusk et al., 2008). However, CYP1A and CYP2 transcripts in *Chaetodon xanthurus, Chaetodon punctatofasciatus, Chaetodon kleinii,* and *C. auriga* following an oral exposure to extracts from the soft coral, *Sinularia sp.* only led to induction of CYP2N7 mRNA expression in *C. punctatofasciatus* (Debusk et al., 2008). Although chemical arms race dogma would suggest consistent benefit to animals with elevated detoxification enzymes, some studies have shown that organism that specifically detoxify toxins associated with their unique diet may be susceptible to novel toxins. For example, when the woodrats *Neotoma stephensi* (specialists) and *Neotoma albigula* (generalists) were exposed to a novel dietary toxin from *Larrea tridentate*, fitness metrics of the specialists were more negatively impacted than the generalists (Sorensen et al., 2005).

Although a variety of positive and negative effects from differing feeding strategies have been observed with dietary specialization among insects and mammals, few examples have been documented in marine organisms (e.g. Debusk et al., 2008, Vrolijk et al., 1995). Furthermore, no studies have directly investigated how butterflyfish specialists survive on diets containing novel coral toxins compared to that of generalists

fed the same coral toxin. Mirroring Berenbaum et al. investigations between plants and insects (Cohen et al., 1992; Berenbaum et al., 1998; Li et al., 2004; McDonnell et al., 2004; Lages et al., 2010), this research seeks to elucidate interactions between corals and butterflyfish.

This study investigates the ability of butterflyfish with different feeding strategies to consume unfamiliar soft coral *Sinularia maxima*. The following questions were asked: (i) whether *S. maxima* or the isolated allelochemical from *S. maxima* 5-episinuleptolide (5ESL) can deter feeding in butterflyfish of different feeding strategies, (ii) whether *S. maxima* or 5ESL can induce CYP in butterflyfish of different feeding strategies, and (iii) whether butterflyfish liver microsomes could biotransform 5ESL into metabolites predicted from CYP(s) that were uniquely induced by 5ESL or *S. maxima* extracts.

Methods

Chemicals

Analytical grade methanol, ethanol and acetonitrile were purchased from Fisher (Pittsburg, PA). Glycerol, Tris(hydroxymethyl)aminomethane 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). MS222, EDTA, gelatin and NAPDH were purchased from Sigma-Aldrich (St. Louis, MO). Tween was purchased from EMD Millipore (Billerica, MA).

5,8-Epoxy-11-hydroxy-18-nor-3,6-dioxo-12-cembradien-15-methyloxirane-20,10-olide (Fig) was synthesized by Dr. John Rimoldi at University of Mississippi. To a

solution of 5ESL (10 mg; 0.003 mmol) in 2 mL of CH_2Cl_2, 3.0 mg (0.032 mmol) of sodium bicarbonate was added and the mixture was cooled to 0°C. A solution of *meta*-chloroperbenzoic acid (7.5 mg; 0.032 mmol; 70%) in 1.0 mL of CH_2Cl_2 was added slowly over a period of 5 minutes. The resulting slurry was stirred for 30 minutes at 0°C, and allowed to stir at ambient temperature for 3 hrs, as the progress of the reaction was monitored by TLC (5% CH_3OH/ 95% CH_2Cl_2; ninhydrin staining) and LC-MS (MNa^+=387). The reaction mixture was diluted with an additional 5.0 mL of CH_2Cl_2 and was washed twice with saturated sodium carbonate solution (10 mL x2). The organic layer was washed twice with saturated $Na_2S_2O_3$ solution. The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated to give 11 mg of crude epoxide product. Purification was accomplished using silica gel and a mobile phase consisting of 5% CH_3OH:95% CH_2Cl_2. Fraction 1 contained unreacted starting material (2.0 mg) and fraction 2 (5.0 mg) corresponded to the epoxide product. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.64 (dd, J = 13.1, 5.6 Hz, 1H), 4.69 (s, 1H), 4.63 (d, J = 7.9 Hz, 1H), 3.89 (d, J = 10.1 Hz, 1H), 3.45 (d, J = 6.8 Hz, 1H), 3.26 (d, J = 6.6 Hz, 1H), 2.49 (dt, J = 17.3, 8.7 Hz, 1H), 2.38 (s, 1H), 2.20 (s, 2H), 2.04 (d, J = 12.7 Hz, 1H), 1.91 (d, J = 3.2 Hz, 1H), 1.51 (s, 1H), 1.39 (s, 1H), 1.25 – 1.13 (m, 7H), 0.90 – 0.72 (m, 3H). LC-MS (ESI+) *m/z* (MNa^+) 387.3.

Animal Collections

Butterflyfish were collected from Kaneohe Bay (*C. auriga* and *C. unimaculatus*) and Yokahama Bay (*C. multicinctus* and *C. kleinii*) reef systems, surrounding the island

of Oahu during June 2012 and 2013. *C. multicinctus* (11 g ±3; 8 mm ± 0.2), *C. kleinii* (23 g ± 7; 9 mm ± 1), *C. auriga* (43 g ± 8; 10 mm ± 1) and *C. unimaculatus* (10.4 g ±6; 7.2 mm ±1.7) were acclimated for two weeks before treatments. Fish were juveniles and sexually immature by visual determination of the gonads after dissection. Fish were held at the Waikiki Aquarium in a large (15 ft diameter and 3 ft height) flow-through tank, with floating cages for separation of doses. *S. maxima* and *S. polydactyla* were collected from Piti Bomb holes, Guam and identified by M. Slattery (Slattery, unpublished).

5-Episinuleptolide Isolation

5-ESL was isolated in the same manner as Kamel et al. (2007). In summary, the *S. maxima* was initially frozen on dry ice. Then it was thoroughly extracted with methanol: dichloromethane (1:1), concentrated under reduced pressure, and subjected to silica gel vacuum liquid chromatography. The column was eluted with hexane, hexane–ethyl acetate, ethyl acetate–methanol to methanol to yield 11 fractions, which were concentrated under reduced pressure. 5-ESL was eluted with 80% ethyl acetate:hexane, recrysalized and washed successively with chloroform and methanol.

Deterrence Assay

The capability of *S. maxima* to deter predators under laboratory and field conditions has been well described (e.g., Slattery et al., 2008), however few studies have looked at the effect on butterflyfish with different feeding strategies. Laboratory feeding assays utilized four butterflyfish species with different feeding strategies under conditions

described above (Slattery et al., 2008); generalist *C. auriga* and *C. vagubundus* and specialist *C. melannotus* and *C. unimaculatus.* Natural concentrations of crude extracts from *S. maxima* x *polydactyla* and were added to agar-based food cubes containing a tropical fish diet. Extract concentrations were based on dry masses. Pairs of pre-weighed control cubes (treated or solvent-only) were offered to replicate butterflyfish (n=15 per assay), along with a set without fish for mass control. A separate assay was conducted for crude extracts and compounds; to prevent learned aversion, fish were only exposed once (Thacker et al., 1997). Each feeding assay ran for 1.75 hr. Cubes were then removed and re-weighed. Butterflyfish consumption of *S. maxima* x *polydactyla* and 5ESL was compared to control food cubes using paired t-tests and the absolute difference in consumption between each replicate paired control and treatment cube was compared using a one-way ANOVA to determine the relative deterrence of extract and compound.

Oral treatments

To examine the effect of *Sinularia maxima* tissue homogenate and 5ESL on survival and hepatic CYP expression, 4-6 fish of each species and each dose were gavaged with either high doses (250mg/kg) or low doses (50mg/kg) of *Sinularia* tissue homogenate (Summer 2010), or high dose (3.0mg/kg), or low dose (1.0mg/kg) of 5ESL (Summer 2011). 1M Tris-buffer (pH 8.2) served as a negative control. Doses were based on average daily consumption rates by *C. multicinctus, C. auriga, C. unimaculatus* and *C. kleinii* [e.g., Pratchett, 2005; Tricas, 1989). Treatments occurred on days 1, 3, and 5. On day 7, fish were euthanized by MS222 overdose and dissected livers were subsequently

maintained at -80 °C until microsomes were prepared for immunoblot and catalytic activity analyses.

Preparation of microsomal fractions

The 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 g for 30 min. Supernatant was collected and centrifuged at 100,000 g for 60 min to obtain microsomal fraction. Supernatant was removed and the microsomal fraction was re-suspended 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 Croomassie Blue method using a commercial kit (Pierce Inc., Rockford, IL) with bovine serum albumin as a standard.

Western immunoblot

CYP3A and CYP2 protein levels were determined by Western blot as described in Maldonado et al. (2015). Microsomal samples were boiled for 5 min in SDS-PAGE buffer (50/50) (Laemmli, 1970) and 10 μg of protein were 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 CYP2K1 or 1:1000 dilution (v/v) of primary rabbit anti-rainbow trout polyclonal CYP3A27 antibodies provided by Dr. Malin Celander University of Gothenburg and Dr. 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). Then the blot was incubated for 1 h with alkaline phosphatase-conjugated anti-rabbit IgG (BioRad). Then excess secondary antibody was removed with two washes of T-TBS. The immunoreactive bands 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 were presented in optical density units per mg protein.

^{14}C Testosterone hydroxylase activity

^{14}C Testosterone hydroxylase activity was measured as described by Martin-Skilton et al. (2006). The incubation contained 200 μg of hepatic microsomal protein with 3.5 μM [^{14}C]-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. An additional 10 μl of NADPH was added, and then incubated for another 30 min at 25 °C. Incubations were stopped with 250 μl of acetonitrile, followed by centrifugation at 10,000 g for 10 min. Then 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 with a radioflow detector β-ram Model 3 (INUS Systems Inc., Tampa, FL) using U-Flow 2:1 (Lablogic Inc.) scintillation liquid. Metabolites 6β-, 16α- and 16β-hydroxytestosterone were identified using authentic standard compounds (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.

5-Episinuleptolide metabolism and metabolite identification

The incubation contained 100 μg of hepatic microsomal protein with 5ESL from 1 to 30 μM and reached enzyme saturation at 11 μM. Final incubations included 7.5 μM NADPH in a final volume of 0.204 mL of 50 mM Tris–HCl pH 7.4. Samples were incubated for 45 min at 30 °C. Incubation reactions were stopped with 100 μl of acetonitrile, followed by centrifugation at 10,000 g for 10 min. Then 1 μM of the internal standard caffeine was added, and finally 40 μl of supernatant was injected onto 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 5ESL metabolites was attained using mobile phase systems of (A) 95% water and 5% acetonitrile and (B) 100% acetonitrile, at a rate of 1 ml/min. The gradient started with 100% A to 50% B in 40-45 min. Chromatographic peaks were monitored with a SPD-10A VP SHIMADZU UV-VS detector (SHIMADZU

Co., Carlsbad, CA) at wavelength 249 nm. 5ESL oxide and the internal standard were quantified by integrating the area under the peaks using a 6 point standard curve, with a limit of detection of 5 pmol/min/mg protein.

C. unimaculatus samples were selected for further metabolite characterization by LCMS/MS in positive electrospray ionization mode using a Agilent 6460 triple quadrupole mass spectrometer at gas temperature 300°C and capillary voltage of 2500V. A 1 µL aliquot of metabolite was loaded onto an Agilent Poroshell 120 SB- C18 column (2.1mm x 50mm, 2.7 µm particle size, Santa Clara, CA) with a mobile phase flow rate of 0.2 mLmin−1. Samples were eluted with a gradient composed of 5 mM ammonium acetate, water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). The linear gradient transitioned from a 5% solvent B to 50% for the first 15min, to a gradient of 50% B over 0.2min. Then the initial solvent (5% B) was restored over a 1min linear gradient and re-equilibrated. The ion trap was programmed to collect a MS2 spectrum from 100 to 1000 m/z, a fragment voltage of 20V and a cell acceleration voltage of 7V from collision- induced dissociation of the most intense target ion from the appropriate product ion spectrum. MS data was analyzed with Agilent Mass Hunter (Agilent Technologies, Santa Clara, CA).

Molecular Modeling

Ligands and crystal structures were prepared for docking using AutoDockTools (v1.5.4; Morris et al., 2009), with the addition of polar hydrogens and the assignment of Gasteiger charges. Ligand structure and charge minimization was performed with semi-

empirical methods (PM6 Hamiltonian in Mopac2009) (Stewart, 2008). Gasteiger-Marsili partial charges were used in the final docking runs. High throughput docking was performed using Autodock Vina (v1.1.2) (Trott &Olson, 2010). Flexible ligands were docked into models with rigid protein backbones and rigid side chains. 100 replicate dockings were performed, retaining a broad range of calculated energies (6 kcal/mol). Docking visualization was performed using PyMol (v. 1.5.0.4, Schrödinger LLC, Portland, OR).

Statistical analyses

Statistical analyses were conducted using Prism5 v5.0a software. Prior to statistical analysis, all data was analyzed to meet the normality and variance assumptions of parametric tests. For normally distributed data, an initial one-way ANOVA analysis 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. If significance was determined, the Tukey's multiple range test was preformed 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 was used.

Results

To investigate feeding deterrence in the hybrid soft coral *S. maxima*, control and treated pellets were offered to the following 4 species of butterflyfish in the field (n=15 replicates of each): *C. auriga* (generalists) and *C. unimaculatus* (specialists). Bars below

the dashed line were significantly different from controls by Fisher's Exact test (Fig. 1). The extract and 5ESL were deterrent to generalists but not specialists.

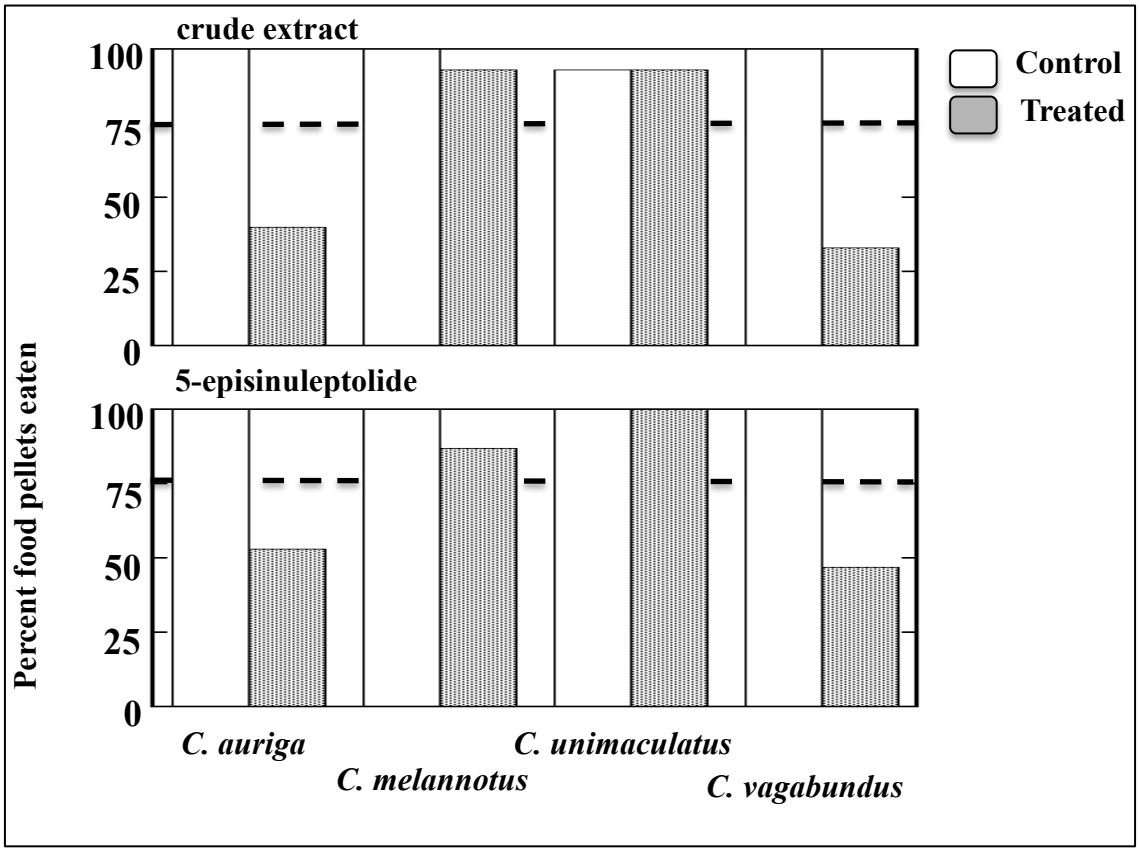

Figure 4.1. Feeding deterrence of *S. maxima* crude extract and 5ESL. Control and treated pellets were offered to 4 species of butterflyfishes in the field (n=15 replicates of each) representing generalist (= *C. auriga* and *C. vagabundus*) and specialist (= *C. melannotus* and *C. unimaculatus*) feeding strategies. Bars below the dashed line are significantly different from controls by Fisher's Exact test.

When comparing the basal CYP3A and CYP2 contents and catalytic activities between the four species, an increasing trend was observed: in increasing order - *C. multicinctus*, < *C. kleinii*, < *C. unimaculatus* < *C. auriga*. In *C. multicinctus,* testosterone hydroxylation levels and content were significantly lower than that of the other three species.

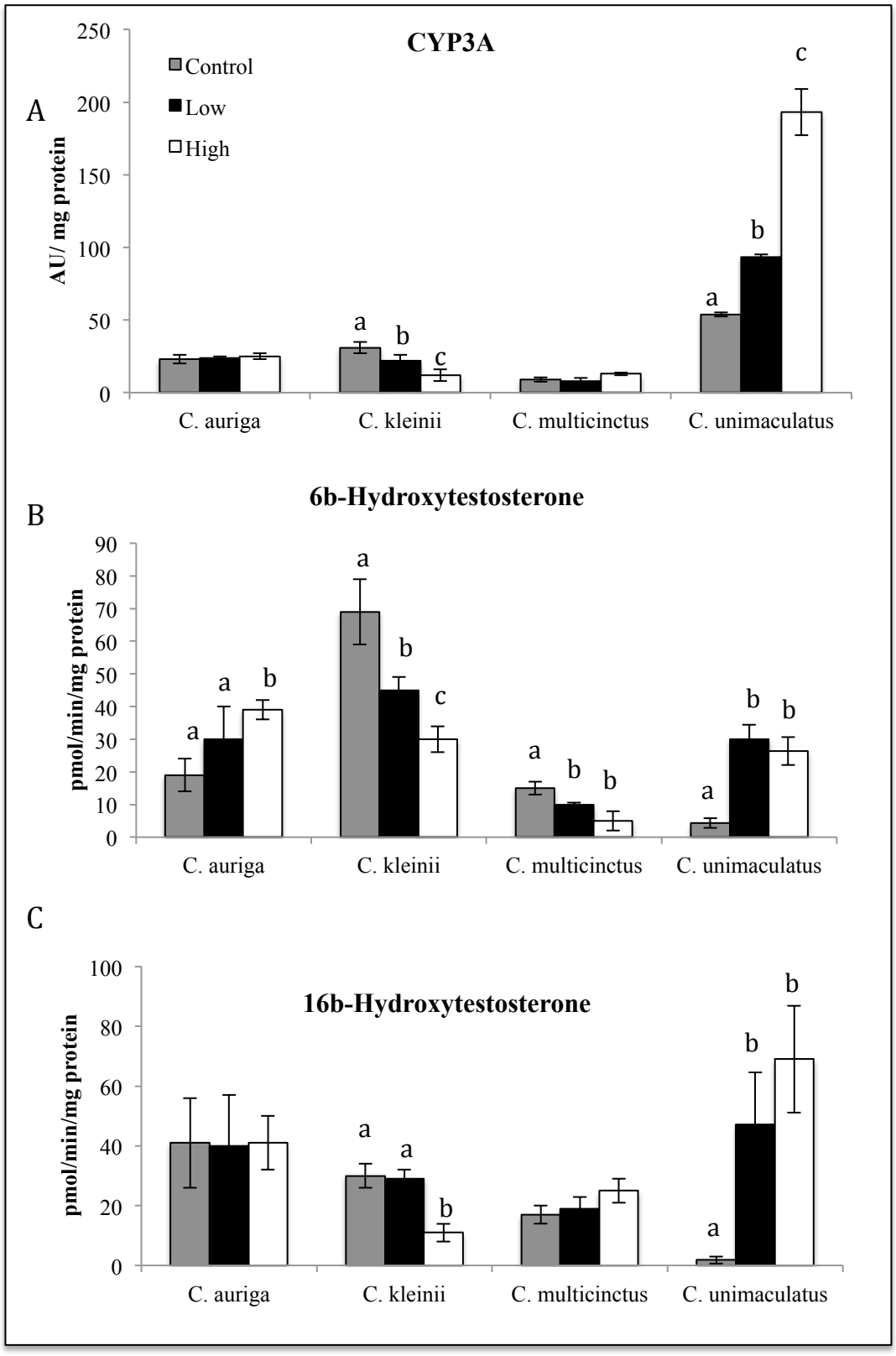
A
CYP3A
Control
Low
High
AU/ mg protein
250
200
150
100
50
0
a
b
c
a
b
c
a
b
c
C. auriga
C. kleinii
C. multicinctus
C. unimaculatus
B
6b-Hydroxytestosterone
pmol/min/mg protein
90
80
70
60
50
40
30
20
10
0
a
a
b
a
b
c
a
b
b
a
b
b
C. auriga
C. kleinii
C. multicinctus
C. unimaculatus
C
16b-Hydroxytestosterone
pmol/min/mg protein
100
80
60
40
20
0
a
a
b
a
b
b
a
b
b
C. auriga
C. kleinii
C. multicinctus
C. unimaculatus

Figure 4.2. Effects of dietary exposure to *S.maxima* tissue homogenate on the hepatic content (A) and catalytic activity (B, C) of and CYP3A in three species of butterflyfish. Different letters indicate significant ($P \leq 0.05$) differences between untreated and treated fish (N=6-8).

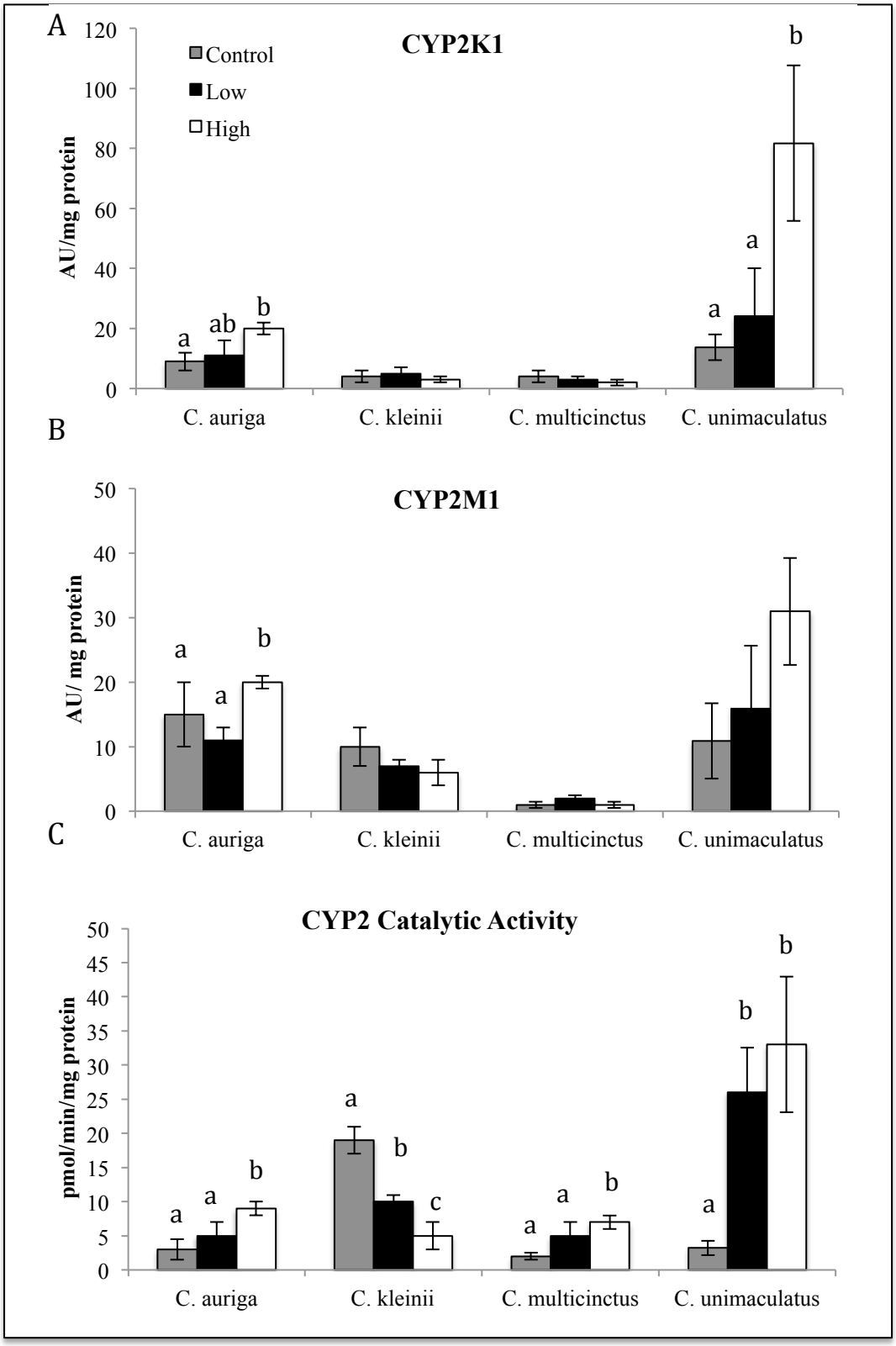
A
CYP2K1
Control
Low
High
AU/mg protein
120
100
80
60
40
20
0
a
ab
b
a
a
b
C. auriga
C. kleinii
C. multicinctus
C. unimaculatus
B
CYP2M1
AU/ mg protein
50
40
30
20
10
0
a
a
b
C. auriga
C. kleinii
C. multicinctus
C. unimaculatus
C
CYP2 Catalytic Activity
pmol/min/mg protein
50
45
40
35
30
25
20
15
10
5
0
a
a
b
a
b
c
a
a
b
a
b
b
C. auriga
C. kleinii
C. multicinctus
C. unimaculatus

Figure 4.3. Effects of dietary exposure to *S.maxima* tissue homogenate on the hepatic content CYP2K1-like and CYP2M1-like (A,B) and catalytic activity (C) of and CYP2 in three species of butterflyfish. Different letters indicate significant ($P \leq 0.05$) differences between untreated and treated fish (N=6-8).

Oral exposure to soft coral *S. maxima* tissue homogenate had no significant effect on survival. However, in *C. kleinii,* oral treatment with the *S. maxima* tissue homogenate caused a statistically significant dose dependent reduction in CYP3A content and catalytic activity (16β hydroxytestosterone formation) ($P \leq 0.05$) (Fig. 2). 6β Hydroxyteststerone formation in *C. kleinii* was also induced by *S. maxima* at the high dose by a factor of 2.3. *C. kleinii's* CYP2K content and CYP2 catalytic activity were significantly induced by *S. maxima* tissue homogenate at the high dose with a 2.33-fold change in content and a 3.2-fold change in catalytic activity ($P \leq 0.05$) (Fig. 3). Oral exposure to *S. maxima* caused a significant 1.5-fold increase in *C. multicinctus* CYP3A content at the high dose. However, protein induction did not correlate with the catalytic activity. *C. multicintus* 6β-hydroxytestosterone formation (CYP3A activity) was significantly decreased by the high dose 2.5-fold and 16α-hydroxytestosterone (CYP2 activity) increased by 3-fold ($P \leq 0.05$). Oral treatment of fish with *S. maxima* tissue homogenate caused a significant induction in the formation of 6β, 16β and 16α hydroxytestosterone (10-20 fold) and CYP3A and CYP2 content (5-11 fold) in *C. unimaculatus*, but caused no significant changes in *C. auriga*. In *C. unimaculatus,* CYP3A catalytic activity (16β and 6β hydroxytestosterone formation) was induced 4-fold in protein and 2-fold in activity ($P \leq 0.05$).

During the oral 5ESL treatments, *C. multicinctus* experienced 100% mortality in both the high and the low doses, with 100% survival in the control. In contrast, 80% survival was observed in *C. auriga*, with 45-60% survival observed for *C. kleinii* after treatment (Fig. 4). *C. unimaculatus* had 100% survival in all treatments. 5ESL caused

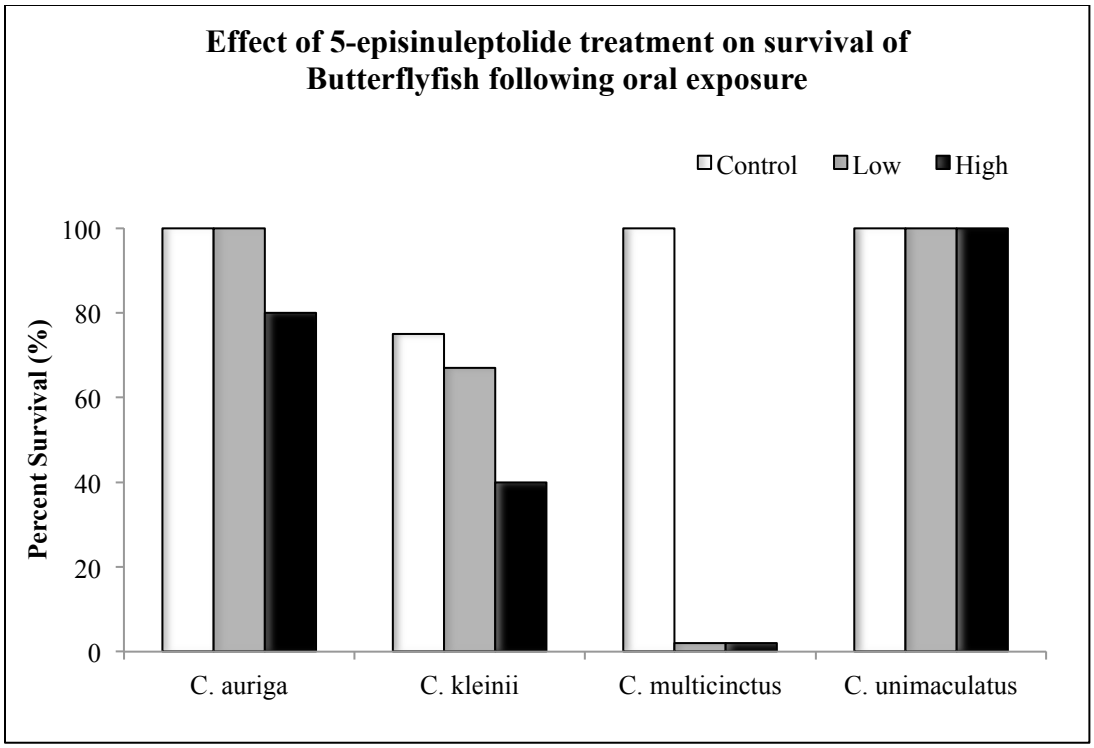

Figure 4.4. The percentage survival of butterflyfish after a 7 day oral exposure to high dose (3.0mg/kg), or low dose (1.0mg/kg) 5ESL. Each value represents the total survival of 6-8 individuals.

1.3-fold induction of CYP3A content in *C. auriga,* ($P \leq 0.05$) but a reduction by a factor of 7.2 in CYP3A catalytic activity (6β hydroxytestosterone) ($P \leq 0.05$) (Fig. 5). 5ESL also caused a reduction in CYP2 catalytic activity by 0.5-fold at the high dose ($P \leq 0.05$) (Fig. 6). In *C. kleinii,* no statistically significant changes in content or catalytic activity were observed after exposure to 5ESL. Due to excessive mortality, liver microsomes from only the *C. multicinctus* control group were evaluated for CYP activity and expression. Hepatic CYP3A content from *C. unimaculatus* had the most significant dose dependent induction following 5ESL treatment compared to the other species, with 8-12 fold times larger values observed after treatment ($P \leq 0.05$). The induction of CYP

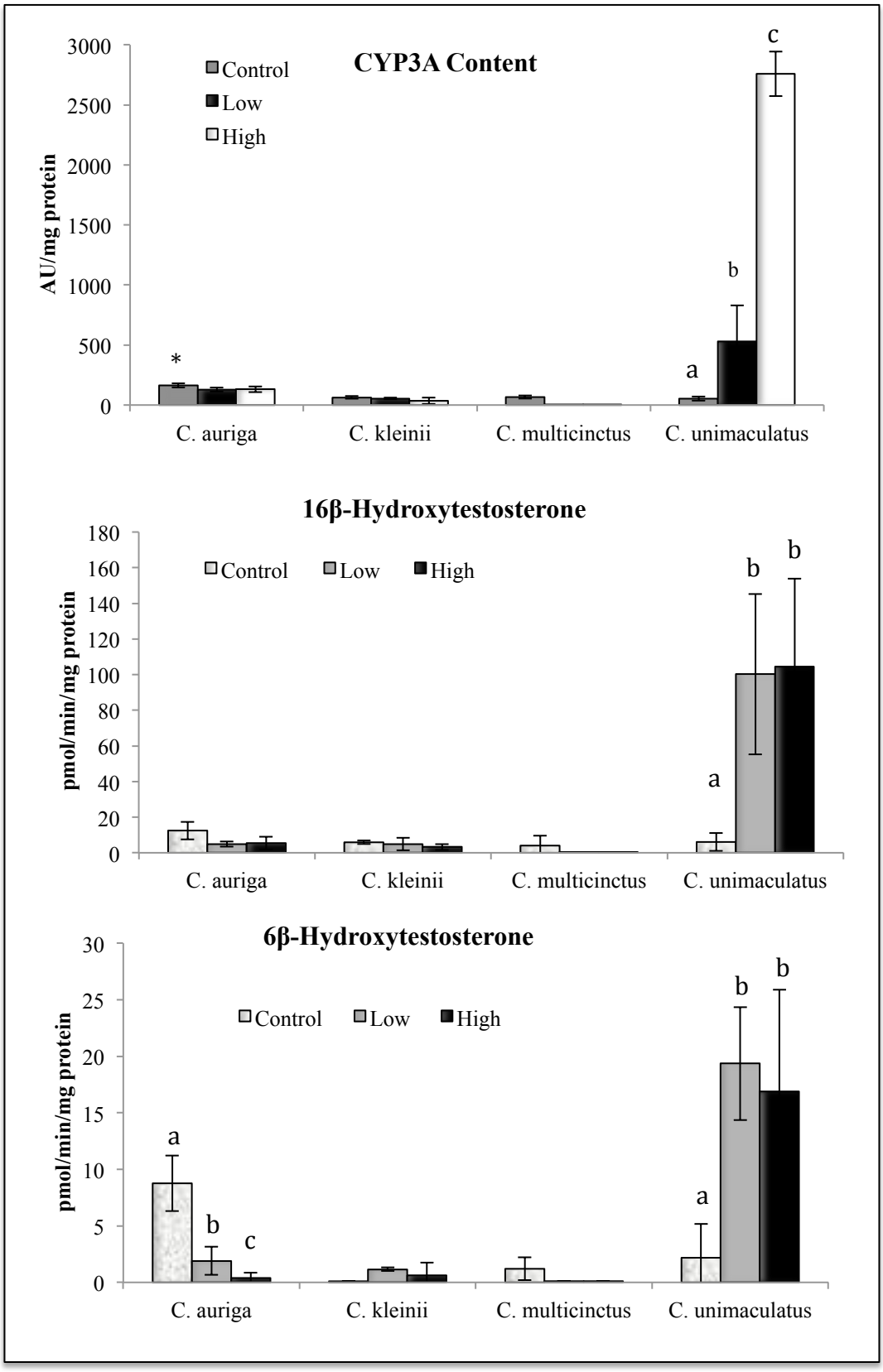
CYP3A Content
AU/mg protein
3000
2500
2000
1500
1000
500
0
Control
Low
High
*
a
b
c
C. auriga
C. kleinii
C. multicinctus
C. unimaculatus
16β-Hydroxytestosterone
pmol/min/mg protein
180
160
140
120
100
80
60
40
20
0
Control
Low
High
a
b
b
C. auriga
C. kleinii
C. multicinctus
C. unimaculatus
6β-Hydroxytestosterone
pmol/min/mg protein
30
25
20
15
10
5
0
Control
Low
High
a
b
c
a
b
b
C. auriga
C. kleinii
C. multicinctus
C. unimaculatus

Figure 4.5. Effects of dietary exposure to 5ESL on the hepatic content (A) and catalytic activity (B,C) of and CYP3A in four species of butterflyfish. Different letters indicate significant ($P \leq 0.05$) differences between untreated and treated fish (N=6-8).

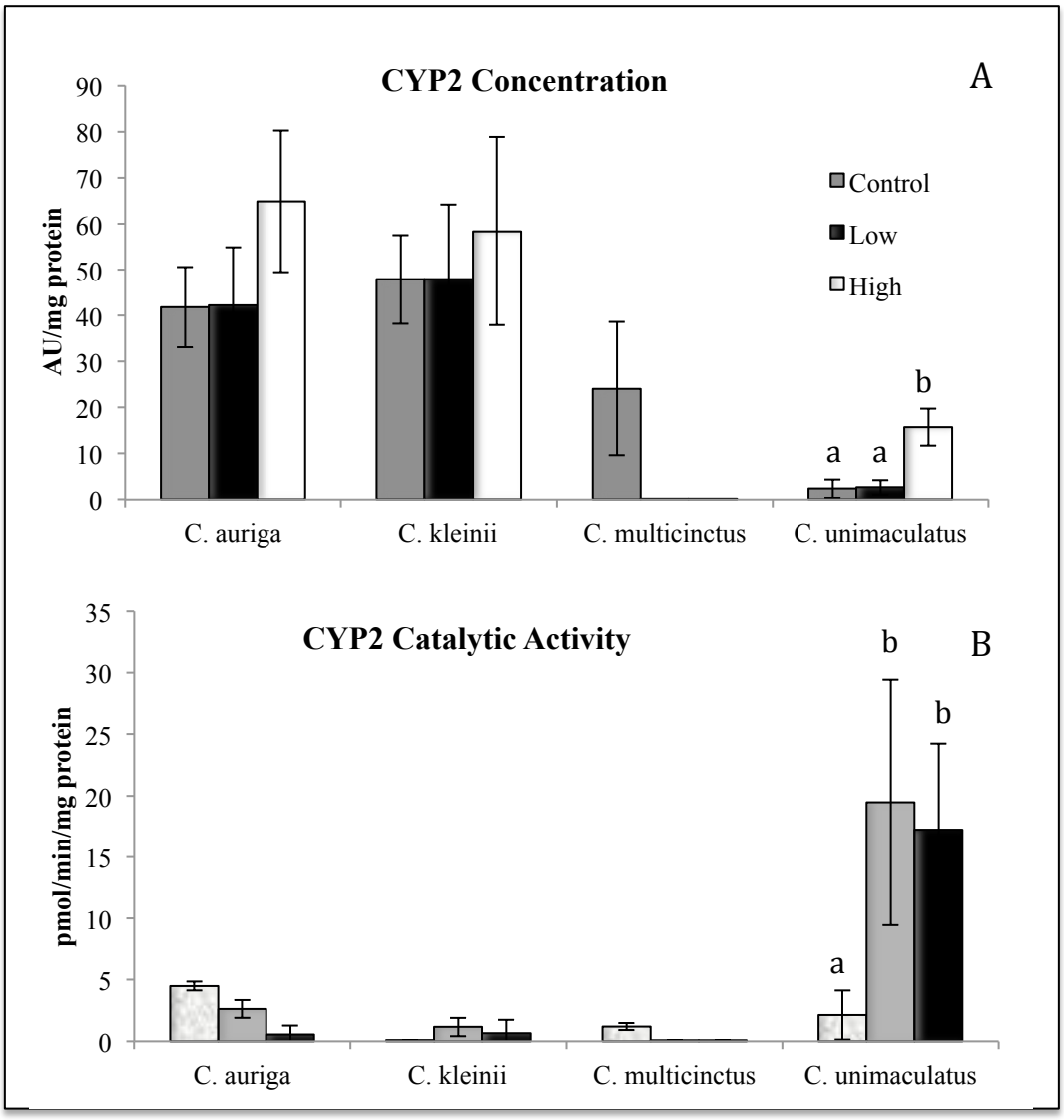

Figure 4.6. Effects of dietary exposure to 5ESL on the hepatic content of CYP2M1-like (A) and catalytic activity (B) of and CYP2 in four species of butterflyfish. Different letters indicate significant ($P \leq 0.05$) differences between untreated and treated fish (N=6-8).

content in *C. unimaculatus* correlated with catalytic activity of CYP3A, increasing 20-25 fold (R^2 =0.66728) (Fig).

5ESL was docked into a crystal structure of CYP3A4, (PDB:3NXU) with the ligand removed. Several low energy (high affinity) positions were found well within the oxidizable distance to a computed Fe-O position. In general, two main docking areas were found – one with an oxidizable carbon within 2-3 A of the Fe-bound oxygen, and the other at about a 7-8 A distant. Both could be occupied at the same time, as the Hill coefficient for CYP3A4 is usually 2 (that is, the reaction rate of CYP3A4 with substrate is dependent on the square of the substrate concentration [S] in Michaelis-Menten type kinetics, implying double occupancy of the active site). There were four different carbons (Fig. C16, C12, C10, C19) that could be oxidized depending on preferred ligand orientation in the active site, on of which was an epoxide formed on the C15-16 (Fig. 7).

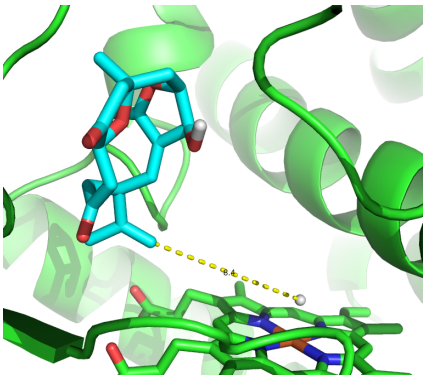

Figure 4.7. One of the top four binding modes of 5ESL with CYP3A4. The computed Fe=O position is shown, assuming a 1.9A Fe-O bond length

Microsomal in-vitro incubations with 5ESL (Fig. 8A) showed NADPH-catalyzed clearance in *C. auriga, C. unimaculatus, C. multicintus* and human CYP3A4 supersomes

(Table 1). An increased trend for biotransformation of 5ESL with NADPH was observed in the following species: in increasing order - *C. multicinctus* < *C. kleinii* < *C. auriga* < *C. unimaculatus;* mimicking the survival trend. Human CYP3A4 completely metabolized 5ESL when incubated with NADPH after 45 min (Table 1). There were three significant peaks at retention times of 2, 3 and 4.5 minutes in the HPLC chromatogram. We further analyzed the peak at retention time 3 min in Fig. 9 using LCMS/MS and compared the chromatogram to the synthesized 5ESL epoxide (5ESLO) (Fig. 8B) based on docking

Table 1. Catalytic activity of hepatic microsomal incubations with 5ESL on four control species of butterflyfish and human isolated CYP3A4 and microsomal 5ESL Epoxide Formation. Different letters indicate significant ($P \leq 0.05$) differences between species and symbols indicate significant ($P \leq 0.05$) (N=6-8)

Species	5ESL Clearance (pmol min^{-1} mg^{-1} protein)	5ESL-O formation (pmol min^{-1} mg^{-1} protein)
C. auriga	266.00±51.2 A	57.39±12.48 A
C. kleinii	-56.61±16.1 B	17.39± 4.92 B
C. multicinctus	13.30±4.22 C	N.D.
C. unimaculatus	490.32±49.3 D	50.43±15.35 A*
C. unimaculatus High		918.75±131.2 *
Human CYP3A4	1125.1±113.4	

5ESL activities were measured using 11 μM NADPH or without NADPH as the electron donor. Values are the mean ± SD (n=6-8).

data. A 365 m/z molecular ion was detected with the metabolite at 3 min and a purified standard of 5ESLO. Loss of 114 and 247 m/z was observed in both compounds from the corresponding pseudomolecular ions (Fig. 10). The structures of the remaining metabolites remain unknown.

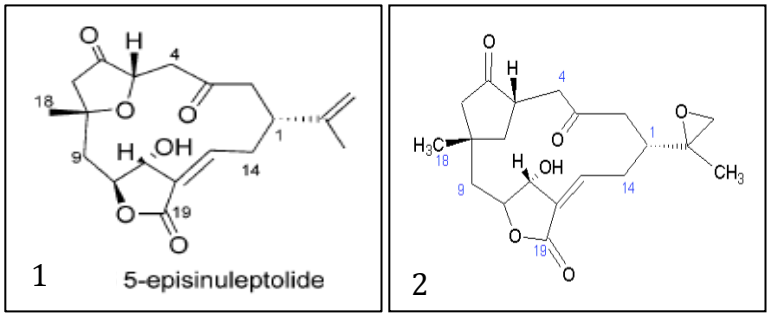

Figure 4.8. Structures of 5ESL (1) from *S. maxima* and 5ESL epoxide (5ESLO) (2).

5ESL epoxide formation correlated with CYP3A activity, with an R^2=0.862. The highest 5ESL formation was seen at the high dose in *C. unimaculatus* compared to control *C. unimaculatus* and the control, *C. auriga* and *C. unimaculatus* compared to *C. kleinii* ($P \leq 0.05$) (Table 1). The 5ESL epoxide formation was below the limit of detection for *C. multicinctus*.

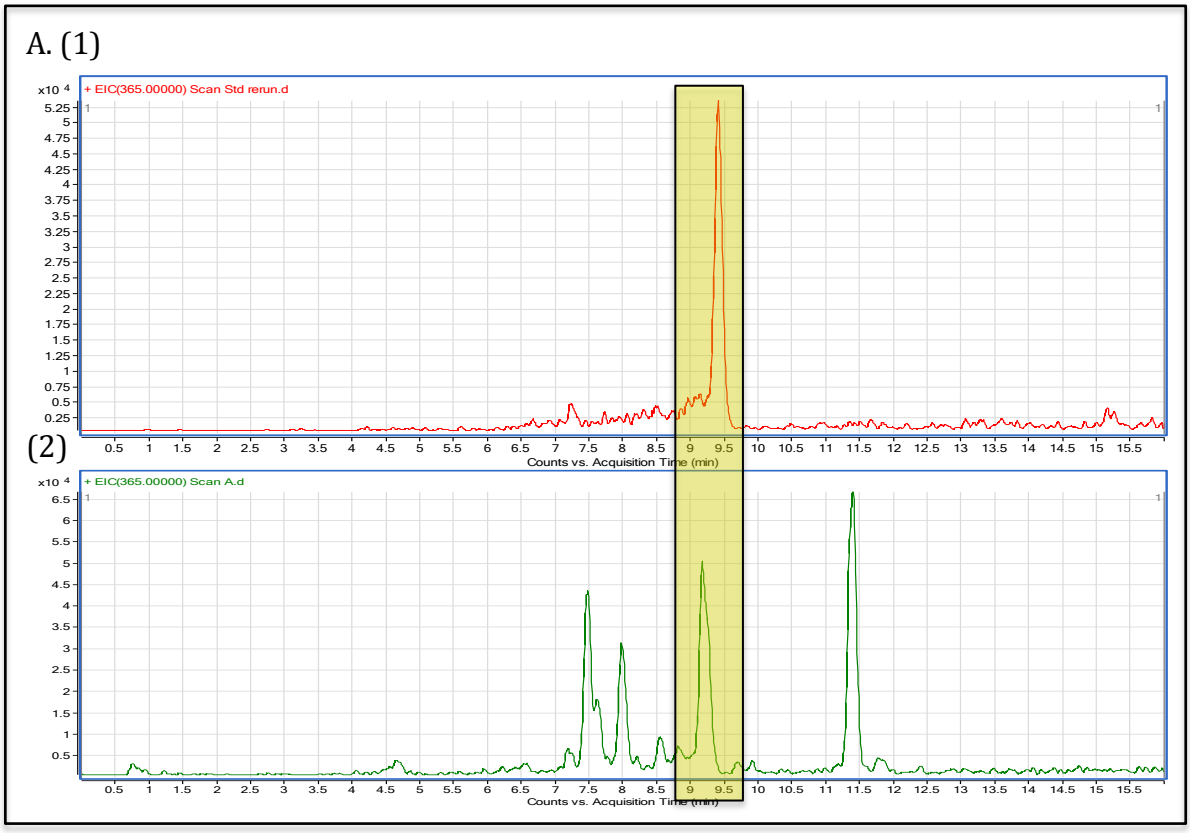

Figure 4.9. Representative liquid chromatograms of standard 5ESL epoxide (1A) and microsomal incubations of 5ESL from livers of *C. unimaculatus* (2A).

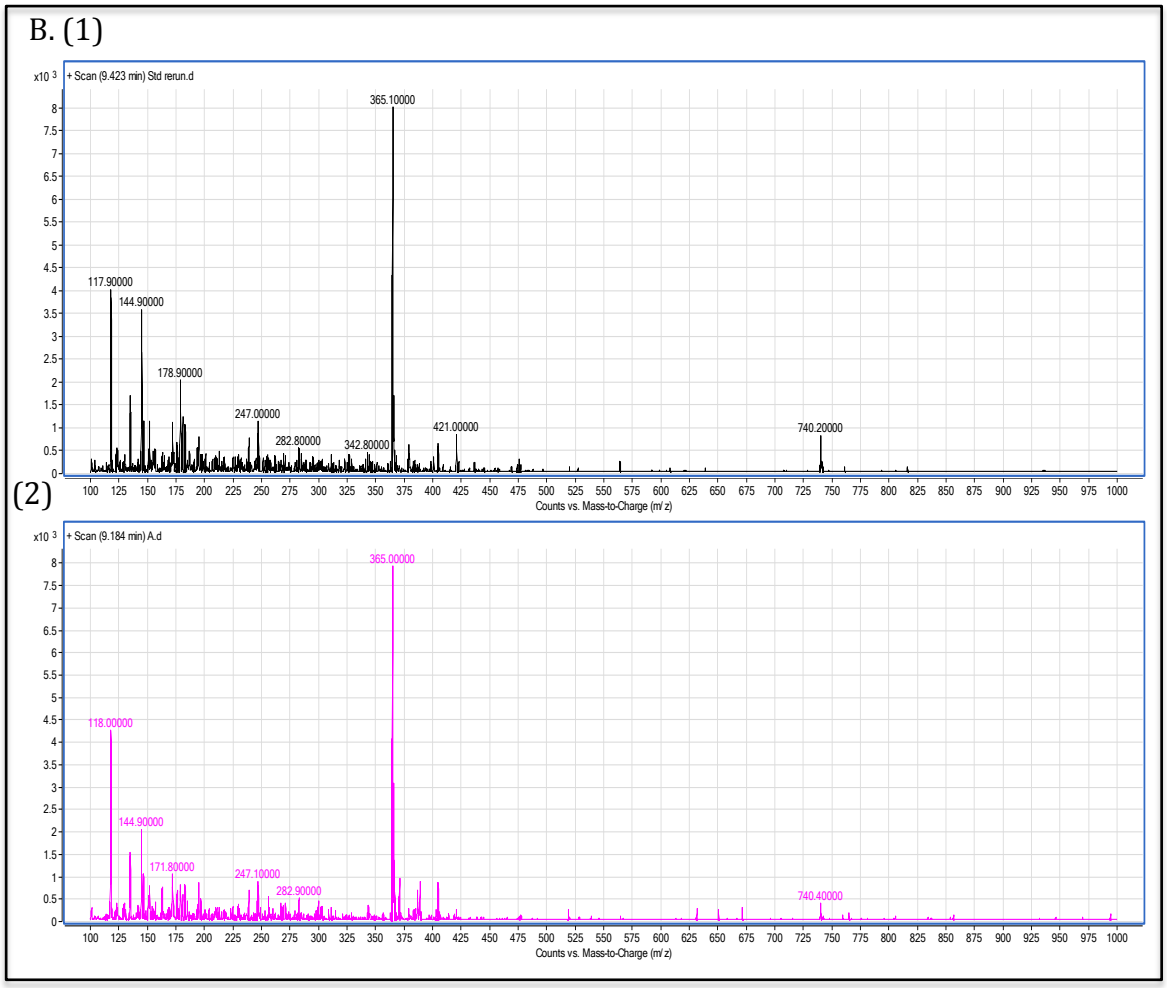

Figure 4.10. Representative mass spectra of the metabolite at 9.3 min (B1) and 5ESL epoxide (B2).

Discussion

Many studies have found that generalist feeding is deterred by allelochemicals, whereas specialists are either unaffected or attracted to the allelochemicals of their preferred diet supported by this experiment (Becerro et al., 1998; Roslin & Salminen et al., 2008). The capability of *S. maxima* to deter predators under laboratory and field conditions has been well described (e.g., Wylie & Paul, 1989; Slattery & Paul 2008). However, few studies have looked at the effect of different feeding strategies within the same genus or species on deterrence. Data indicated the extract and 5ESL were deterrent to generalists but not specialists. Deterrence in the generalists may indicate possible toxicity by learned avoidance in the wild or distastefulness during exposure [20-22]. In contrast, specialist species of butterflyfish were not deterred, which may indicate an evolved adaptation to the toxins in the soft coral *S. maxima*. *C. unimaculatus* preferentially feed on the soft coral *S. maximus* in Guam (Wylie & Paul, 1989) but not in Hawaii because it does not occur there (Li et al., 2007).

In Hawaii, exposure to soft coral *S. maxima* tissue homogenate had no significant effect on survival of the 4 species treated. Similarly, noteworthy effects on CYP2 or CYP3A were not detected. In contrast, 5ESL treatment caused 100% mortality in, *C. multicinctus* given both doses. Only 20% mortality was observed in *C. auriga*, 45-60% for *C. kleinii* and 0% for *C. unimaculatus* after treatment (all controls had 100% survival). Survival following 5ESL exposure correlated to CYP3A and CYP2 protein content. Exposure to 5ESL in the specialist *C. unimaculatus* caused a significant dose dependent induction of CYP3A and CYP2 catalytic activities and protein (Fig. 5 and 6).

C. auriga, the generalist, had higher survival than *C. multicinctus* and *C. kleinii* following exposed to 5ESL, potentially a result from having the highest basal CYP3A and CYP2 content and catalytic activities compared to the other species. The CYP3A and CYP2 of *C. auriga* may function as multipurpose detoxification enzymes, capable of detoxifying a broad range of lipophilic compounds, thereby allowing this fish to exploit a large range of prey (Dearing & Cork, 1999; Li et al., 2000; Sorensen & Dearing, 2003; Sorensen et al., 2005). Conversely, *C. multicinctus,* with a limited range of available food may have CYP's with greater specificity (Cohen et al., 1989; Cohen et al., 1992; Li et al., 2004). A potential negative effect of this trait is that the specialists are less energetically and/or mechanistically efficient at eliminating novel toxins (Sorensen et al., 2005). Consistent with this hypothesis, *C. multicinctus* had the lowest survival of all four species and the lowest CYP2 and CYP3A basal content and activities. There are several cases where specialist herbivores are negatively impacted by novel defense compounds; such as *N. stephensi* reduced locomotor activity when exposed to novel plant toxins from *Larrea tridentata* (Berenbaum, 1989; Sorensen et al., 2005).

Since there were multiple CYP2 isoforms observed in western blots and catalytic activites associated with CYP2 (16 a testosterone hydroxylase) were not correlated with survival, focus was placed upon CYP3A as a potential enzyme for detoxification. 5ESL docking into the crystal structure of CYP3A4 showed several low energy (high affinity) positions. There are four different carbons that could be oxidized, depending on preferred ligand orientation in the active site. CYP3A enzymes are functionally among the most versatile forms of CYP's. Mutation and docking studies have demonstrated that CYP3A

proteins have a large substrate-binding pocket in comparison to other members of the CYP superfamily (Khan & Halpert et al., 2000). This large pocket may enable CYP3A enzymes to catalyze biotransformation of the large 5ESL molecule. Further analysis revealed C15-16 epoxide (Fig. 8B) to be the most likely CYP3A metabolite. To confirm the 5ESLO metabolite, microsomal in vitro incubations were conducted. Incubations showed NADPH dependence and, as predicted by CYP3A4 molecular modeling, identification of 5ESLO (Fig. 7). LCMS chromatograms and spectra of the collected metabolite formed by liver microsomes and a synthesized standard were identical (Fig. 9 and 10). In addition, 5ESLO formation correlated with hepatic CYP3A content and as well as survival in all species examined. This is consistent with the possibility that CYP3A 5ESLO formation is a detoxification pathway.

The corresponding pattern of CYP3A content induction and catalytic activity in the liver enzymes of *C. unimaculatus* dosed with 5ESL is similar to cases in plant-insect interactions, where black swallowtails (*Papilio polyxenes*) where exposed to the plant alleochemical, xanthotoxin that induced CYP activity in a dose-dependent manner, increasing seven fold (Cohen et al., 1989). Allelochemicals often act as ligands to induce their own CYP metabolism, (Crwford, 1980; Cohen et al., 1989; Li et al., 2004; McDonell et al., 2004). Although it is unclear how 5ESL regulates CYP3A, other studies have demonstrated that CYP3A can be induced by allelochemicals that bind the nuclear pregnane X receptor (PXR) (Dresser et al., 2003; Kullak-Ublick & Becker, 2003; Moore et al., 2000). Further research is needed to elucidate this mechanism.

Just as insects evolved specific CYP's to detoxify dietary plant allelochemicals, butterflyfish have CYP's capable of metabolizing coral allelochemicals. The results of this study also demonstrate that CYP functional versatility in generalists may facilitate consumption of a variety of prey as well as novel dietary allelochemicals, whereas functional specialization of certain CYP genes in specialists may promote susceptibility to unfamiliar dietary allelochemicals. However, certain specialists such as *C. unimaculatus* seem to have evolved CYP enzymes that are particularly tolerant to highly toxic coral allelochemicals. This idea is supported by the inducability of CYP3A and CYP2 in *C. unimaculatus* after exposure to 5ESL, with which they have never been exposed, due to Hawaii's geographical isolation.

CYP analysis in butterflyfish suggested many commonalities and several potential differences between the specialist and generalist CYP proteins. Further studies, closely analyzing species that diverge in their degree of feeding specialization, may resolve remaining questions related to generalists' coping mechanisms with respect to their diverse feeding habits. Moreover, understanding the biochemical and molecular mechanisms underlying diet choice of butterflyfish has broad implications for identifying the role of the environment in determining gene function in the co-evolutionary relationship between corals and butterflyfish.

C. unimaculatus had the greatest clearance of 5ESL, the highest induction of CYP3A content as well as activity, and the highest relative formation of 5ESLO which were all coordinately related This species preferentially feeds on *S. maxima* in Guam and Australia, which may indicate this species generally has a high tolerance for soft coral

toxins regardless of current feeding preferences. Induction of CYP3A after exposure to a dietary item that is geographically removed from Hawaii suggests that *C. unimaculatus* may have evolutionarily migrated from Guam (where the fish feeds on the soft coral) to Hawaii. The Biodiversity Feedback model indicates that biodiversity flows eastward from the indo-pacific archipelago to peripheral habitats in Hawaii (Cowman & Bellwood, 2013). The Central Pacific area is characterized by low species departure (10–16% Central Pacific), but high dispersal of lineages into the region, from the Indo-Pacific Archipelago (Cowman & Bellwood, 2013). In terms of global diversity for Chaetodontidae, the Indo-Pacific Archipelago stands out as a significant source of diversity in terms of both origination within the region and the expansion of lineages into adjacent regions (Cowman & Bellwood, 2013). Gene flow of two related coral-feeding butterflyfish was found to be high, across the Pacific Ocean over both recent and historical timeframes, in the specialist, *Chaetodon trifascialis* and more stable in the generalist, *C. lunulatus* (Lawton et al., 2011). It is possible that *C. unimaculatus* has gene flow between Hawaii and the rest of the Pacific or historically migrated from Guam eastward to Hawaii, however future studies on *C. unimaculatus* should be conducted to support or refute the evolutionary migration from Guam to Hawaii.

C. multicinctus had the least metabolism of all species tested, and it is endemic to Hawaii, with no evolutionary history with *S. maximus*. The distinct levels of allelochemical tolerance may explain coral-feeding butterflyfish highly contrasting levels of dietary specialization, since nutritional value of corals has yet to explain these differences (Berumen & Pratchett, 2008). The risks associated with dietary specialization

are thought to be offset by increased nutritional benefits (e.g growth) when feeding on preferred prey over generalist feeding on the same prey (Schoener, 1971). However a study conducted on coral-feeding butterflyfish found that the more specialized species (*Chaetodon trifascialis*) did not outperform the generalist species (*Chaetodon plebeius*) when both consumed their preferred prey (Berumen & Pratchett, 2008). In the current study *C. unimaculatus* appears to have an innate ability to tolerate toxic dietary allelochemicals allowing them to explore an uncompetitive diets of soft corals throughout the Pacific (LaJeunesse et al, 2004). Losing this advantage may have led *C. unimaculatus* to consume hard coral *Monipora spp.* in Hawaii (Cox, 1983). This may also explain the innate inability of *C. multicinctus* to tolerate the soft coral toxin 5ESL, and its preference for hard corals. These differences in dietary choices may be explained by their characteristic ability to tolerate dietary allelochemicals.

Interactions between corals and coral-feeding fishes are particularly important in the context of coral reef decline; contributing factors to this decline include: climate-induced coral bleaching, ocean acidification, coral disease and direct anthropogenic stresses such as sedimentation, eutrophication and over-fishing (Bellwood et al., 2004; Hoegh-Guldberg et al, 2007; Anthony et al., 2008). Coral feeding butterflyfish are particularly sensitive to the declining abundance of corals and are frequently among the first and worst affected fish during extensive coral loss (Jones et al., 2004; Cole et al., 2008; Prachette, 2007). Increased knowledge in allelochemical tolerance can hold the potential to improve conservation efforts of certain butterflyfish species that are potentially incapable of switching prey (e.g. *C. multicinctus*) and thus more susceptible to

extinction. Butterflyfish play a significant role in coral reef ecosystems as one of the few trophic levels responsible for exchanging nutrients and energy between corals and predators, thus a better understanding of their biochemical ecology is necessary and may provide insight into the protection of coral reef ecosystems throughout the ocean.

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Chapter 5: Conclusion

Butterflyfish are an important component of the ichthyofauna of tropical coral reefs as they are among the few taxa specialized to feed on scleractinian corals (Glynn, 1990). The goal of this project was to further knowledge related to CYP expression and differentiation and the role of biotransformation enzymes in corallivorous fish species, in the genus *Chaetodon*. This study enhanced our knowledge of the biochemical and molecular mechanisms underlying the ability of *Chaetodon* spp. to consume corals and how they relate to feeding strategies and diet choice. This information on the coevolutionary relationship between corals and butterflyfish provided critical insight into the susceptibility of endemic specialist *C. multicintus* to survive while feeding on preferred and non-preferred coral prey. Chemical defense is generally considered to be a major selective force driving the evolution of several CYP gene families, and this research increased our understanding of the role of allelochemical biotransformation and detoxification in marine organisms; holding significance for understanding patterns of predation and herbivory in the marine environments. Previous research has failed to relate butterflyfish-feeding preference to nutritional value of corals. This research begins to explain feeding preference through molecular ability to consume certain prey species of corals.

C. auriga, C. lunulatus, C. kleinii and *C. unimaculatus* in Australia and Hawaii represent an ideal model system for studying dietary detoxification concept because we are able to study the same species in similar environments with the same reproductive strategy, but significantly different feeding preferences. The Hawaiian *C. auriga* had

significantly higher CYP1A mRNA, EROD rates and GST activity than Australian *C. auriga,* which may suggest exposure to aromatic hydrocarbons. Feeding on allelochemically-rich prey by the Australian *C. auriga, C. kleinii* and *C. unimaculatus* is well documented, and they express qualitatively higher levels of CYP2 and CYP3A isozymes and GST activity, when compared to their Hawaiian counterparts that avoid allelochemically-rich prey. These research findings suggest that biotransformation enzymes may be involved in detoxification of dietary allelochemicals in butterflyfish. Bioactivity profiles of extracts from species of scleractinian coral in order to determine potential for chemical defense showed that extracts from *Porities spp.* showed significant toxicity to mice, fish and cytotoxicity, whereas *Acroporidae spp.* only showed significant cytotoxicity (Gunthorpe & Cameron 1990). Therefore, *Porities spp.* may contain more allelochemicals that require *C. lunulatus* from Hawaii to have higher CYP2, CYP3 and GST levels than *C. lunulatus* from Australia.

To further understand the toxic nature of *Porites spp.* exposure to *Porites lobata* and the effects isolated allelochemical homarine has on *C. auriga, C. kleinii* and *C. multicinctus* (preferred prey *P. lobata*) further experiments were conducted. Similar with insect exposures to preferred prey, significantly induced CYP3A and CYP2 was measured in *C. multicinctus.* These CYP levels were lower when compared to *C. auriga* and *C. kleinii,* which do not preferentially feed on *P. lobata*. These results may indicate a unique adaptation allowing consumption of *P. lobata* and related corals in *C. multicinctus* diet. Isolated *P. lobata* allelochemical homarine caused dose dependent lethality in *C. kleinii.* In addition, homarine exposure caused CYP3A induction in the generalist *C.*

auriga. Hormesis occurred in *C. multicinctus* and decreased activity was observed in *C. kleinii.* Both *C. auriga* and *C. kleinii* are generalists but have different dietary histories and feeding strategies, possibly influencing differences in response to coral homogenate and homarine. The mechanism for homarine toxicity is as yet unknown, and although it is not known to be a substrate for CYP, homarine does alter CYP regulation in butterflyfish.

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, the different responses to these coral tissues may have resulted from other unknown compounds within the homogenate that potentially served to diminish homarine toxicity relative to the tested fish species. Acclimation is necessary for controlled laboratory experiments to provide normalizing conditions for comparisons, but it may be another possible explanation for the differences between the three species due to the concurrent change of feeding to the same dietary items (brine shrimp). 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 butterflyfish.

After identifying higher CYP levels in butterflyfish that preferentially feed on chemically defended soft corals, impacts of soft coral *S. maxima* and isolated allelochemical 5ESL were examined on butterflyfish *C. auriga, C. multicinctus, C. kleinii* and *C. unimaculatus*. *S. maxima* and isolated 5ESL toxin were deterrent to butterflyfish generalist feeders but not to specialists. Exposure to 5ESL, which is not normally

encountered in their Hawaiian diet, endemic specialist *Chaetodon multicinctus* experienced 100% mortality compared to generalist *Chaetodon auriga* which had significantly more CYP3A (3-6 times higher) basal content and catalytic activity. Hawaiian specialist *Chaetodon unimaculatus*, which preferentially feed on *S. maxima* in Guam, but not in Hawaii, had 100% survival, a significant induction of 8-12 times more CYP3A and the ability to metabolize twice the amount of 5ESL. Several lines of evidence are provided implicating butterflyfish CYP3A in the mechanism of adaptation to soft coral allelochemical 5ESL. The corresponding pattern of CYP3 content responsiveness and catalytic activity in the liver enzymes of butterflyfish dosed with 5ESL, coupled with NAPDH-dependent metabolism and the identification of CYP3 as a potential metabolite suggests that CYP3A plays a role in mediating the metabolism of dietary 5ESL coral allelochemicals in butterflyfish. Just as insects evolved specific CYP's to detoxify dietary plant allelochemicals, butterflyfish have CYP's capable of metabolizing potent coral allelochemicals.

The results of this study also demonstrate that CYP functional versatility in generalists may facilitate consumption of a variety of prey as well as novel dietary allelochemicals, whereas functional specialization of certain CYP genes in specialists may promote susceptibility to unfamiliar dietary allelochemicals. However, certain specialists such as *C. unimaculatus* seem to have evolved CYP enzymes that are particularly tolerant to highly toxic coral allelochemicals, which may have evolved in Guam and later migrated to Hawaii. This idea is supported by the inducability of CYP3A and CYP2 in *C. unimaculatus* after exposure to 5ESL, with which they have never been

exposed, due to the geographical isolation. This research shows that Berenbaum's discoveries are not excusive to insect prey interaction but may apply to marine ecosystems as well.

This research sought to elucidate the nature of the tight coevolutionary relationship between butterflyfish and corals, by understanding the mechanisms that allow butterflyfish to feed on chemically defended corals. A mechanism we have identified to consume coral allelochemicals is cytochrome P450 enzymes, similar to insects. We have multiple lines of evidence that implicate CYPs in the detoxification of coral allelochemical in butterflyfish, such as: dose dependent induction of CYP3A and CYP2 in obligate coral feeding butterflyfish, butterflyfish CYP3A invitro metabolism of coral alleochemicals and higher levels of CYP2, CYP3, and GST in butterflyfish that preferentially feed on allelochemically-rich corals.

In summary, this project has broadened our knowledge of novel biotransformation and detoxification pathways, not previously identified in butterflyfish. Increasing the knowledge of metabolic pathways in butterflyfish has enabled us to better understand these fish and consequently protect them in the future. Obligate corallivores in many locations may be threatened with extinction, given the present state and predicted impacts of climate change on coral reefs. We have a better understanding of the susceptibility of endemic specialist *C. multicinctus* to novel toxins, potentially indicating aninability to switching prey and thus a higher susceptibility to extinction. A key challenge still facing marine ecologists, seeking to explain the vast differences in consumer tolerance of dietary allelochemicals, remains the lack of understanding regarding biochemical and

molecular mechanisms underlying diet choice. Coral–feeding organisms often exhibit significant feeding selectivity, but the underlying basis of prey preference is not based on nutritional value and is still unknown. One explanation could be their detoxification abilities. In this research we show varying abilities to detoxify preferred and novel allelochemicals based on feeding preferences. The ability of some butterflyfish to consume toxic allelochemicals is supported by the results of *C. unimaculatus* from Hawaii. This species had a 100% survival rate and complete invitro metabolism of 5ESL, whereas other butterflyfish species in the trial exhibited less desirable results. Their detoxification ability could be the basis for prey preference. Further studies on the molecular mechanisms governing allelochemical resistance is crucial for understanding the genetic basis of adaptation in consumers like butterflyfish that regularly feed on chemically defended prey