THE PHYSIOLOGICAL ECOLOGY OF UV-ABSORBING COMPOUNDS FROM THE MUCUS OF MARINE FISHES

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ABSTRACT

This dissertation details my investigation into the physiological ecology of UV-absorbing compounds found in the mucus of marine fishes. In a survey of over 200 species of fishes from around the Pacific, I found that approximately 90% of fishes possess mucus that absorbs strongly in the UV. High-performance liquid chromatography of selected mucus confirmed that the UV-absorbing compounds in the mucus are mycosporine-like amino acids, or MAAs.

I determined that the mucus of experimentally UV-exposed *Thalassoma duperrey* absorbs more strongly in the UV than the mucus of those protected from UV by UV-opaque (but visible light transparent) plastic. However, this difference in mucus absorbance only occurs if fish are provided a dietary source of MAAs. Furthermore, I found that males have higher mucus absorbance than females, and females exposed to UV suffer high rates of skin damage. Females also sequester MAAs in their eggs, and may suffer a conflict of interest between providing sunscreen protection for their eggs vs. their own skin.

Three coral reef fish species (*Canthigaster jactator*, *Chaetodon multicinctus*, and *Thalassoma duperrey*) were sampled over a depth gradient, and shallow water fish generally had superior sunscreen, both in terms of magnitude and short-wavelength spectral shifting, as compared with deeper water individuals of the same species.

Temperate tidepool sculpins (Family: Cottidae) showed a significant loss of UV-absorbing compounds with increasing north latitude, and overall, fishes from higher tidepools had more sunscreen than fishes from low tidepools.

Behavioral experiments with *Thalassoma duperrey* showed no dietary or UV-induced differences in weight loss or swimming behavior, and the results on shade-seeking behavior were equivocal.

Thus, sunscreening compounds seem to be ubiquitous among marine fishes. The correlations I have found between the UV absorbance of mucus and the depth, latitude, or UV exposure of the sampled individual lead me to believe that mucus UV absorbance is an adaptive defense against UV for fishes.

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CHAPTER 1:

GENERAL INTRODUCTION

Ultraviolet radiation (UV, =280-400 nm) is damaging to organisms, from cyanobacteria (Garcia-Pichel et al. 1993) to humans (Marks 1989). Solar UV can damage DNA directly via formation of cyclobutane pyrimidine dimers (Setlow 1968) and 6-4 photoproducts (Vincent and Neale 2000). Solar UV can also induce cellular damage due to the formation of reactive oxygen species such as singlet oxygen and superoxide radicals (Dunlap et al. 2000). Another consequence of UV is the formation of "sunburn cells", damaged cells "possessing a densely staining, glassy, homogeneous cytoplasm and a pyknotic nucleus most often in association with a perinuclear or pericellular oedematous halo" (Bullock 1988).

Solar UV radiation penetrates clear water in quantities sufficient to be biologically harmful, and negatively affects aquatic organisms such as dinoflagellates (Jeffrey et al. 1999), phyto- and bacterio-plankton (Buma et al. 2001), zooplankton (Browman et al. 2000), amphibians (e.g., Fite et al. 1998, Kiesecker et al. 2001) and larval (e.g., Vetter et al. 1999, Lesser et al. 2001) and adult fishes (e.g., Ahmed & Setlow 1993, Bullock 1981). In Antarctica, Karentz and Lutze (1990) found biological effects of UVB (280-320 nm) at 30 m depths. Utilizing a deep submersible, Frank and Widder (1996) measured 380 nm irradiance, in the UVA (321-400 nm) range of the spectrum, sufficient for detection by crustacean visual systems at 500-600 m depth. Tropical marine fishes living in shallow water are exposed to extremely high and variable levels of UV -- much higher levels than are experienced by animals

under the Antarctic "ozone hole" (Smith et al. 1992; Stolarski et al. 1992; Gleason et al. 1993; Sasaki et al. 1993).

Since 1930, we have known that fishes exposed to high levels of UV suffer fatal "sunburn" (Crowell and McCay 1930), a condition characterized by dorsal skin lesions or necrotic areas and congestion of the fins (Bullock et al. 1983; Bullock and Coutts 1985; McArdle and Bullock 1987). These lesions are evidence of radiation trauma (Bullock & Coutts 1985) and if allowed to continue, they generally become infected and eventually cause death (McArdle & Bullock 1987). Mortality due to sunburn continues to be a problem for aquaculture projects worldwide (Bullock 1988). Fishes also suffer corneal damage and permanent lenticular damage (cataracts) as a result of chronic UV exposure (Cullen & Montith-McMaster 1993, Cullen et al. 1994).

As global ozone levels continue to decrease at all latitudes, allowing more UV to strike the earth's surface and penetrate into its oceans (Smith et al. 1992; Stolarski et al. 1992; Gleason et al. 1993; Sasaki et al. 1993), defending against this harmful radiation may be expected to become a higher priority for fishes and other aquatic organisms. The most severely stressed fishes are likely to be those living in shallow clear water such as pelagic larvae, juveniles found in inshore and tidepool nursery areas, shallow coral reef fishes, and commercially important near-surface open ocean fishes.

Aquatic organisms have several strategies for defending against damaging UV radiation. Hammerhead sharks are known to "suntan", or increase melanin production, in response to UV radiation (Lowe and Goodman-Lowe 1996). Marine

organisms such as corals, echinoderms, molluscs, and algae, synthesize or sequester UV-absorbing chemical compounds (mycosporine-like amino acids, or MAAs) in response to changing UV levels (Dunlap and Shick 1998; Cockell and Knowland 1999; Shick and Dunlap 2002). Fishes also possess MAAs, particularly in the ocular tissues (Chioccara et al. 1980; Dunlap et al. 1989). Animals derive these compounds from dietary sources (Shick and Dunlap 2002), and Mason et al. (1998) demonstrated this experimentally with the medaka fish. Freshwater fishes possess a UVB-absorbing (therefore not an MAA), possibly protective substance in their skin (Fabacher & Little 1995).

Mycosporine-like amino acids are a class of chemical compounds with absorbance maxima ranging from 309 to 360 nm (Shick and Dunlap 2002). Gadusol and deoxygadusol are closely related compounds (with absorbance maxima of 294 and 268nm, respectively) that have been found in the roe of marine fishes (Plack et al. 1981) and in brine shrimp (Grant et al. 1985). There are 19 known MAAs, and they are found in marine organisms and also terrestrial fungi (Shick and Dunlap 2002). Palythine and mycosporine-glycine (absorbance maxima of 320 and 310nm, respectively) are the most prevalent MAAs found in corals (Shick and Dunlap 2002). Palythine, palythene, palythinol and asterina 330 (absorbance maxima of 320, 360, 332 and 330 nm, respectively) were the only MAAs found in a broad taxonomic survey of ocular tissues of Great Barrier Reef fishes (Dunlap et al. 1989).

A number of studies have investigated how MAA concentrations vary with environmental parameters. Bathymetric correlations (decreasing MAA concentrations with increasing depths) have been shown in corals, algae and echinoderms (Shick et

al. 1992; Karentz et al. 1997; Dunlap and Shick 1998; Karsten et al. 1998a; Dunlap et al. 2000). A latitudinal effect (decreasing MAA concentrations with increasing N latitude) has been demonstrated in red algae (Karsten et al. 1998a; Karsten et al. 1998b). The concentration of MAAs in the mucus of Tahitian corals was shown to positively correlate with ambient solar UV radiation over a period of 18 months, with a lag time of one week (Drollet et al. 1997). A correlation between MAA concentrations and temperature, as well as UV irradiance, was shown for soft corals (Michalek-Wagner 2001).

In addition to possessing MAAs, organisms may mitigate UV-induced damage by behavioral avoidance of harmful radiation. Sea urchins exhibit negative phototaxis and covering behavior (Adams 2001; Verling et al. 2002). Several *Daphnia spp.* migrate significantly deeper when exposed to UV radiation as opposed to human-visible radiation (Rhode et al. 2001). UV-exposed newts became more active, and UV-exposed toads and frogs show reduced anti-predator behavior (Blaustein et al. 2000; Kats et al. 2000).

The purpose of my doctoral research was to investigate sunscreening compounds found in fish mucus. After discovering the existence of these compounds, I first conducted a broad survey of coral reef fish taxa and found that UV-absorbing compounds are ubiquitous among diurnal coral reef fishes (Chapter 2). I then proceeded to investigate the effects of UV exposure regime (Chapters 2-3), diet (Chapter 3), bathymetry (Chapter 4), latitude and height in the intertidal zone (Chapter 5) on the spectral qualities and (putative) concentration of MAAs in the

mucus of fishes. Finally, I examined the effects of UV regime and diet on the behavior of a coral reef fish (Chapter 6).

Briefly, I found that UV-exposed fish sequestered more MAAs in the mucus than those protected from UV, but only if provided a dietary source for these compounds. Three coral reef fish species were sampled over a depth gradient, and shallow water fish generally had superior sunscreen, both in terms of overall absorbance and short-wavelength spectral shifting, as compared with deeper water individuals of the same species. Temperate tidepool fishes showed a significant loss in UV-absorbing compounds with increasing north latitude, and fishes from high tidepools had more sunscreen than fishes from low tidepools. Behavioral experiments showed no dietary or UV-induced differences in weight loss or swimming behavior, and the results on shade-seeking behavior were equivocal.

CHAPTER 2:

BROAD SURVEY AND EFFECTS OF UV REGIME

Ultraviolet radiation absorbance by coral reef fish mucus:

sunscreen protection and visual communication

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by

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ABSTRACT

Tropical reef fishes are exposed to high levels of damaging ultraviolet radiation. Here we report the widespread distribution of both UVA- and UVB- absorbing compounds in the mucus of these fishes. Mucus from 137 reef fish species was examined by spectrophotometry and 90% were found to have strong absorbance peaks between 290 and 400 nm. Most fish species (78%) had more than one peak, that suggests a broad-band ultraviolet screening function for their mucus. *Thalassoma duperrey*, a tropical wrasse, was able to alter the absorbance of its epithelial mucus in response to both naturally and experimentally manipulated UV regimes. Visual modelling suggests that a fish with UV vision, such as *Dascyllus albisella*, could detect the changes in mucus spectra of *Thalassoma duperrey* that occurred in these experiments.

INTRODUCTION

Animals inhabiting high light intensity environments may be damaged by short wavelength radiation, and as these wavelengths can penetrate water, this includes fishes (Calkins 1982, Bullock 1988, Winckler & Fidhiany 1996). The detrimental effects of ultraviolet radiation (UV=280-400 nm), particularly UVB (280-320 nm), on aquatic ecosystems have been well documented (Holm-Hansen et al. 1993, Häder et al. 1998). A UVB-absorbing compound was recently found in skin extractions of some freshwater fishes (Fabacher & Little 1995). Here we report the discovery of UVA- and UVB-absorbing properties of mucus from the body surface of tropical marine fishes. Furthermore, we found that the absorbing compounds are not

contained within the skin cells, but are secreted into the epithelial mucus, and thus provide a protective coating to the animal. We show that at least one species, *Thalassoma duperrey*, can alter the absorbance of its mucus in response to changes in solar UV radiation in a natural environment. We also demonstrate that *Dascyllus albisella*, a Hawaiian damselfish with a UV-sensitive visual system (Losey et al. 2000, McFarland et al. unpubl.), should be able to visually detect these differences in mucus absorbance.

Shallow-dwelling tropical marine organisms are subjected to extremely high (several times that experienced by organisms under the Antarctic 'ozone hole') and changeable levels of UVA and UVB, both on a daily cycle and during different seasons (Smith et al. 1992, Stolarski et al. 1992, Sasaki et al. 1993). Many invertebrate organisms, such as corals and echinoderms, synthesize or sequester UV-absorbing chemical compounds (often in the form of mycosporine-like amino acids, or MAAs), in response to changing UV levels (Dunlap & Shick 1998, Cockell & Knowland 1999). Some of this 'natural technology' is already in use commercially (Australian Institute of Marine Science Media release, February 2000. Http://www.aims.gov.au/news/pages/media-release-20000222.html). Here we demonstrate that vertebrates, specifically reef fishes exposed to high intensity UV radiation in tropical marine habitats, possess a similar line of defense.

The UV world of coral reef fishes has only recently begun to be studied in detail (Losey et al. 1999). Of particular interest have been the UV visual capabilities of many of these coral reef fishes (Dunlap et al. 1989, Lythgoe et al. 1994, McFarland & Loew 1994, Losey et al. 2000, Siebeck & Marshall 2001, Marshall 2000a,b). Losey

et al. (1999) estimate that, for an animal looking horizontally or downward through shallow clear water, 40% of the photons available for vision are in the UVA portion of the spectrum. We utilized known irradiance, ocular transmission, and visual pigment data in an existing visual model, and determined that a Hawaiian damselfish at 3.5 m depth might perceive the changes in mucus absorbance documented for *Thalassoma duperrey*. It has been suggested that many of the colors we humans see are almost certainly perceived differently by coral reef fish (Losey et al. 1999, Marshall 2000a,b). Now, we must consider that reef fishes might 'tint' their colors with a mucous layer of variable absorbance on a weekly, or perhaps even daily, basis.

MATERIALS AND METHODS

Fishes were captured by hook-and-line or barrier net at depths of 1-16 m at Heron and Lizard Islands, Great Barrier Reef, Australia; Oahu, Hawai'i; and Johnston Atoll. In most cases animals were kept alive, sampled, and released. Experimental parrotfish were held in individual tanks at the Heron Island Research Station, and provided with plastic tubes of appropriate size for nocturnal shelter. Parrotfish often secrete "mucous cocoons", envelopes of mucus that completely surround the body of the fish, which they remain inside overnight. These cocoons are produced by goblet cells in a folded epithelium in the opercular gland in the gill cavity (Videler et al. 1999). The morning after capture, tubes containing parrotfish cocoons were removed and poured into a small pail, to be sampled in the lab. The fishes were sampled later in the day for epithelial mucus. If a fish did not produce a cocoon the first night, it was simply sampled for epithelial mucus (see below) and released.

Epithelial mucus for all species was sampled by scraping a dull scalpel blade along the dorsal flank of live fishes, anterior to posterior. Collected mucus was squashed to 0.25 mm thickness between two ultraviolet-transparent (50% transmission cutoff = 266 nm) slides with coverslip spacers at either end. When possible, fishes were kept in UV-transparent aquaria and visualized with a UV-sensitive image-intensified CCD camera taking digital video through selected optical filters prior to epithelial mucus sampling. One individual of each species was euthanized and ocular media transmission measured following Losey et al. (2000). Briefly, the eye was excised, and a small window cut in the back just through the retina. The eye was placed on a UV-transparent slide and transmission through the whole eye was measured with the system described below.

Light absorbance of, or transmission through, the mucous layer was measured with an Ocean Optics S-2000 spectrophotometer (Losey et al. 2000). Briefly, illumination provided by a Deuterium-Tungsten source (Analytical Instruments Inc. Model DT-1000) was shone through a UV-transmitting 400 micron fiber optic probe, through the sample, into a UV-transmitting collimating lens, through another 400 micron fiber optic cable and into the spectrometer (all equipment specifications can be found at: www.oceanoptics.com). The source and pick-up for the system were close enough together (~1cm) to negate any effects of surface scatter from the sample or the slide. Eight to ten measurements taken through each mucous sample were averaged in order to compensate for possible heterogeneity of the sample. Some absorbance measurements were converted to transmission for ease of comparison with ocular transmission data.

Absorbance data are discussed in terms of peak wavelength (λ_{max}), and transmission data in terms of 50% transmission cutoff wavelength (T_{50}). Absorbance peaks were separated into three categories by approximate λ_{max}, ca.: (1) 290-295 nm, (2) 320-335 nm, and (3) 360 nm (Figure 2.1a). These categories are designated for ease of description and each category may include more than one chemical compound. Category 3 peaks were often 'swamped' by a larger category 2 peak (Figure 2.1a), making the precise λ_{max} difficult to determine, however presence/absence of a category 3 peak was easy to discern.

Twenty-four Hawaiian saddleback wrasse, *Thalassoma duperrey*, were collected from 1-3 m depths, and 11 were collected from 14-16 m in order toinvestigate the effects of depth on mucus T_{50}. Sixteen of the shallow-caught individuals were then used for solar UV manipulation experiments. Four 1 x 1 x 0.25 m deep outdoor tanks at the Hawai'i Institute of Marine Biology were equipped with plexiglass covers which either transmitted (UVT, T_{50} = 293 nm) or blocked (UVO, T_{50} = 408 nm) UV radiation. Fish were randomly assigned a treatment, controlling for size, housed four to a tank, and fed chopped squid daily. Mucus was sampled initially, and once per week for 5 weeks thereafter. At the conclusion of the study, fish were killed by overdose of MS-222 (Tricaine methanesulphonate), gender determined, and ocular transmission measured. Analysis of this data set was performed by repeated measures ANOVA with autoregressive covariance structure and Satterthwaite determination of degrees of freedom (SAS systems, version 8).

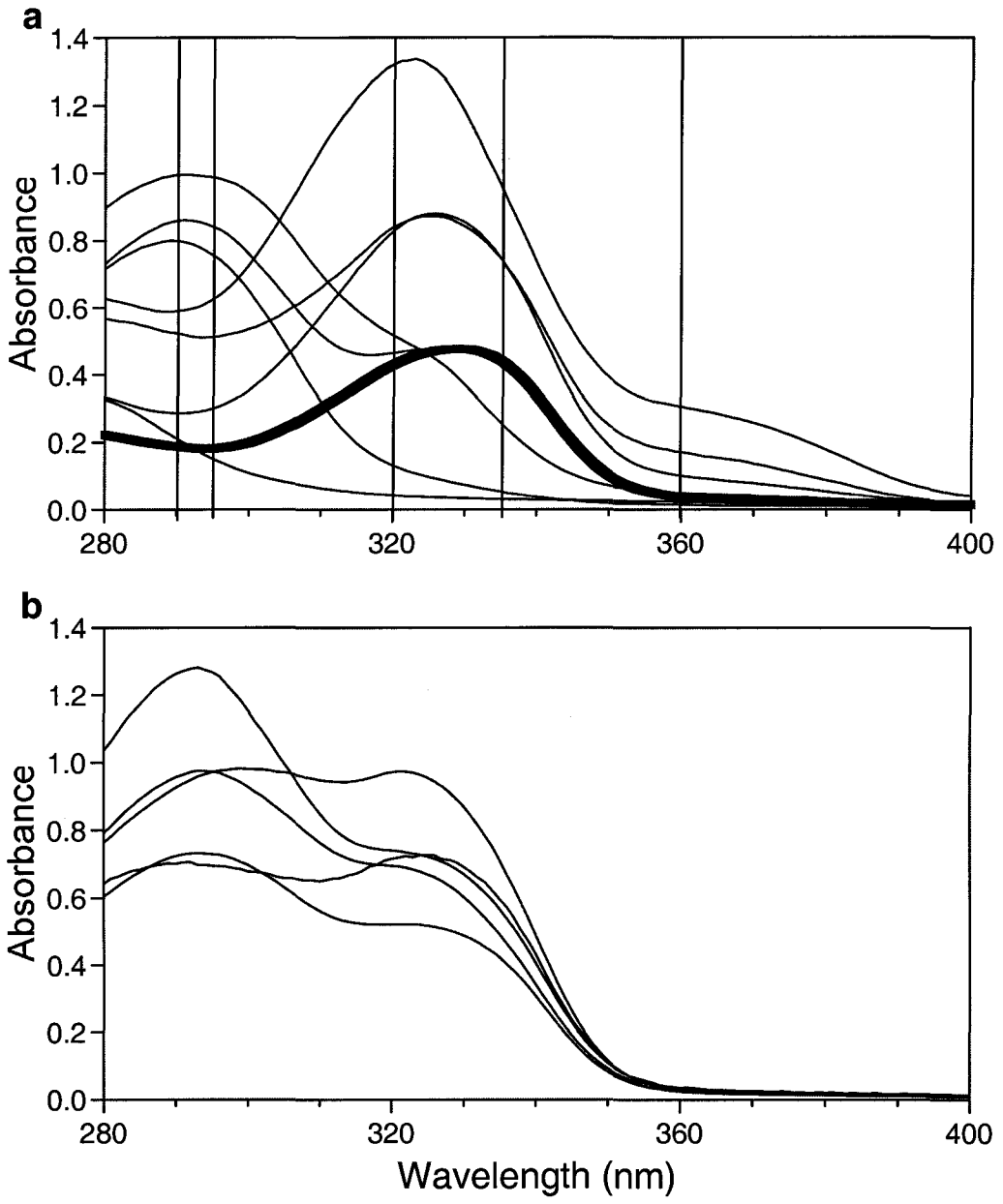

Figure 2.1. Absorbance spectra of fish mucus of several Hawaiian coral reef fish species. a- Examples of mucus absorbance spectra taken from the Hawaiian fish fauna. Each line represents a different species; the heavy line is *Canthigaster jactator*. Peak categories 1 (290-295 nm), 2 (320-335 nm), and 3 (~360 nm) are delineated by vertical lines. b- Absorbance spectra of *Thalassoma duperrey* caught at 1-3 m depth. Each line is a separate individual. The absorbance spectra indicate the presence of UV-absorbing compounds in categories 1 and 2.

A typical irradiation measurement taken just below the ocean's surface at the Hawai'i Institute of Marine Biology (Figure 2.2a) was used to estimate the total energy between 300 and 400 nm that would penetrate the mucus to strike the skin of a representative individual of each treatment group at the end of the experiment. As the tanks housing the fish were only 0.25m deep, attenuation by this layer of water would be minimal; therefore we considered this subsurface measurement a reasonable eestimate of the irradiance striking an experimental fish.

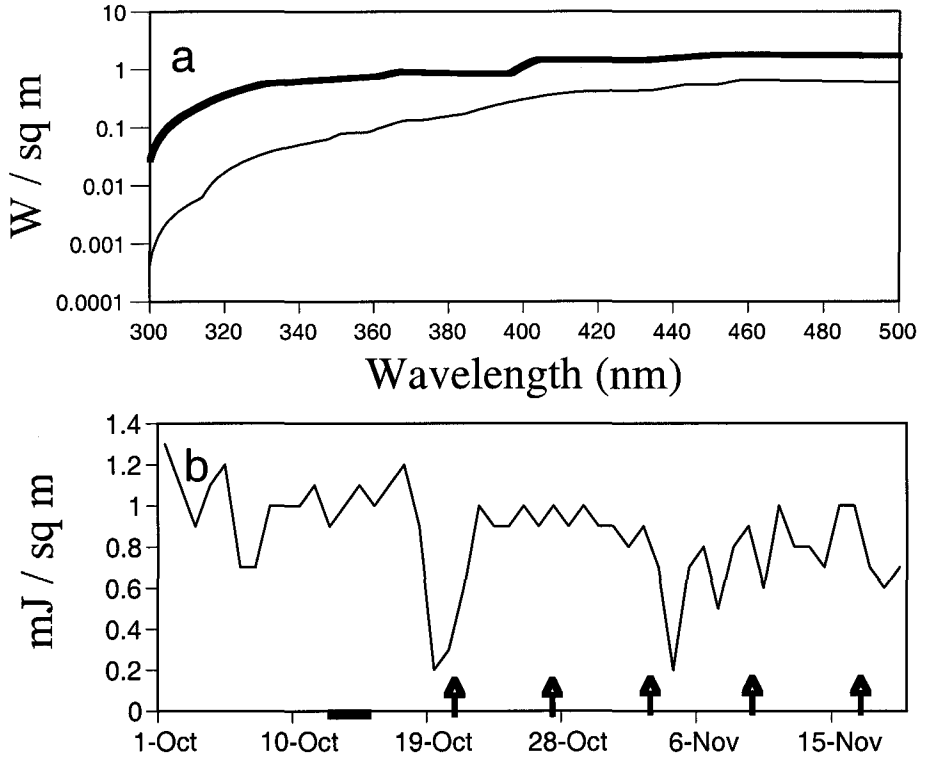

Figure 2.2. Irradiance measurements from Kaneohe Bay, Oahu, Hawaii. a- Irradiance measurements taken around midday on Coconut Island in Kaneohe Bay (thick solid line = solar irradiance just beneath the surface, thin solid line = irradiance at 3.5 meters depth). b- Average daily UV irradiance over the period of the study (solid bar on x-axis indicates the fish collection period and beginning of the experiment, bold arrows indicate the sampling dates for each week).

We utilized known underwater irradiance spectra from 3.5m depth (Figure 2.2a), ocular transmission (Losey et al. 2000), and visual pigment data (McFarland et al. unpubl.) in a well-established visual model (Vorobyev & Osorio 1998) to estimate whether the absorbance properties of mucus could be visually detected by *Dascyllus albisella*, a common Hawaiian damselfish. Our calculations slightly modified equation 6 in Vorobyev's model, briefly, we estimated the Weber fraction (a visual contrast estimate) while taking into account the fact that that the number of each photoreceptor type is unknown, and light in shallow water on a coral reef is assumed to be bright. All work was performed under IACUC Animal Care Protocol 95-012.

RESULTS

Epithelial mucus from 137 species (39 families) of tropical marine fishes was sampled, and 90% of the species were found to have strong absorbance peaks between 290 and 400 nm (Table 2.1). All samples absorbed light below 290 nm (Figure 2.1a). The absorbance spectra of most species' mucus (78%) contained more than one UV-absorbing peak.

Nine species of parrotfish (Scaridae) were sampled, and all possessed UV-blocking compounds in their mucus (Figure 2.3). Many species of parrotfish and wrasse (Labridae) sleep in mucous 'cocoons', secreted nightly, presumably to provide protection from parasites and deter nocturnal predators (Winn & Bardach 1956, Videler et al. 1999) . Samples from mucous cocoons of the same individuals sampled for skin mucus UV-blockers showed no discernable peaks of UV-absorbing compounds.

Table 2.1. Fish species sampled for mucus transmission/absorbance as of June, 2003. Species are listed roughly in phylogenetic order.

Family	Species	Number sampled
Carcharhinidae	*Carcharhinus melanopterus*	2
	Carcharhinus plumbeus	1
	Negaprion acutidens	1
Sphyrnidae	*Sphyrna lewini (tanned)*	1
Dasyatidae	*Dasyatus brevis*	1
	Taeniura lymma	1
Synodontidae	*Synodus variegatus*	1
Brotulidae	*Brotula multibarbata*	1
Antennariidae	*Histrio histrio*	1
Hemiramphidae	*Hyporamphus acutus*	1
Holocentridae	*Neoniphon sammara*	1
	Sargocentron spiniferem	1
Aulostomidae	*Aulostomus chinensis*	1
Scorpaenidae	*Scorpaenodes parvipinnis*	1
	Scorpaenopsis brevifrons	1
	Sebastipistes coniorta	1
Platycephalidae	*Thysanophrys otaitensis*	1
Serranidae	*Cephalopholis argus*	1
	Diploprion bifasciatum	1
	Diploprion bifasciatum	1
	Liopropoma colletti	1
	Pseudanthias squamipinnis	1
	Pseudanthias tuka	1
	Pseudogramma polyacantha	1
	Plectropomus leopardus	1
Pseudochromidae	*Congrogadus subducens*	1
	Pseudochromus fuscus	1
	Pseudochromis pacagnellae	1
Kuhliidae	*Kuhlia sandvicensis*	1
Priacanthidae	*Priacanthus meeki*	1
Apogonidae	*Apogon compressus*	1
	Apogon fragilis	1
	Apogon kallopterus	1
	Apogon leptacanthus	1
	Cheilodipterus quinquelineatus	1
	Foa brachygramma	1
Echeneidae	*Echeneis naucrates*	1
Rachycentridae	*Rachycentron canadum*	1
Carangidae	*Caranx sexfasciatus*	1
	Gnathonodon speciosis	1
	Trachinotus botla	1

Table 2.1. (Continued) Fish Species Sampled as of June, 2003

Family	Species	Number sampled
Lutjanidae	*Lutjanus bohar*	1
	Lutjanus carponotatus	3
Caesionidae	*Caesio teres*	1
Haemulidae	*Diagramma pictum*	1
Lethrinidae	*Gymnocranius audleyi*	1
	Lethrinus miniatus	1
	Lethrinus nebulosus	1
Nemipteridae	*Scolopsis margaritifer*	1
	Scolopsis bilineatus	1
Mullidae	*Mulloidichthys flavolineatus*	2
	Parupeneus cyclostomus	1
	Parupeneus multifasciatus	1
Chaetodontidae	*Chaetodon aureofasciatus*	1
	Chaetodon auriga	3
	Chaetodon baronessa	1
	Chaetodon ephippium	1
	Chaetodon kleinii	1
	Chaetodon melannotus	2
	Chaetodon miliaris	12
	Chaetodon plebeius	1
	Chaetodon rainfordi	1
	Chaetodon trifasciatus	2
	Chaetodon ulietensis	1
	Chaetodon unimaculatus	1
	Chelmon rostratus	1
	Forcipiger flavissimus	1
Pomacanthidae	*Centropyge bicolor*	1
Pomacentridae	*Abudefduf abdominalis*	1
	Abudefduf whitleyi	1
	Acanthochromis polyacanthus	1
	Amblyglyphididon curacao	1
	Chromis ovalis	1
	Chromis ternatensis	2
	Chromis xanthura	1
	Chrysiptera cyanea	1
	Dascyllus albisella	6
	Dascyllus aruanus	4
	Dascyllus reticulatus	4
	Dischistodus perspicillatus	1
	Dischistodus prosopotaenia	3
	Neoglyphididon melas	1

Table 2.1. (Continued) Fish Species Sampled as of June, 2003

Family	Species	Number sampled
Pomacentridae	*Neopomacentrus azysron*	1
	Pomacentrus amboensis	1
	Pomacentrus chrysurus	1
	Pomacentrus moluccensis	1
Cirrhitidae:	*Paracirrhites arcatus*	1
Sphyraenidae	*Sphyraena flavicauda*	1
Labridae:	*Anampses neoguinaicus*	2
	Bodianus axillarus	1
	Choerodon cyanodus	1
	Choerodon fasciatus	2
	Choerodon venustus	1
	Cirrhilabrus punctatus	2
	Coris aygula	1
	Coris batuensis	2
	Coris venusta female	2
	Epibulus insidiator female	2
	Gomphosus varius female	3
	Halichoeres chloropterus	1
	Halichoeres margaritaceus	2
	Halichoeres melanurus	2
	Halichoeres prosopeion	1
	Hemigymnus fasciatus	1
	Hemigymnus melapterus	4
	Labrichthys unilineatus	5
	Labropsis australis	2
	Macropharyngodon choati	1
	Macropharyngodon geoffroy	1
	Novaculichthys taeniorus	1
	Oxycheilinus digrammus	3
	Pseudocheilinus octotaenia	2
	Pseudocheilinus tetrataenia	1
	Pseudojuloides cerasina	1
	Stethojulis bandanensis	1
	Stethojulis balteata	1
	Stethojulis strigiventer fem	2
	Thalassoma duperrey	52
	Thalassoma lunare	6
	Thalassoma lutescens	1
Scaridae	*Calotomous carolinus*	1
	Chlorurus frontalis	1

Table 2.1. (Continued) Fish Species Sampled as of June, 2003

Family	Species	Number sampled
Scaridae	*Chlorurus sordidus*	6
	Scarus frenatus	1
	Scarus ghobban	1
	Scarus globiceps	1
	Scarus longipinnis	1
	Scarus niger	1
	Scarus psittacus	1
	Scarus rivulatus	1
Blenniidae	*Blenniella paula*	1
	Istiblennius edentulus	1
	Istiblennius zebra	1
	Omobranchus rotundiceps	4
	Salarius fasciatus	2
Gobiidae	*Callogobius sclateri*	1
Acanthuridae	*Acanthurus achilles*	1
	Acanthurus blochii	1
	Acanthurus nigrofuscus	1
	Acanthurus xanthopterus	1
	Zebrasoma veliferum	1
Siganidae	*Siganus doliatus*	1
	Siganus punctatissimum	1
Balistidae	*Balistoides conspicillum*	1
	Sufflamen bursa	1
	Sufflamen chrysopterus	1
Monacanthidae	*Paraluteres prionurus*	1
Tetraodontidae	*Arothron hispidus*	1
	Arothron meleagris	1
	Canthigaster coronata	1
	Canthigaster jactator	15
	Canthigaster papua	1

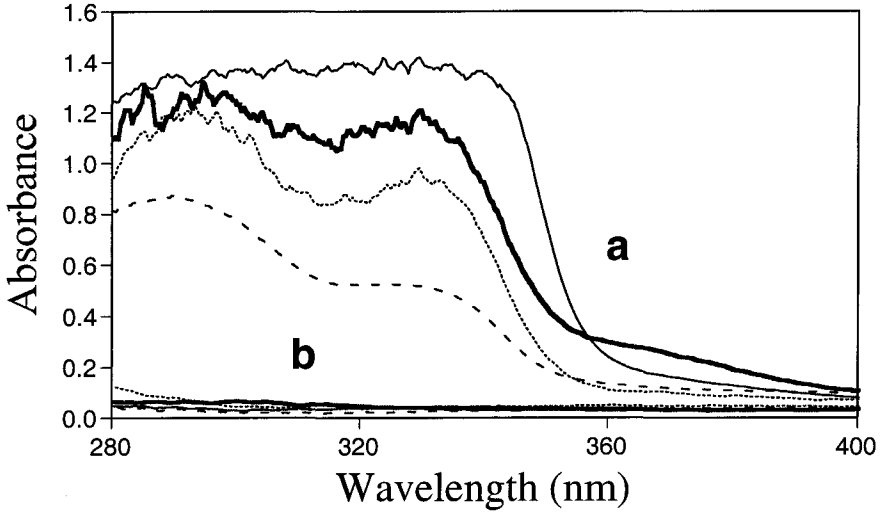

Figure 2.3. Absorbance spectra of four species of Australian parrotfish mucus. Thick solid line = *Scarus globiceps*, thin solid line = *Chlorurus sordidus*, dashed line = *Scarus niger*, dotted line = *Scarus psittacus* (a = skin mucus sampled in the daytime, showing considerable absorbance in categories 1 and 2, b = nocturnal 'cocoon' mucus from the same individuals, showing no discernable UV-absorbing peaks).

Deep-caught *T. duperrey* had significantly shorter wavelength mucus T_{50} cutoffs than shallow-caught fish (Figure 2.4b, 2-sample T-test: T=3.32(16), p<0.01).

The change in T_{50} cutoffs of *T. duperrey* mucus differed significantly between experimental treatments (Figure 2.4a, Repeated measures ANOVA, F(1,10)=10.35, p=0.009). The change in T_{50} values within both groups was also significantly different between weeks, (Repeated measures ANOVA, F(4,32)=6.25, p=0.0008). T_{50} values between treatment groups were not significantly different at the start of the experiment (2-tailed t-test, T=0.34(13), p=0.74). The change in ten-percent mucus cutoffs (T_{10}) also differed significantly between UV treatments (Repeated measures ANOVA; F(1,15)=26.7, p=0.0001) and by week (Repeated measures ANOVA; F(4,41) = 6.15, p=0.0006). Additionally, T_{10} cutoff values showed a significant

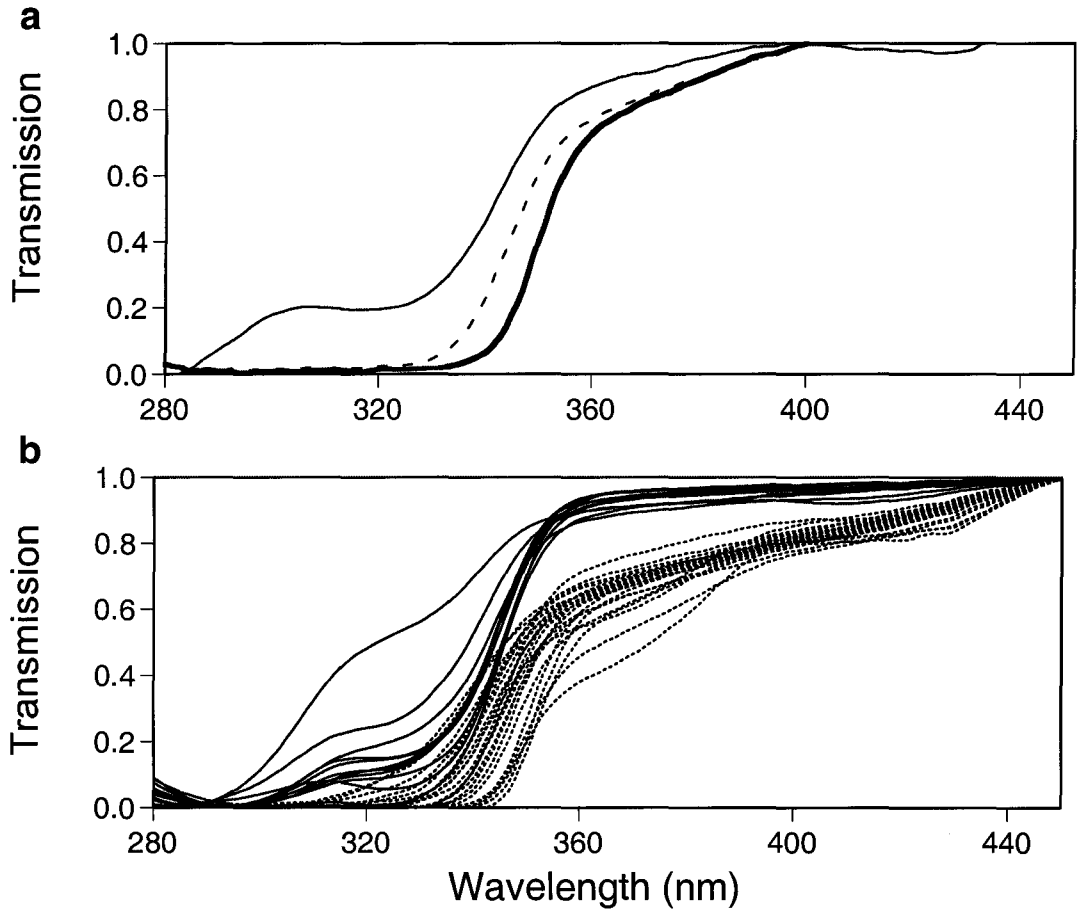

Figure 2.4. Mucus transmission of experimental and wild-caught *Thalassoma duperrey*. a - Typical mucus transmission curves of experimental *Thalassoma duperrey* (thick solid line = mucus from a fish subjected to UV-transmitting experimental conditions for five weeks, thin solid line = mucus from an individual living under experimental UV-opaque conditions for five weeks). Dashed line = wild-caught individual. b- Mucus transmission curves from *T. duperrey* from different depths (thin solid lines = fish caught from 14-16 m depths, dotted lines = fish caught at 1-3 m depths) .

difference between individual fish, nested within treatment (Repeated measures ANOVA, $F(14,12)=3.17$, p 0.03). Due to the shapes of transmission curves, the T_{10} value may be a more sensitive measure of change than the T_{50} value (Figures 2.4a,b).

Both groups showed a net decrease in transmission cutoff values over the course of the experiment (Table 2.2). Some fish involved in this study acquired lesions consistent with epithelial 'sunburn' damage (Bullock 1982) in the UVT treatment.

Table 2.2. Average cumulative change in T_{50} cutoff value for each treatment. The initial T_{50} value, in nanometers, was subtracted from the value for each week for each fish, and then averaged over the treatment group. Values are mean +/- standard error (nm).

Treatment	Week 1	Week 2	Week 3	Week 4	Week 5
UVT	-1.9+/-0.85	-3.8+/-1.2	-5.4+/-2.2	-3.9+/-1.1	-2+/-1.1
UVO	-2.8+/-1.0	-9.9+/-1.6	-8.4+/-1.7	-5.1+/-2.0	-5.3+/-1.0

Total daily UV irradiation over the 37 days of the experiment varied between .23 and 1.18 MJ/m^2, averaging .81 +/- .04 MJ/m^2 (x +/- SE) (Figure 2.2b). A typical sub-surface mid-day irradiance measure (Figure 2.2a) was used to calculate the total UV (300-400 nm) irradiance as 62.9 W/m^2. After mucus adaptations, at the end of the experiment, total UV irradiation penetrating the mucus layer of a fish (under full solar irradiance and in shallow water) would be 37.1 W/m^2 for UVT-treatment fish, and 47.0 W/m^2 for UVO-treatment fish, assuming a 0.25 mm layer of mucus. The thickness of the mucus layer on the outside of a fish has been surprisingly difficult to determine (Shephard 1994), however we believe that 0.25 mm is a reasonable thickness estimate for a relatively mucus-rich fish such as *T. duperrey*. No difference in mucus production between treatments was evident. The lenses and corneas of *T. duperrey* contain UV-absorbing compounds (T_{50} = 427nm), and no difference in transmission of these ocular media was found at the end of the study (2-tailed T-test: T=1.09(8), p=0.31).

The appearance of several species, as visualized by the UV camera, was strikingly different at short (340-360 nm) wavelengths. For example, the absorbance properties of the mucus of *Canthigaster jactator*, the Hawaiian white-spotted toby (Figures 2.1a, 2.5a), render the fish completely 'black' when seen through the 340 +/- 5nm half-band width filter (Figure 2.5b), only to have their UV-reflective spots 'reappear' when the mucus is removed by sampling (Figure 2.5c). It took the animal approximately 24 hours to replace the mucus, such that the spots once again 'disappeared' (Figure 2.5b).

Visual contrast as estimated by the Weber fraction from our visual model (see Methods) between UVT and UVO treatment *T. duperrey* at a depth of 3.5 meters is 0.18 for an adult *D. albisella* observer and 0.40 for a juvenile whose ocular media have higher transmission at short wavelengths (Losey et al. 2000).

DISCUSSION

Most species' mucus contained more than one UV-absorbing peak; these fishes are likely combining several compounds to create an effective broad-band ultraviolet filter (Cockell & Knowland 1999). Absorbance peaks between 310-360 nm resulting from MAAs have been documented from fish lenses, including 13 of the species sampled in this study (Dunlap et al. 1989). Of the four MAAs commonly found in fish lenses, palythine (λ_{max} 320nm), asterina-330 (λ_{max} 330nm), and palythinol (λ_{max} 332nm) would fall into category 2, and palythene (λ_{max} 360nm) into category 3. The UV-absorbing compound(s) in category 1 may be related to gadusol (λ_{max} 294nm), a

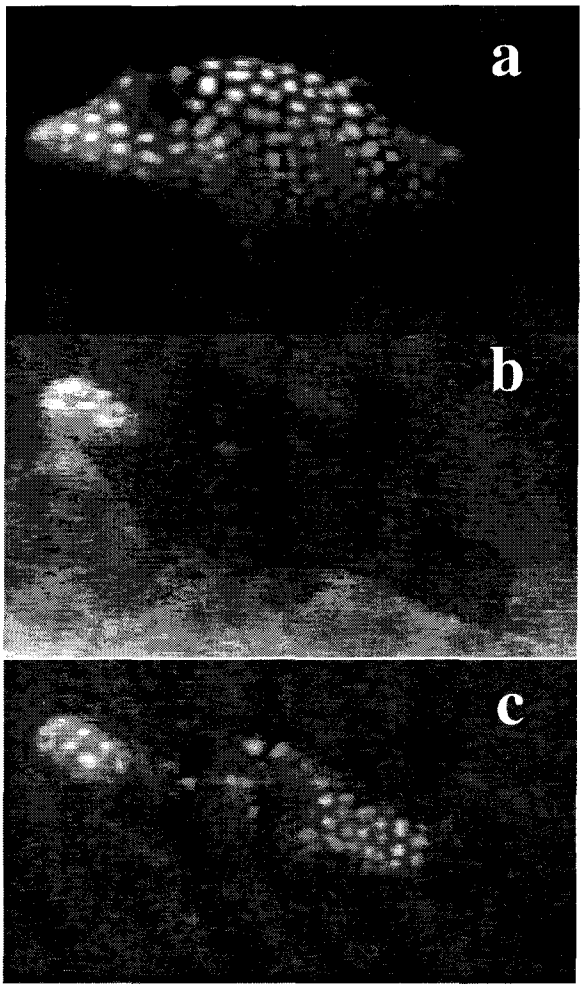

Figure 2.5. *Canthigaster jactator*, the Hawaiian white-spotted toby, as seen through a UV-sensitive video camera. a - A frame from video taken through a 360-nm (+/-5 nm half-bandwidth) optical 'notch' filter. b - The same individual as seen through a 340-nm (+/- 5 nm half-bandwidth) optical 'notch' filter. c - The same individual, seen as in b, after sampling of mucus for spectrophotometric analysis. The pectoral fin, which was not sampled, is visible as a dark blotch on the animal's side.

compound similar to MAAs that has been found in fish roe (Plack et al. 1981). Category 1 compound(s) may also have some relation to an unidentified compound (λ_{max} 292nm) extracted from the skin of a few freshwater fish species (Fabacher & Little 1995). The fishes presumably acquire these compounds from dietary sources, as does the medaka fish (Mason et al. 1998). The diet for fish in both

treatments in our experiment was squid. This indicates that the fish were able to differentially sequester UV-blocking compounds from their food source, and were not dependent on passive uptake according to dietary availability. The absorbance of all mucus below 290 nm is likely caused by structural components of the mucus, such as nucleic acids and proteins (Douglas & Marshall 1999).

Parrotfish sequester UV-absorbing compounds only in skin mucus, that could serve a UV-protective function. Mucous cocoons are secreted each evening by goblet cells in a folded epithelium in the opercular gland in the gill cavity (Videler et al. 1999), whereas skin mucus is secreted primarily by external epithelial goblet cells (Shephard 1994). Both types of mucus possess biochemical antibiotic compounds (Shephard 1994, Videler et al. 1999), but only epithelial mucus possesses UV-absorbing compounds (Figure 2.3). This may indicate that UV blocking compounds are energetically costly to sequester, in limited supply, or both. The cells involved in mucus production may differ in their ability to sequester UV-blockers.

Deeper-caught *T. duperrey* mucus T_{50} cutoff wavelength values were significantly shorter than those of shallower caught fish (Figure 2.4b). The fact that the mucus from deep-caught fish blocked less of the UV portion of the spectrum than shallow-caught individuals is likely due to the smaller amounts of UV radiation at depth resulting in less need for photo-protection. The significant difference shown in UV-blocking between treatments over time (Figure 2.4a) indicates that *T. duperrey* is able to respond to ambient UV irradiation conditions. The fish may be able to manipulate the proportions of the UV-blocking compounds found in their mucus (Figure 2.1b), as has been shown to occur in dinoflagellates within hours of a change

in light intensity (Carreto et al. 1990). The decrease in mucus T_{50} and T_{10} values for both groups over the weeks of the experiment (Table 2.2) may have been in response to considerable fluctuations in ambient solar radiation in this time frame (Figure 2.2b). It may also have been due to stress of capture, or perhaps insufficient concentrations or proportions of UV-blocking compounds in the diet provided.

Sunburn of fishes is a well-established problem for aquaculture projects worldwide (Bullock & Coutts 1985, Bullock 1988). As global ozone depletion continues to allow greater amounts of UV to strike the earth's surface at arctic, temperate and tropical latitudes (Stolarski et al. 1992), fish sunburn may also become a problem for tidepool nursery areas and commercially important near-surface open ocean fishes. The rapid loss of UV-protective compounds demonstrated by the UVO-treatment fishes in this study suggests that there is a cost associated with sequestering these compounds that must outweigh their benefits in the absence of UV radiation. In a pilot study similar to the present study but performed under significantly higher irradiation conditions (P. Nelson, unpubl.), differential mortality of UVT-treatment *Thalassoma duperrey* occurred. These organisms may be near the limits of their tolerance to UV exposure.

We determined that *Dascyllus albisella* should be able to discern the difference between UVT-treatment and UVO-treatment *T. duperrey* at a depth of 3.5 meters. Each of the calculated Weber values is at least one order of magnitude larger than published values of visual contrast threshold for fishes (Douglas & Hawryshyn 1990). This suggests that *D. albisella* and other fish with UV-sensitive vision may be able to visually detect differing levels of UV absorbing compounds in the mucus of

other fishes, i.e., one fish may see another as 'tinted' vs. 'untinted'. Numerous implications for fish biology and behavior arise from this suggestion. In terms of visual communication, mucus may indicate an individual's recent UV exposure, or may be used as a measure of fitness (e.g., a measure of the ability of an individual to gain food sources rich in UV-blockers). Minor wounds or other mucus disturbances may also be easily visible to a UV-sensitive species, as they are to our UV-sensitive camera system (Figure 2.5), and serve as a fitness indicator.

The skin of fishes is more delicate than that of higher animals (Bullock 1988), but it is far from defenseless. Fish mucus provides a formidable suite of defensive mechanisms, including antibiotics, resistance to abrasion and protection from heavy metals (Shephard 1994). With this study, we add blocking of damaging UV radiation, as well as a possible role in visual communication, to the list.

CHAPTER 3:

EFFECTS OF DIET, UV EXPOSURE AND GENDER

Effects of diet, ultraviolet exposure and gender on the ultraviolet absorbance of fish mucus and ocular media

Manuscript

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ABSTRACT

Ultraviolet radiation can be damaging to fish skin and ocular components. Coral reef fishes are regularly exposed to potentially harmful radiation. It was recently discovered that tropical marine fishes possess UV absorbing compounds in their mucus. This experiment demonstrates significant independent effects of both diet and ultraviolet exposure on the UV-absorbing compounds in the mucus of a tropical wrasse, *Thalassoma duperrey*. Fish that are exposed to UV radiation increase the UV absorbance of their mucus only if UV-absorbing compounds are provided in their diet. Fish that are protected from UV radiation decrease the UV absorbance of their mucus regardless of diet. Mucus from female *T. duperrey* absorbed less UV and females had higher rates of skin damage than males. Females may sequester UV-absorbing compounds for their pelagic eggs as well as their epithelial mucus. Spectral transmission through the whole eye was not affected by diet or UV manipulations, but corneal tissue transmission showed a significant effect of UV exposure. These results demonstrate that coral reef fish can adapt to environmental irradiance conditions, so long as UV absorbing compounds are available in the diet.

INTRODUCTION

Coral reef systems are exposed to extremely high and variable levels of ultraviolet radiation on a daily basis (Stolarski et al. 1992; Madronich et al. 1998). For example, on any summer day, organisms living in the shallow waters of the Great Barrier Reef may experience more than 30 times the minimum dose capable of causing human sunburn (Dunlap et al. 2000). The damaging biological effects of

ultraviolet radiation on organisms in the marine environment occur even at tens of meters in depth (Karentz and Lutze 1990; Lyons et al. 1998; Buma et al. 2001). Both UVA (320-400 nm) and UVB (290-320 nm) radiation have significant effects on fish egg and larval survival, length at hatching, DNA damage, and oxidative stress levels (Vetter et al. 1999; Lesser et al. 2001; Zagarese and Williamson 2001). Adult fishes may be more resistant to UV-induced damage than their small, transparent larvae, but they are nonetheless susceptible to DNA damage (Ahmed and Setlow 1993), cataracts and corneal damage (Cullen and Monteith-McMaster 1993; Cullen et al. 1994),'sunburn' of the skin (Bullock et al. 1983; Ramos et al. 1994) and subsequent infections (Roberts 1989; Zagarese and Williamson 2001).

Marine organisms employ two common strategies for avoiding UV damage. Some avoid UV exposure (e.g., living under rocks, in caves, in an opaque shell/test or at great depths), and some synthesize or sequester UV-absorbing "sunscreens" (Jokiel 1980; Cockell and Knowland 1999; Adams 2001). These two strategies are not necessarily mutually exclusive. In marine environments, UV-absorbing mycosporine-like amino acids (MAAs) appear to be ubiquitous, occurring in organisms from dinoflagellates and algae to corals and fishes (Carreto et al. 1990; Banaszak et al. 1998; Dunlap and Shick 1998). MAA concentrations have been shown to positively correlate with UV exposure in organisms such as corals and diatoms (e.g., Dunlap et al. 1986; Shick et al. 1991; Shick et al. 1995; Helbling et al. 1996). Mycosporine-like amino acids apparently cannot be produced by animals (Shick et al. 2000), and have been shown to be accumulated via trophic transfer in marine invertebrates and a freshwater fish (Carroll and Shick 1996; Mason et al. 1998; Carefoot et al. 2000;

Newman et al. 2000; Whitehead et al. 2001). To my knowledge, simultaneous effects of diet and UV exposure on MAA concentrations have not been demonstrated in any animal.

Tropical marine fishes commonly possess four MAAs in their ocular media: palythine, palythene, palythinol, and asterina-330 (Dunlap et al. 1989; Thorpe et al. 1993). Mason et al. (1998) showed that the medaka fish selectively accumulated palythine and asterina-330 from a diet containing these compounds in trace amounts. Shinorine, the predominant MAA in the diet, was not sequestered by the fish (Mason et al. 1998). It was recently demonstrated that the epithelial mucus of tropical marine fishes have absorbance spectra that are consistent with the four MAAs found in the lenses of tropical fishes (Zamzow and Losey 2002), but the origin of these mucus compounds remains to be determined.

This study will test two hypotheses regarding the UV-absorbing compounds in the mucus of coral reef fishes. First, I hypothesize that the UV-absorbing compounds in the mucus of coral reef fishes is derived from the diet ("dietary origin hypothesis"). Second, I hypothesize that fishes are able to change the amount of sunscreen in the mucus in response to variations in UV radiation ("variable sunscreen hypothesis").

MATERIALS AND METHODS

The effects of both diet and UV exposure on the UV absorbance of fish epithelial mucus were tested simultaneously with a two-factor design. Fish were either exposed to (UV+) or protected from (UV-) UV radiation, and half the fish in each UV treatment were provided a diet rich in MAAs (MAA+), while half received a

nutritionally complete, but MAA-free diet (MAA-). My hypotheses (see Introduction) lead me to several predictions of the experimental outcome. Following the dietary-origin hypothesis, I predict that that both groups of MAA- fish will not be able to sequester sunscreen in their mucus. The variable sunscreen hypothesis leads me to predict that mucus from UV+ MAA+ fish will absorb the greatest amount of UV radiation, and I expect that the UV absorbance of the mucus from the UV-MAA+ group will be intermediate between the UV+MAA+ and the two MAA- groups. In addition, if the dietary origin hypothesis is correct and UV+MAA- fish are unable to increase the UV absorbance of their mucus, I expect them to suffer higher rates of skin damage or mortality than the three other groups.

Thirty-two Hawaiian saddleback wrasse, *Thalassoma duperrey*, were collected with hook and line from 1-3 m depths in Kaneohe Bay, and transported to the Hawaii Institute of Marine Biology (HIMB). Eight 1 x 1 x 0.25 m deep fiberglass outdoor tanks at HIMB were equipped with acrylic covers that either transmitted (50% transmission cutoff [T_{50}] = 293 nm) or blocked (T_{50} = 408 nm) UV radiation (Figure 3.1). Fish were randomly assigned to treatments while controlling for size, housed four to a tank, and fed one of the experimental diets daily. Fish in each tank differed from one another by at least 1 cm in length, and could be individually identified. Tanks were cleaned at least every other day in order to eliminate fouling organisms as a possible food source. Tanks were located adjacent to an Eppley Total Ultraviolet Radiometer, which monitored UV irradiance and recorded hourly and daily UV dosages (mJ/m^2) for the duration of the experiment (Figure 3.2).

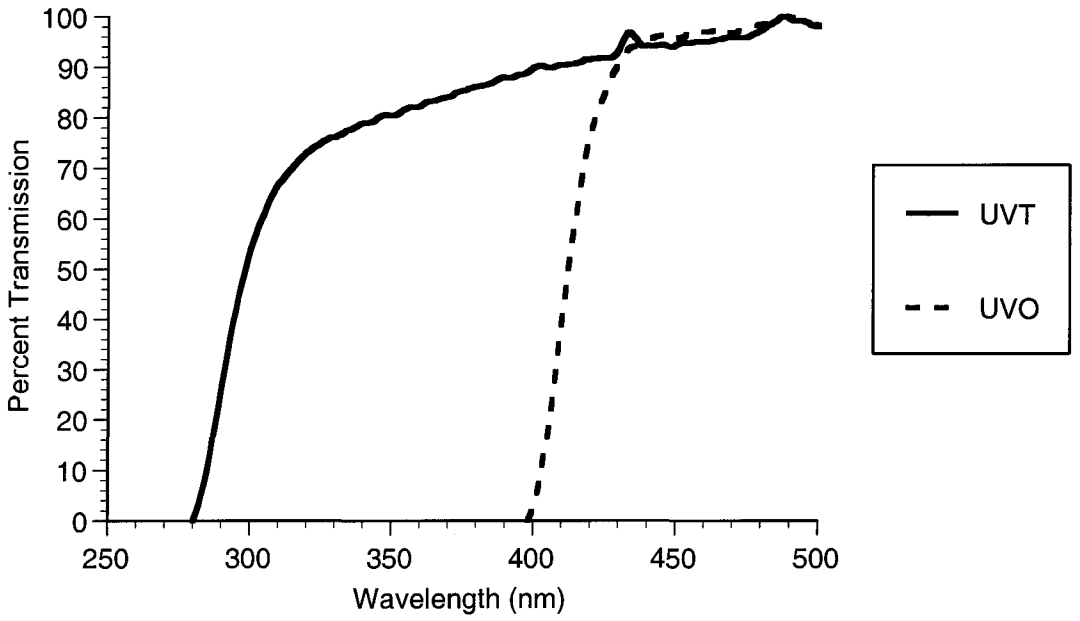

Figure 3.1. Transmission spectra of acrylics used to create experimental UV treatment. UVO = UV-opaque acrylic (UV- treatment), UVT = UV-transparent acrylic (UV+ treatment).

The experimental diets consisted of a nutritionally complete, MAA-free purified-casein based diet (MAA-, (DeKoven et al. 1992; Mason et al. 1998), or purified casein diet plus *Acanthophora spicifera* collected from 0.5 m depth (MAA+). Freeze-dried, ground *A. spicifera* was substituted for 10% (by weight) of the dry ingredients of the diet. *Acanthophora spicifera* is an extremely abundant Kaneohe Bay alga that is highly preferred by herbivorous fishes in preference tests (Stimson et al. 2001). An earlier study in Kaneohe Bay showed that this alga contained 12.5 nanomoles/mg protein palythine, 14.64 nanomoles/mg protein asterina 330, and 3.91 nanomoles/mg protein palythinol as well as mycosporine-glycine, shinorine, porphyra-334, and an unknown pigment (Banaszak and Lesser 1995). A methanol extract of Caribbean *A spicifera* showed a sizable unidentified absorption peak at

~290 nm, and HPLC analyses of the alga demonstrated the presence of at least six MAAs (Carefoot et al. 1998).

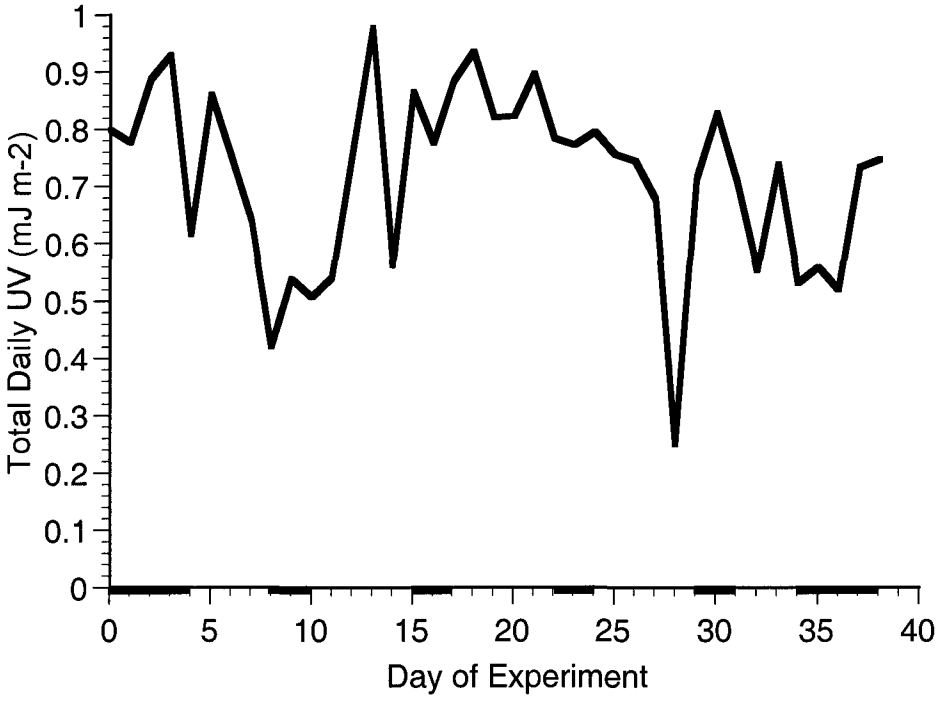

Figure 3.2: Total daily UV doses (mJ/m^2) over the course of the experiment, as recorded by the Eppley Cell Total Ultraviolet Radiometer. Thickened abscissa indicates sampling periods.

Mucus was sampled from each fish on the day of capture, and once per week for 5 weeks thereafter, following Zamzow and Losey (2002). Epithelial mucus was collected by gently scraping a dull scalpel blade along the dorsal flank of live fishes, anterior to posterior. Samples were squashed to 0.25 mm thickness between two ultraviolet-transparent (T_{50} = 266 nm) slides. Each week, prior to sampling mucus, a "damage score" was tabulated for each fish, with one "point" assigned for each of the following impairments: scale or patch of scales missing, erosion of the dorsal or

caudal fin, erosion of the upper lip, eye infection, or visibly pale appearance. Any fish that died was assigned a damage score of 5. At the conclusion of the study, fish were euthanized by overdose of tricaine methanesulphonate and sexed. One eye was excised, and ocular transmission measured. Following Losey et al. (2000), a small window was cut in the back of the eye just through the retina, and the eye was placed on a UV-transparent slide. Light transmission through the whole eye, isolated lens, and isolated cornea was measured. Corneal measures were taken through the central portion of the cornea while avoiding patches of carotenoid pigments that produced a distinctive series of absorbance maxima from 400-500 nm (Orlov and Gamburtzeva 1976; Siebeck and Marshall 2000). Carotenoid pigments were avoided as this study was designed to investigate shorter-wavelength UV-absorbing pigments.

Light absorbance of the mucus 'squash' and transmission of the ocular components was measured with an Ocean Optics S-2000 spectrophotometer with illumination provided by a Deuterium-Tungsten source (Losey et al. 2000, Zamzow and Losey 2002). Light was shone through a UV-transmitting fiber optic probe, through the sample, into a UV-transmitting collimating lens, through another fiber optic cable and into the spectrophotometer. Eight measurements taken through different points in each mucous sample were averaged in order to compensate for possible heterogeneity of the sample.

Reflectance spectra of the dorsal skin just behind the head were taken at the beginning and the end of the experiment. For this measurement, the S-2000 was fitted with a bifurcated fiber optic reflectance probe kept at 45 degrees to the skin by a custom holder. Reflectance measurements were taken relative to a Spectralon

standard. The difference between initial and final reflectance for each fish was calculated by integrating the area under each spectrum and subtracting the initial measurement from the final measurement.

Two measures were calculated from each mucus absorbance spectrum: integrated absorbance and center of absorbance. Integrated absorbance was calculated by adding the absorbance values (units = optical density, O. D.) for each wavelength from 280-400 nm, thereby approximating the area under the curve. Center of absorbance was calculated by multiplying the absorbance value (O. D.) at each wavelength by its corresponding wavelength (nm), then dividing by the integrated absorbance (O. D.). This yielded a wavelength value indicative of the relative contributions of the peak(s) and shoulder(s) of the absorbance curve (Figure 3.3).

These measures were averaged over the eight replicate spectra from each mucus sample. The initial measure at time zero was subtracted from each subsequent measure, and analyses were performed on the resultant variables: change in integrated absorbance, and change in center of absorbance. Figure 3.4a shows change in integrated absorbance as a proportion of initial absorbance. Each of the above absorbance measures from the weekly mucus samples, as well as the damage score data (as change from initial damage score), was used as the dependent variable in a repeated measures linear model. Factors in the analysis were UV treatment, diet treatment, gender, fish standard length, and interactions (see tables for individual analyses).

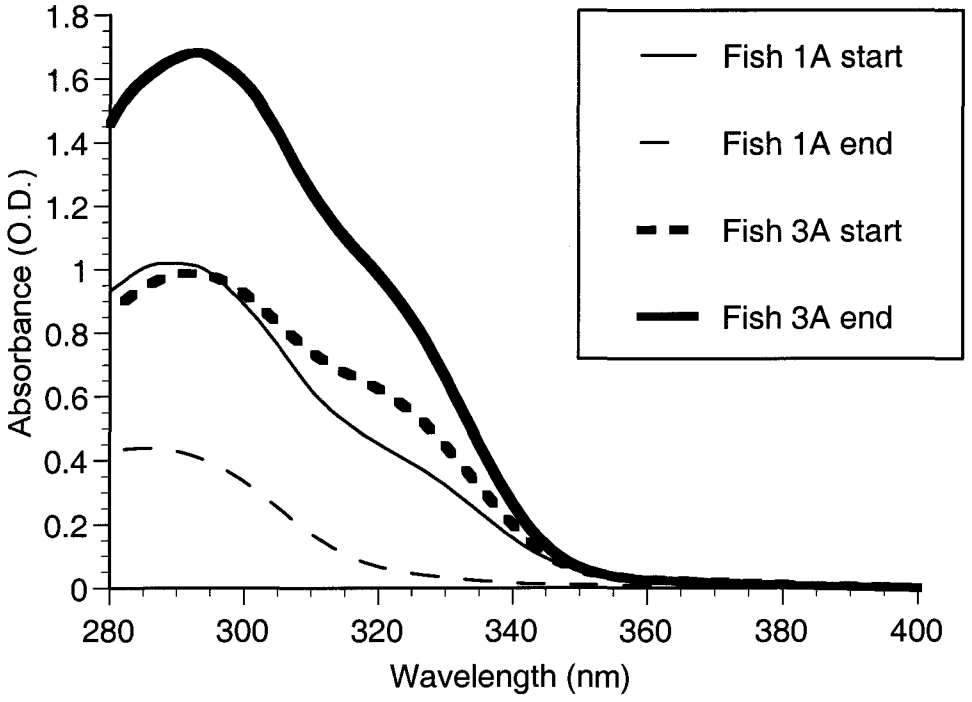

Figure 3.3: Representative mucus absorbance spectra for two experimental fish before and after the experiment. Fish 1A was in the UV- MAA- treatment, and Fish 3A was in the UV+ MAA+ treatment.

"Tank effects" were eliminated as significant factors ($p>>0.05$) before performing the final repeated-measures analysis. Estimation and hypothesis testing in these models used likelihood-based methods, and an autoregressive covariance structure was assumed, with Satterthwaite determination of degrees of freedom (PROC MIXED, SAS systems, version 8.2). Both integrated absorbance and center of absorbance were also tested for an effect of gender by t-test at the beginning and end of the experiment (initial and final mucus samples).

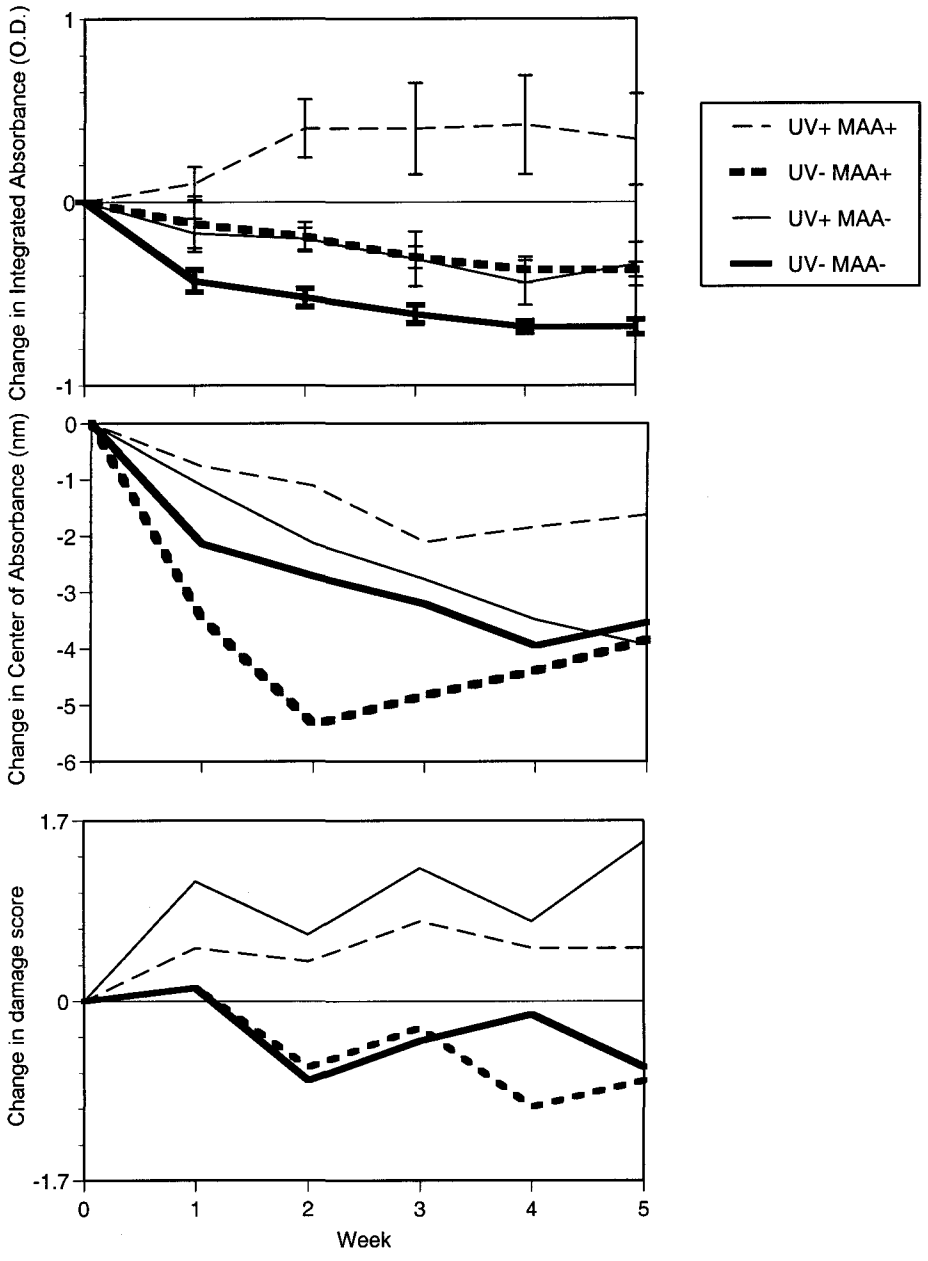

Figure 3.4: Changes shown by experimental fish over the course of the experiment. a) change in integrated absorbance as a proportion of initial measurement. Mean +/- standard error. b) change in center of absorbance as a proportion of initial measurement. Error bars are highly overlapping and have been omitted for clarity. c) change in damage score. Error bars are highly overlapping and have been omitted for clarity. UV+ = exposed to UV, UV- = protected from UV, MAA+ = diet with UV-absorbing compounds, MAA- = diet without UV-absorbing compounds.

Whole eye, lens, and cornea T_{50} values were analyzed separately by general linear model (GLM) ANOVAs. The average T_{50} value of each component was used as the dependent variable and tested for effects of UV treatment, diet treatment, gender, fish standard length and interactions (SAS systems, version 8.2). Skin reflectance data were similarly analyzed by GLM ANOVA for effects of UV treatment, diet treatment, gender, fish standard length, and interactions.

RESULTS

The repeated measures linear model of change in integrated absorbance showed significant effects of diet treatment, ultraviolet treatment, and time (Table 3.1; Figure 3.4a). Neither fish length nor gender had a significant effect, and there were no significant interactions. The change in integrated mucus absorbance for

Table 3.1: Effects of UV treatment, diet treatment, and time on the change in integrated UV absorbance (O.D.) of *Thalassoma duperrey* mucus. Repeated-measures linear model results, Type III tests

Effect	DF	F value	P value
UV treatment	1,24	6.1	0.02
Diet treatment	1,24	8.1	<0.01
Gender	1,24	0.16	0.70
Fish length	1,23	0.57	0.46
Week	4,89	2.53	<0.05
UV * Diet	1,24	1.34	0.26
UV * Gender	1,24	0.39	0.54
Diet * Gender	1,24	0.37	0.55
UV * Week	4,89	1.24	0.30
Diet * Week	4,89	0.80	0.53
Gender * Week	4,89	1.06	0.38
UV * Diet * Gender	1,23	2.02	0.17
UV * Diet * Week	4,89	1.28	0.29
UV * Gender * Week	4,89	0.91	0.46
Diet * Gender * Week	4,89	1.18	0.32

MAA+ fish (averaged over UV treatments and time) was –1.72 +/- 3.0 O. D. (mean+/- s.e.) as opposed to –13.5 +/- 2.8 for MAA- fish. The change in integrated mucus absorbance for UV+ fish (averaged over diet and time) was –2.6 +/- 2.9 O. D., and UV- fish averaged –12.6 +/- 2.9 O. D.

Female mucus absorbed less UV than male mucus both before ($T = -3.13$, $DF = 27$, $P = 0.004$) and after ($T = -2.60$, $DF = 26$, $P = 0.015$) the experiment. Overall integrated female mucus absorbance averaged 27.1 +/- 2.0 O. D. before, and 15.01 +/- 3.3 O. D. after the experiment, whereas mean male mucus absorbance was 36.1 +/- 2.1 O. D. before and 29.1 +/- 4.3 O. D. after the experiment.

The repeated measures linear model of change in center of absorbance found significant effects of UV treatment and time (Table 3.2; Figure 3.4b). However, diet, gender, and fish length had no effect, and there were no significant interactions. The

Table 3.2: Effects of UV treatment and time on the change in Center of absorbance (nm). Repeated-measures linear model results, Type III tests.

Effect	DF	F value	P value
UV treatment	1,25	4.41	<0.05
Diet treatment	1,25	0.27	0.61
Gender	1,25	0.28	0.60
Fish length	1,24	0.28	0.60
Week	4,87	4.04	<0.01
UV * Diet	1,25	1.58	0.22
UV * Gender	1,25	0.36	0.56
Diet * Gender	1,25	0.43	0.52
Diet * Week	4,87	1.36	0.26
UV * Week	4,87	1.91	0.12
Gender * Week	4,87	0.77	0.55
UV * Diet * Gender	1,24	0.57	0.46
Gender * Diet * Week	4,87	2.36	0.06
UV * Diet * Week	4,87	1.11	0.36
Gender * UV * Week	4,87	0.39	0.81

change in center of absorbance for UV-exposed fish (averaged over diet treatment and time) was –1.9 +/- 0.6 nm vs. –3.5 +/- 0.5 nm for UV-protected fish.

The repeated-measures ANOVA model of change in UV damage score showed a significant effect of UV treatment (Table 3.3; Figure 3.4c). There was no effect of diet, fish length, gender or time. The average UV-exposed fish damage score increased 0.76 +/- 0.3 as opposed to a decrease of 0.44 +/- 0.3 for UV-protected fish. There was a significant interaction between gender and UV treatment, as the damage score of females exposed to UV increased by much more than that of UV-exposed males, whereas the damage score of females protected from UV decreased by more than that of UV-protected males (Figure 3.5). There was also a significant 3-way interaction between gender, UV treatment and week (Figure 3.5).

Table 3.3: Effects of gender and UV treatment on change in damage score. Repeated-measures linear model results, Type III tests.

Effect	DF	F value	P value
UV treatment	1,25	8.42	<0.01
Diet treatment	1,25	<0.01	0.94
Gender	1,25	1.77	0.20
Week	4,83	0.46	0.77
Fish length	1,26	0.15	0.70
UV * Diet	1,25	1.07	0.31
UV * Gender	1,25	6.72	0.02
Diet * Gender	1,25	0.35	0.56
Diet * Week	4,83	0.72	0.58
UV * Week	4,83	0.68	0.61
Gender * Week	4,83	2.02	0.09
UV * Diet * Gender	1,26	0.06	0.82
Gender * Diet * Week	4,83	1.77	0.14
UV * Diet * Week	4,83	0.30	0.88
Gender * UV * Week	4,83	2.63	0.04

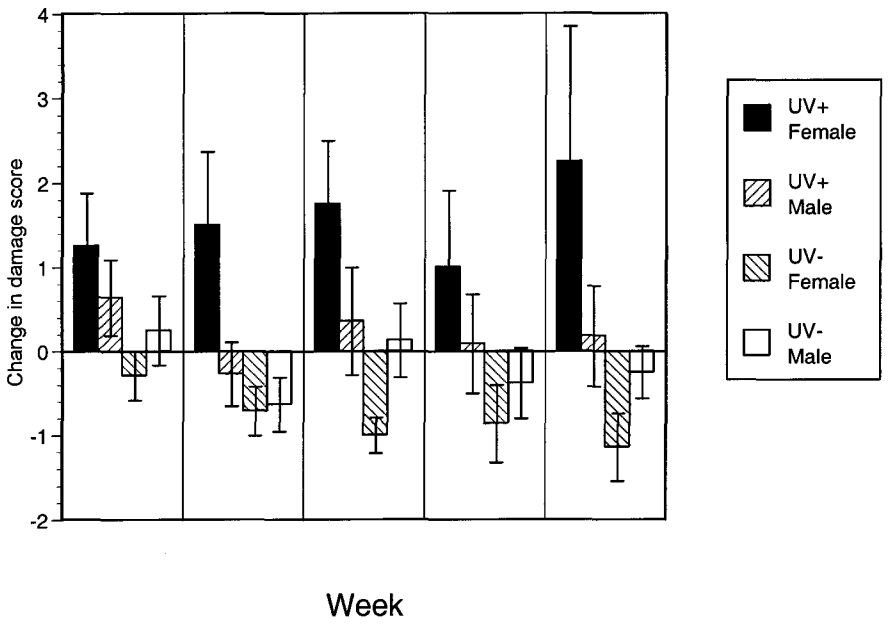

Figure 3.5: Change in damage score by gender and UV treatment. The significant UV*gender interaction in the damage score analysis is due to females showing higher damage scores than males in the UV+ treatment, and lower damage scores than males in the UV- treatment. The significant 3-way interaction between UV, gender and time signifies that the UV*gender interaction intensifies with time as female damage scores continue to change over time whereas males show little change with time.

Thalassoma duperrey whole eye transmission T_{50} values were affected by fish length (Table 3.4, Figure 3.6a), but not diet treatment, UV treatment, or gender. The equation for the regression of whole eye T_{50} value on standard length is $y = 0.5389x + 420.9$. None of the independent variables tested had a significant effect on the isolated lens T_{50} value (data not shown). The regression equation for whole lens T_{50} value on standard length is $y = 0.391x + 421.7$ (Figure 3.6a). Corneal transmission T_{50} values showed statistically significant effects of both fish length and UV treatment, but not diet treatment or gender (Table 3.4, Figure 3.6b). Larger fish had higher T_{50} values than smaller fish for all tissues, and the corneas of UV-exposed fish

Table 3.4. Effect of fish length on whole eye transmission T_{50} values, and significant effects of UV treatment and fish length on corneal T_{50} values. General linear model analysis results, Type III tests.

	Whole eye Transmission			Corneal Transmission		
Effect	DF	F value	P value	DF	F value	P value
UV treatment	1,18	<0.01	0.99	1,19	7.37	0.01
Diet treatment	1,18	<0.01	0.95	1,19	0.50	0.49
Gender	1,18	0.08	0.79	1,19	0.05	0.82
Fish length	1,18	6.54	0.02	1,19	6.83	0.02
UV * Diet	1,18	0.12	0.73	1,19	0.49	0.49
UV * Gender	1,18	0.05	0.82	1,19	1.66	0.21
UV * Fish length	1,18	0.01	0.93	1,19	5.96	0.03
Diet * Gender	1,18	1.16	0.30	1,19	1.04	0.32
Diet * Fish length	1,18	0.01	0.94	1,19	0.50	0.49
Gender * Fish length	1,18	0.11	0.74	1,19	0.15	0.70

had higher T_{50} values, thus absorbing more UV radiation than the corneas of UV-protected fish. A significant interaction between UV treatment and fish length indicated that the increase in T_{50} value with size was greater for fish in the UV- treatment than for those in the UV+ treatment (Figure 3.6b). The regression equations of corneal T_{50} values on standard length for UV+ and UV- treatments are $y = 0.3395x + 381.97$ and $y = 3.256x + 341.05$, respectively.

All but one fish became lighter in color (greater % reflectance) over the course of the experiment (mean +/- SE = 58 +/- 6%). The degree of lightening was significantly related to the diet of the fish, but not to the length of the fish, UV treatment, or gender (Table 3.5). The color of fish fed the MAA- diet changed more than did the color of MAA+ fish. The marginally non-significant interaction between diet and fish length stems from the fact that MAA- fish show a negative trend between degree of lightening and size, whereas MAA+ fish show essentially no trend with size (Figure 3.7). The regression equations for degree of lightening on standard

length for MAA- and MAA+ fish are y = -445.09x + 7062.9 and y = 0.4289x + 1019.2, respectively.

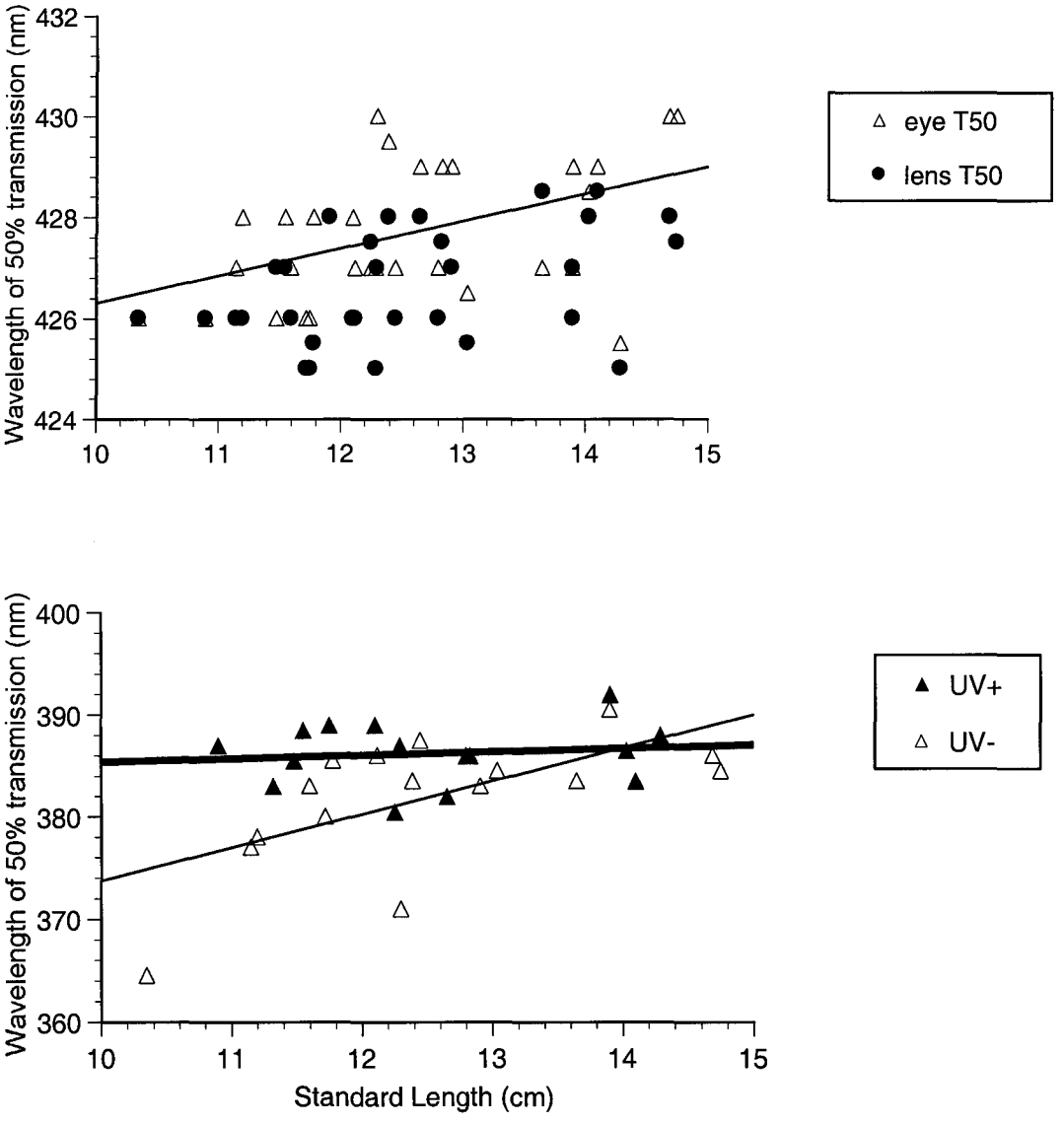

Figure 3.6: Ocular transmission T_{50} values vs. fish standard length. a) whole eye and lens transmission vs. standard length. Linear regression lines for both data sets are shown (heavy line = lens, thin line = eye). b) corneal transmission vs. standard length, split by UV treatment. Linear regression lines for both treatments are shown, heavy line = UV+, thin line = UV-.

Table 3.5. Effect of diet treatment on change in skin color. General linear model analysis results, Type III tests.

Effect	DF	F value	P value
UV treatment	1,18	0.15	0.70
Diet treatment	1,18	4.52	<0.05
Gender	1,18	1.38	0.26
Fish length	1,18	1.87	0.19
UV * Diet	1,18	0.08	0.79
UV * Fish length	1,18	0.17	0.69
Diet * Gender	1,18	0.04	0.84
Diet * Fish length	1,18	3.93	0.06
Gender * Fish length	1,18	1.38	0.26

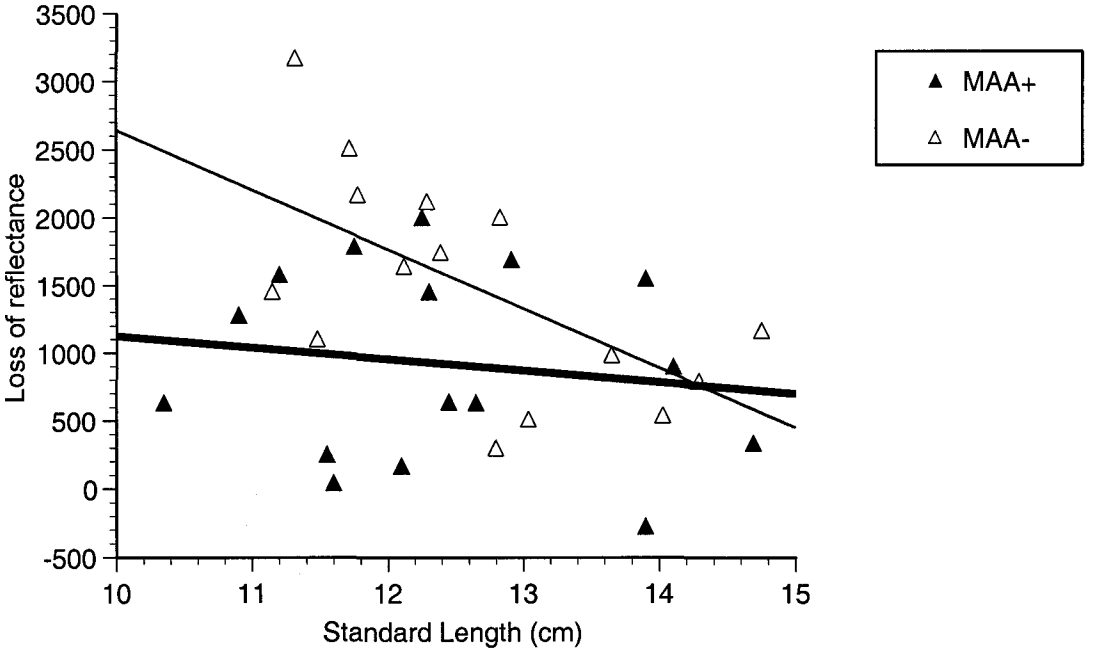

Figure 3.7: Change in reflectance (difference between the initial and final areas under reflectance curves) vs. standard length, split by diet treatment. MAA+ = UV-absorbing compounds in the diet, MAA- = no UV-absorbing compounds in the diet Linear regression lines are shown (heavy line = MAA+, thin line = MAA-).

DISCUSSION

Thalassoma duperrey is clearly able to adapt its mucus sunscreen to environmental UV exposure, but only when provided a dietary source of sunscreen compounds (Figure 3.4a). Integrated UV absorbance of the mucus increased in the UV-exposed fish, but only when *Acanthophora spicifera* was provided in the diet. This indicates that fish are sequestering the UV absorbing compounds from the diet, as has been found previously for invertebrates and a freshwater fish (Carroll and Shick 1996; Mason et al. 1998; Newman et al. 2000; Whitehead et al. 2001).

However, an increase in the integrated absorbance of the mucus occurred only in the presence of ultraviolet radiation. If fish were given the MAA+ diet and protected from UV exposure, the integrated absorbance of their mucus did not increase (Figure 3.4a). This indicates that there may be a physiological cost to sequestering UV absorbing compounds in the mucus, perhaps a tradeoff between using the compounds for epithelial sunscreen or for ocular tissues, or perhaps catabolizing them for energy. It would seem that *T. duperrey* have developed the ability to react to their environment so that they are protected when necessary but do not sequester UV-absorbing compounds into the mucus when they are not needed. Mucus is a renewable surface, continually dissolved into the water column and resupplied from the epidermis (Shephard 1994; Zamzow and Losey 2002), so MAAs or other compounds sequestered in the mucus are likely lost within days after entering the mucus layer.

The changes found in integrated absorbance occurred relatively quickly and appear to have leveled off after 3 weeks of experimental treatment (Figure 3.4a). The

proximate mechanisms by which *T. duperrey* are sensing environmental conditions (e.g., skin damage or photoreception) are unknown. The lens of *T. duperrey* blocks UV radiation from reaching the retina, so a retinal photoreceptor mechanism is unlikely. However, there may be an extraretinal photoreceptor mechanism, perhaps involving the pineal photoreceptors often found in fishes and other non-mammalian vertebrates, if there is a "window" allowing UV penetration to the pineal gland (Shand and Foster 1999). Alternatively, the increase in UV-absorbing compounds in the mucus could result directly from epidermal or other cellular damage. The quick response to environmental conditions suggests the possibility of 'storage depots' for UV-absorbing compounds, as have been suggested for sea cucumbers and sea hares (Bandaranayake and Des Rocher 1999; Carefoot et al. 2000).

Female mucus absorbed significantly less UV than male mucus both before and after the experiment, and females suffered more skin damage than males under the UV+ treatment (Table 3.3, Figure 3.5). The lack of gender effect in the integrated absorbance and center of absorbance analyses (Tables 3.1, 3.2) is due to the fact that these analyses dealt with **change** in absorbance. Female mucus was always absorbed less UV, hence there was no significant change over the course of the experiment. The lack of a significant gender factor in the change in damage score analysis is due to the significant three-way Gender*UV*Week interaction: females protected from UV became less damaged with time, whereas females exposed to UV became more damaged with time (Figure 3.5) and males showed no differences. UV-absorbing compounds have been found in the eggs of several marine fishes (Chioccara et al. 1980; Plack et al. 1981), and preliminary high performance liquid chromatography

(HPLC) analyses indicated the presence of the MAAs palythine and asterina-330 in both *T. duperrey* epithelial mucus and ovaries, but not testes (Zamzow unpubl). This raises the possibility that females may be experiencing different selection pressures than males, and the sequestration of UV-absorbing compounds into the eggs may compete with their own epithelial mucus protection. Female *T. duperrey* are year-round broadcast spawners (Ross and Losey 1983), and may be faced with a continual demand for UV-absorbing compounds for both their eggs and their own epithelial mucus that cannot be adequately met at high UV levels such as those experienced in this experiment. *Thalassoma duperrey*, unlike *Poecilia reticulata*, are probably unable to visually perceive UV wavelengths due to the UV-absorbance of their lenses (Figure 3.7a). Therefore, it is unlikely that the gender differences in mucus UV-absorbance (and thus UV-appearance, see Zamzow and Losey 2002) of *T. duperrey* play a role in their mate selection, as was recently found for *P. reticulata* (Smith et al. 2002). In *P. reticulata*, the factor causing differential mate preference was not elucidated, but females preferred males seen through UV-transparent filters whereas males preferred females seen through UV- filters.

The center of absorbance was significantly affected by UV treatment, but not diet (Table 3.2). Mucus from all treatment groups showed decreases in the wavelength of center of absorbance, but fish in the UV+ treatments decreased the center of absorbance of their mucus less (Figure 3.4b). This may indicate that a longer-wavelength center of absorbance allows broader absorbance of damaging UV radiation than a shorter-wavelength center of absorbance. *Acanthophora spicifera* has been shown to contain a sizable absorbance peak at ~290 nm (Carefoot et al. 1998).

The compound(s) responsible for this absorbance peak may have been preferentially sequestered from the experimental diet into the mucus of MAA+ *T. duperrey*; this could also account for the shift in center of absorbance of the MAA+ fish. The fact that MAA- fish also shifted the center of absorbance of their mucus suggests the possibility of the conversion of UV-absorbing chemical compounds already present in the fish when captured to others with a lower center of absorbance. A lowered center of absorbance could also result from depletion of a reservoir of longer wavelength UV absorbing compounds, or the use of longer wavelength compounds elsewhere in the body (e.g., in the ocular media).

The whole eye and cornea of *T. duperrey* showed a significant effect of fish standard length, with larger fishes having higher ocular media T_{50} values. A positive correlation between ocular transmission cutoff and fish size has also been shown in previous studies (Thorpe and Douglas 1993; Losey et al. 2000; Siebeck and Marshall 2001). As fish length had no effect on mucus absorbance, the ocular increase may be due to increased pathlength through the eye rather than an ontogenetic change in either the diet or the ability to sequester UV absorbing compounds.

There was no effect of diet or UV exposure on the whole eye or lens T_{50} values. Krill and sea hares have shown the ability to retain MAAs though deprived of any dietary source for months (Carefoot et al. 2000; Newman et al. 2000); fish may also have this ability when it comes to their ocular tissues. Corneal transmission, however, showed a significant effect of UV exposure, with UV-exposed corneas transmitting less UV light than UV-protected corneas (Figure 3.6b). The tissues of the corneal epithelium turn over rapidly (on the order of days) in humans (Hogan et al.

1971), and perhaps also in fishes (Losey et al. 2000), thus the cornea may be able to react more quickly than the lens to the ambient environment. This corneal adaptation is likely irrelevant in terms of vision, as the lens is the primary filter in the eye of *T. duperrey*. However, by adapting to ambient UV exposure the cornea may be able to protect the lens from more of the shortest-wavelength, most damaging radiation that causes ocular cataracts (Cullen and Monteith-McMaster 1993; Cullen et al. 1994; Zigman 1995). As mucus is sloughed into the water column, the mucus layer is therefore turned over rapidly (replacement of lost mucus occurs in approximately 24 hours, Zamzow and Losey 2002), and we would not expect a similar MAA-retention mechanism to function in the mucus.

In the wild, dietary MAA presence is not likely to be a limitation. *Thalassoma duperrey* has an extremely varied diet, including algae, crustaceans, molluscs, corals, echinoids and fish eggs (Hobson 1974), and MAAs are nearly ubiquitous among tropical marine organisms (Dunlap and Shick 1998). UVB-absorbing compounds, such as gadusol, have not been as well studied, but may also be present in the natural diet, as they are in sea cucumbers, brine shrimp, *Acanthophora spicifera* and fishes (Chioccara et al. 1980; Plack et al. 1981; Grant et al. 1985; Carefoot et al. 1998; Bandaranayake and Des Rocher 1999). Additionally, MAAs and other UV-absorbing compounds may be derived from closely related compounds by chemical reactions or bacteria in the gut, as has been proposed for sea cucumbers (Bandaranayake and Des Rocher 1999; Dunlap et al. 2000). Trophic co-acclimation to the UV environment, as has been proposed for Antarctic pteropods and krill, clearly does not apply in this instance (Newman et al. 2000; Whitehead et al. 2001). *T. duperrey* react to their

environmental UV dosage when given a steady dietary supply of algae, rather than simply sequestering in the mucus all of the available UV-absorbing compounds. Whether the UV-absorbing compounds found in the algae are used for another purpose or excreted in the feces is unknown.

Thalassoma duperrey does not show protective melanic darkening, as do hammerhead sharks (Lowe and Goodman-Lowe 1996). All fish became more reflective over the course of the experiment, and the diet (particularly the MAA- diet) was presumably lacking in carotenoids or other necessary pigments. Small fish lost more color than larger fish, particularly on the MAA- diet. Small fish also showed lower corneal T_{50} values than larger fish when protected from UV, but equal T_{50} values when exposed to UV. It would seem that small fish react more quickly to experimental conditions than larger individuals, although not in terms of mucus absorbance.

This study demonstrates both the dietary origins, and UV-induction, of UV absorbing compounds in the mucus of *Thalassoma duperrey.* Furthermore, unexpected differences were found between the genders in both the UV absorbance of the mucus and the degree of damage experienced by the fish. These results lead to further questions: if MAAs are plentiful in the natural diet, why do females sequester less UV-absorbing compounds in the mucus than males? Is there a physiological limitation to the amount of compounds that may be transported from the gut in any one day? The answers to these questions remain obscure. It should also be noted that some other strategy for UV protection, such as behavioral regulation of UV exposure, may be utilized by free-living *T. duperrey.* Regardless, it appears that epithelial

mucus provides adequate UV protection for *T. duperrey* males - females seemed to have been challenged by the extremely high UV irradiation conditions used in these experiments.

CHAPTER 4:

ONTOGENETIC AND ENVIRONMENTAL EFFECTS

Ontogenetic changes and environmental effects on UV absorbance of epithelial mucus from three species of coral reef fishes

Manuscript

by

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INTRODUCTION

Ultraviolet radiation is abundant in the tropics due to naturally low ozone levels and a small solar zenith angle (Cutchis 1982). UV penetrates seawater quite well, particularly in clear tropical waters, but is attenuated with increasing depth (Jerlov 1976). The shortest ultraviolet wavelengths are attenuated most quickly, so the spectrum of ambient UV radiation also shifts to longer wavelengths with depth (Jerlov 1976). Marine organisms are damaged by UV and nearly all marine species synthesize or sequester putative sunscreen compounds, mycosporine-like amino acids (MAAs) and related gadusols (Cockell and Knowland 1999; Dunlap et al. 2000; Shick et al. 2000; Karentz 2001; Shick and Dunlap 2002). Coral reef fishes have been shown to possess MAAs in their ocular tissues (Dunlap et al. 1989; Thorpe et al. 1993) and their epithelial mucus (Zamzow and Losey 2002; Zamzow in review).

Bathymetric trends in MAA concentrations have been demonstrated for corals (Dunlap et al. 1986; Dunlap and Shick 1998) and red macroalgae (Karsten et al. 1998a). Antarctic sea urchins have higher ovarian MAA concentrations in shallower water (Karentz et al. 1997), and coral reef holothuroids may also show depth-related patterns of MAA concentrations (Shick et al. 1992). The ocular media of coral reef fishes show no obvious trend with depth, but increased UV absorbance of the eyes occurs with increasing fish size for some coral reef fishes (Siebeck and Marshall 2001; Nelson et al. 2003).

In this study, I measured the UV absorbance of the mucus of three coral reef fish species over a range of depths (3-12m) and fish sizes. The three species chosen (*Canthigaster jactator*, *Chaetodon multicinctus*, and *Thalassoma duperrey*) are

ecologically and taxonomically diverse. *Canthigaster jactator* are poor swimmers and *C. multicinctus* are territorial and site-attached, so it is likely that the depth of capture truly reflected the previous experience of these fishes. *Thalassoma duperrey* is a more vagile species. *Chaetodon multicinctus* feeds predominantly on coral polyps, whereas *C. jactator* and *T. duperrey* are both generalist invertebrate/algae feeders (Hobson 1974). *C. jactator* consumes coralline and filamentous algae, corals, sponges, ascidians, bryozoans, and a variety of other invertebrates whereas *T. duperrey* consumes some algae and invertebrates, predominantly gastropods, echinoids, and crustaceans (Hobson 1974).

Depth of capture consistently significantly affected the magnitude of the UV absorbance of the three species' mucus, and size never had a significant effect. The spectrum of mucus absorbance shifted significantly toward the longer UV wavelengths with increasing depths for *T. duperrey*, and a similar trend was seen for *C. multicinctus*. *C. multicinctus* had higher mucus absorbance values than the other two species, and *T. duperrey* blocked shorter-wavelength radiation than did *C. jactator* or *C. multicinctus*.

MATERIALS AND METHODS

Fishes were captured by SCUBA divers with barrier nets and held in buckets of aerated seawater for no more than 3h post-capture. Mucus was sampled following Zamzow and Losey (2002). Briefly, epithelial mucus was collected by gently scraping a dull scalpel blade along the dorsal flank of live fishes, anterior to posterior. Samples were squashed to 0.25 mm thickness between two ultraviolet-transparent

(T_{50} = 266 nm) slides. Light absorbance of the mucus 'squash' was measured with an Ocean Optics S-2000 spectrophotometer with illumination provided by a Deuterium-Tungsten source. Eight absorbance measurements were taken through different areas of each sample in order to compensate for possible heterogeneity of the mucus.

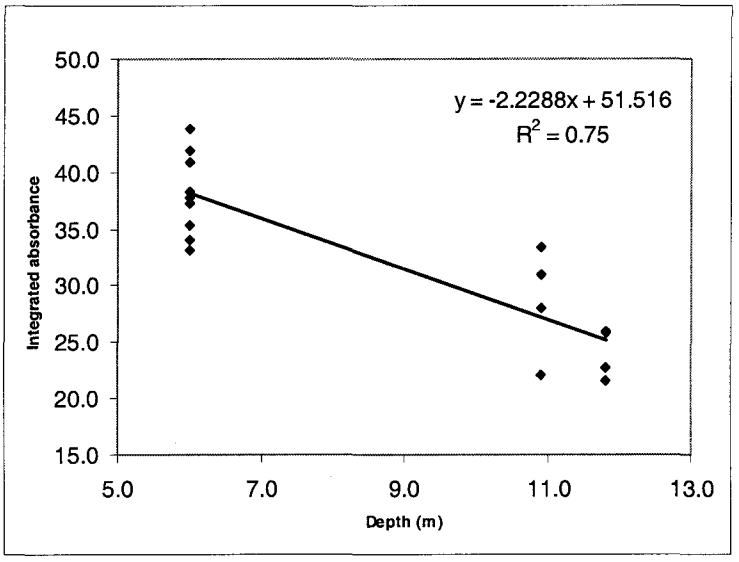
Two measures were calculated from each mucus absorbance spectrum: integrated absorbance and center of absorbance. Integrated absorbance was calculated by adding the absorbance values (units = optical density, O. D.) for each wavelength from 280-400 nm, thereby approximating the area under the curve. Center of absorbance was calculated by multiplying the absorbance value (O. D.) at each wavelength by its corresponding wavelength (nm), then summing these and dividing the resultant number by the integrated absorbance (O. D.). This yielded a wavelength value indicative of the relative contributions of the various peaks of the absorbance curve. These measures were averaged over the eight replicate spectra from each mucus sample.

I used multiple regression (SAS systems, v. 8.2) to test the hypotheses that integrated absorbance and center of absorbance were determined by the depth of capture and/or the standard length of each species of fish. I used a general linear model to determine whether there were between-species differences in integrated absorbance or center of absorbance. I accounted for fish length and depth by including them as independent variables in the model, and then tested for an effect of species type.

RESULTS

Canthigaster jactator

Depth of capture had a significant effect on the integrated absorbance of *C. jactator* mucus (Figure 1a; $t_{1,14} = -7.24$, $p < 0.0001$), whereas standard length did not (Figure 1b; $t_{1,14} = -1.66$, $p = 0.12$). The overall model explained much of the variation in the data (adj. R-squared = 0.76).



a

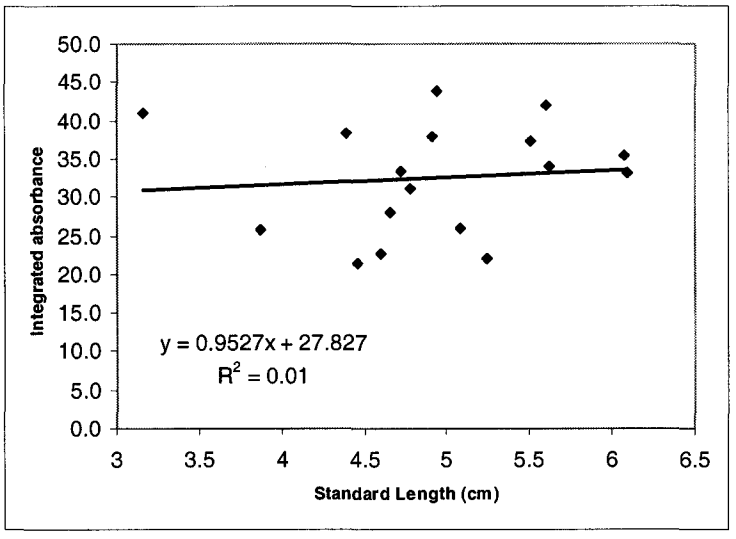


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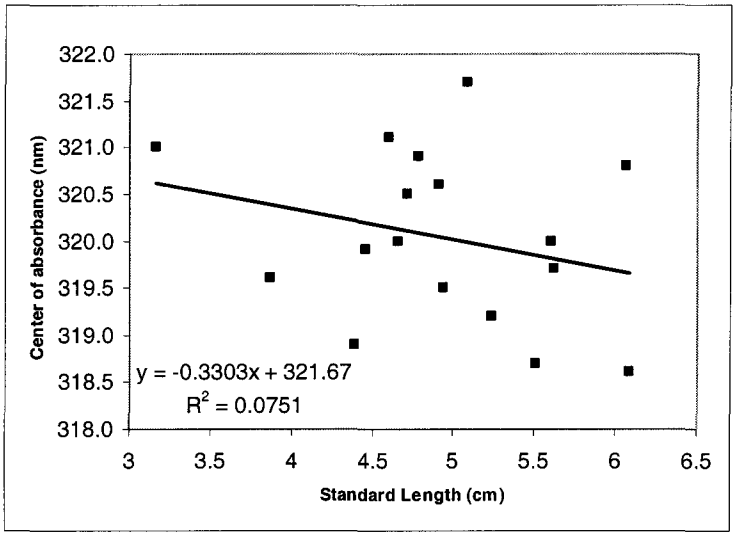
Figure 4.1. Integrated absorbance of *Canthigaster jactator* mucus. a) Integrated absorbance vs. depth of capture, b) Integrated absorbance vs. standard length

Neither depth nor standard length had an effect on the center of absorbance of *C. jactator* mucus (Figures 2a, 2b; Depth: $t_{1,14} = 1.17$, $p = 0.26$; Length: $t_{1,14} = -0.66$, $p = 0.52$). The overall model did not explain much of the variation in the data (adj. R-squared = 0.04).



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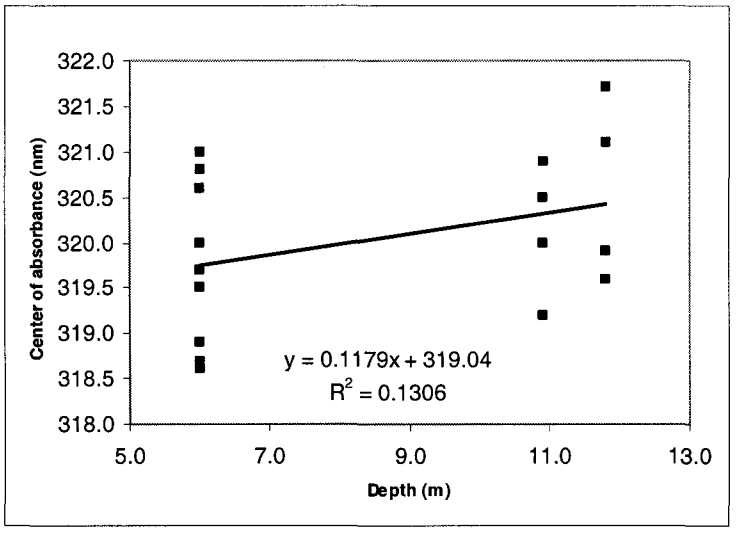

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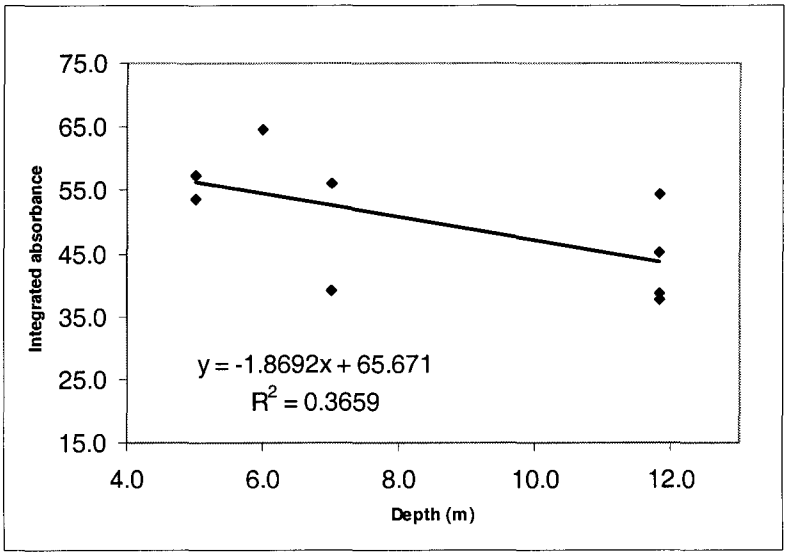
Figure 4.2. Center of absorbance of *Canthigaster jactator* mucus. a) Center of absorbance vs. depth of capture, b) Center of absorbance vs. standard length

Chaetodon multicinctus

Depth of capture had a significant effect on the integrated absorbance of *C. multicinctus* mucus (Figure 3a; $t_{1,6} = -2.93$, $p < 0.05$), whereas standard length did not (Figure 3b; $t_{1,6} = 1.93$, $p = 0.10$). The overall model explained about half of the variation in the data (adj. R-squared = 0.48).



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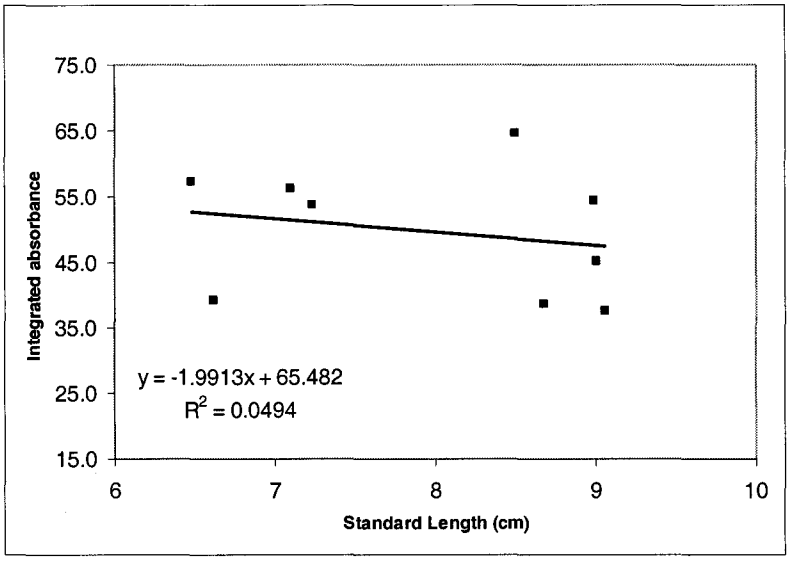


b

Figure 4.3. Integrated absorbance of *Chaetodon multicinctus* mucus. a) Integrated absorbance vs. depth of capture, b) Integrated absorbance vs. standard length

Neither depth nor standard length had an effect on the center of absorbance of *C. multicinctus* mucus in the original analysis (Figures 4a, 4b; Depth: $t_{1,6} = 1.68$, $p = 0.14$; Length: $t_{1,6} = 1.73$, $p = 0.13$). However, when considered separately, both depth and standard length have effects on the center of absorbance (Linear regression;

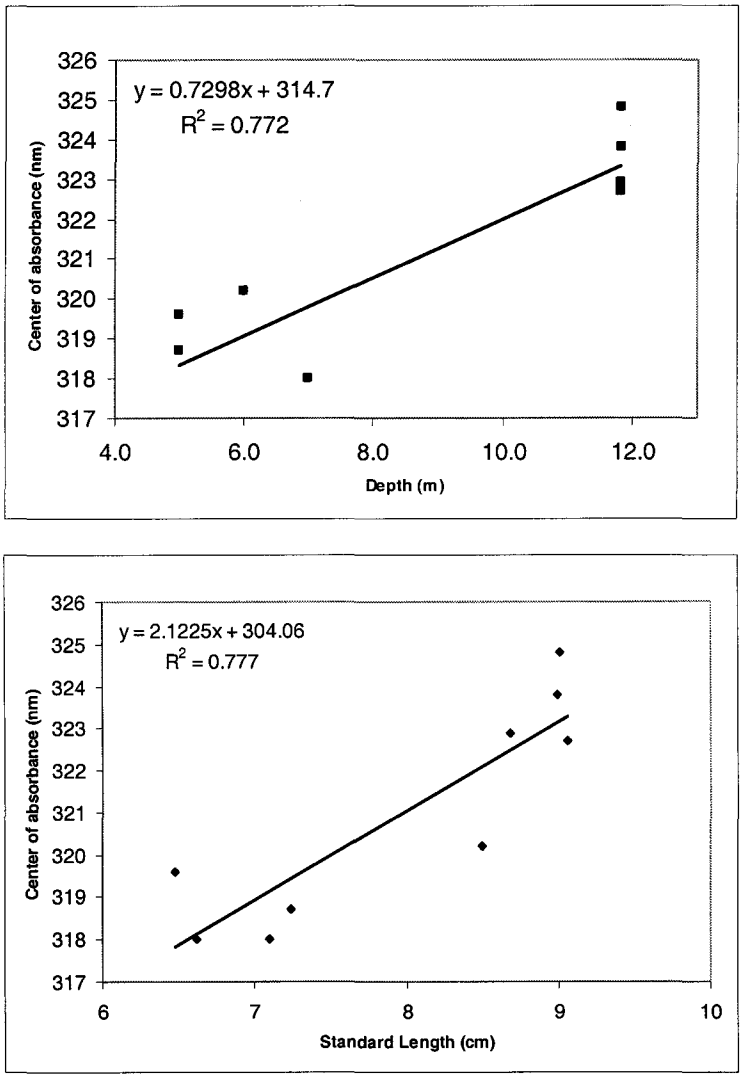


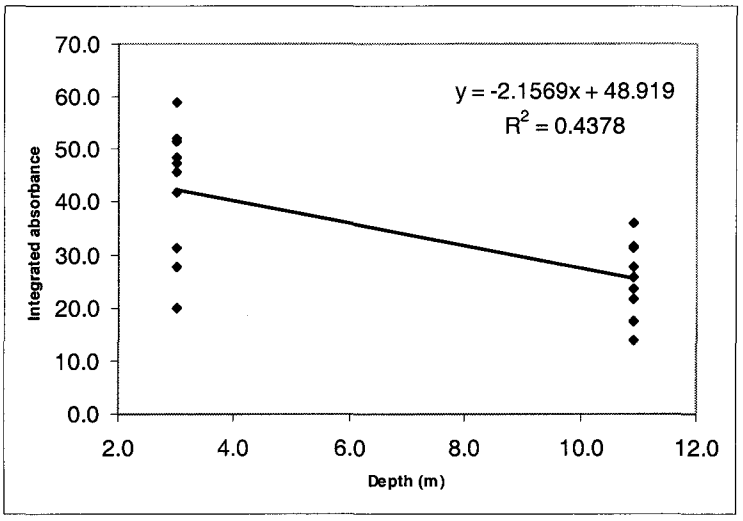
Figure 4.4. Center of absorbance of *Chaetodon multicinctus* mucus. a) Center of absorbance vs. depth of capture, b) Center of absorbance vs. standard length

Depth: $F_{1,7} = 42.47$, $p = 0.002$; Length: $F_{1,7} = 24.39$, $p = 0.002$). Depth and length are also highly correlated (Pearson correlation = 0.83), so the initial analysis could not

detect a **separate** effect of either independent variable. The original overall model explained much of the variation in the data (adj. R-squared = 0.80).

Thalassoma duperrey

Depth of capture had a significant effect on the integrated absorbance of T. duperrey mucus (Figure 5a; $t_{1,16} = -3.31$, $p = 0.005$), whereas standard length did not



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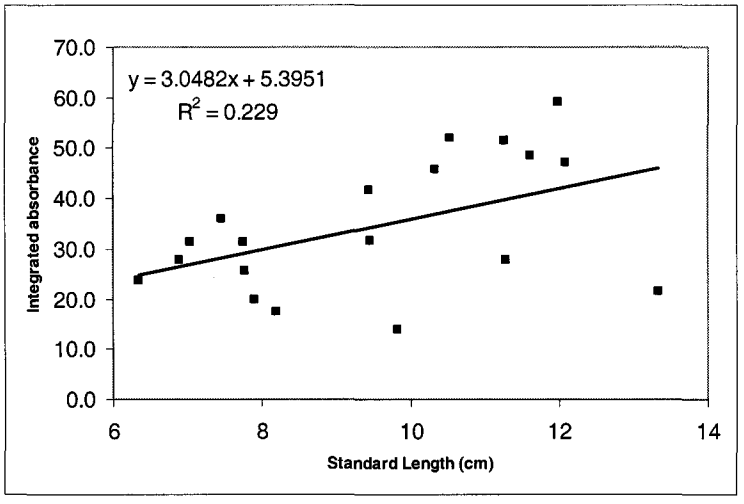
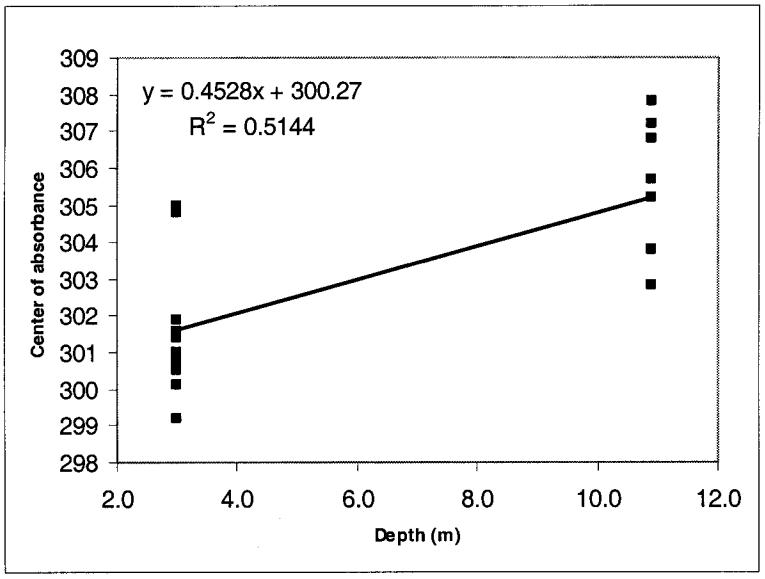


b

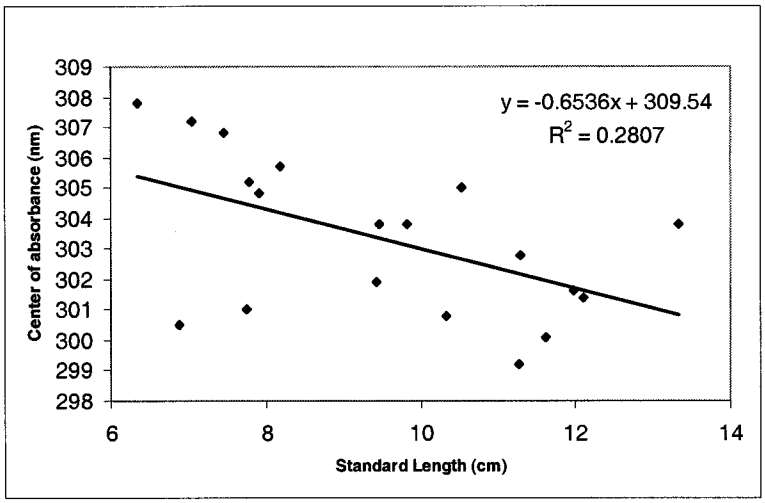
Figure 4.5. Integrated absorbance of *Thalassoma duperrey* mucus. a) Integrated absorbance vs. depth of capture, b) Integrated absorbance vs. standard length

(Figure 5b; $t_{1,16} = 1.91$, $p = 0.07$). The overall model explained about half of the variation in the data (adj. R-squared = 0.48).

Both depth of capture and standard length had significant effects on the center of absorbance of *T. duperrey* mucus (Depth: Figure 6a; $t_{1,16} = 4.05$, $p < 0.001$; Standard Length: Figure 6b; $t_{1,16} = -2.43$, $p = 0.03$). The overall model explained much of the variation in the data (adj. R-squared = 0.60).



a

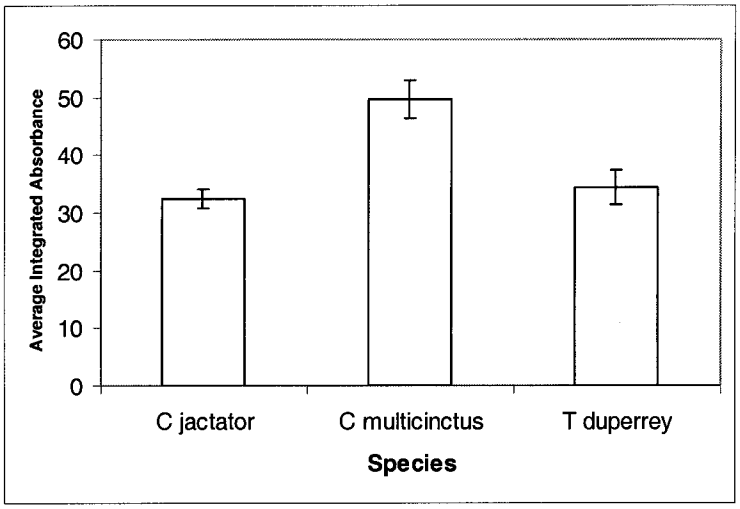


b

Figure 4.6. Center of absorbance of *Thalassoma duperrey* mucus. a) Center of absorbance vs. depth of capture, b) Center of absorbance vs. standard length

General trends

Overall, *Chaetodon multicinctus* had significantly higher integrated absorbance values than either *T. duperrey* or *C. jactator* (Figures 7a, 8a-c; General linear model, Species effect $F_{2,40} = 23.53$, $p < 0.0001$, Tukey post-hoc comparisons, $p < 0.05$); the latter two could not be distinguished from one another (pairwise comparisons, Tukey adjustment, $p > 0.05$).



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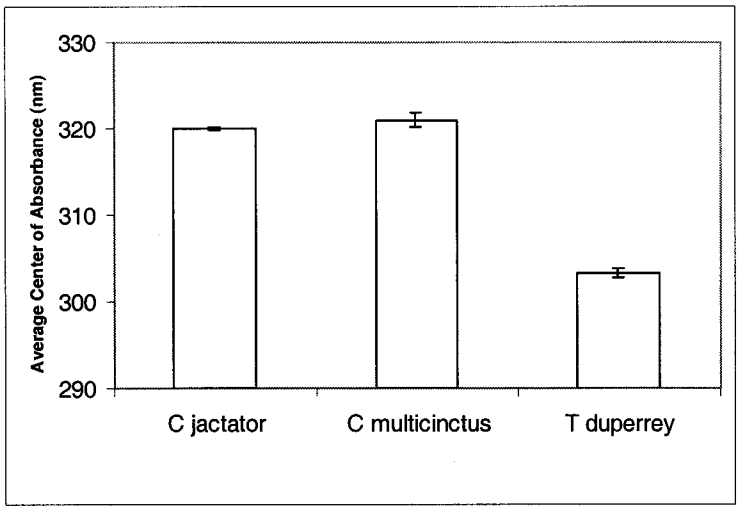

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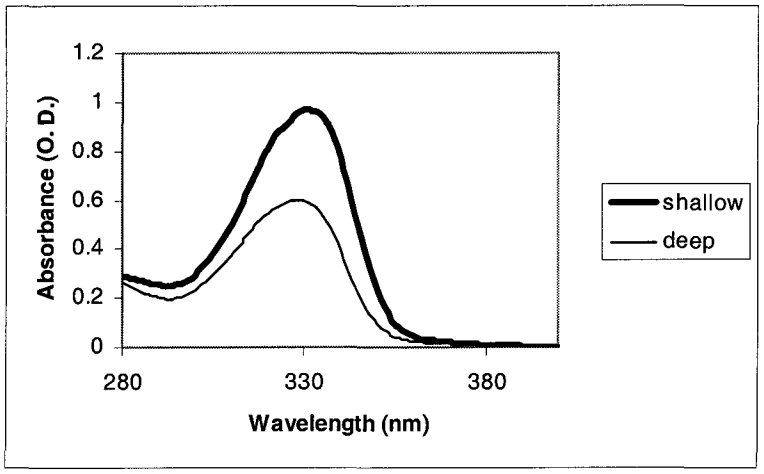
Figure 4.7. Mean absorbance values for each species a) Average integrated absorbance, b) Center of absorbance



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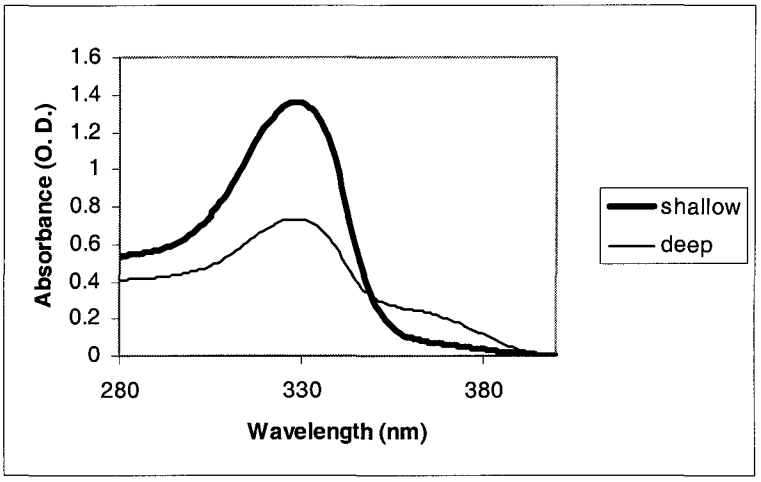

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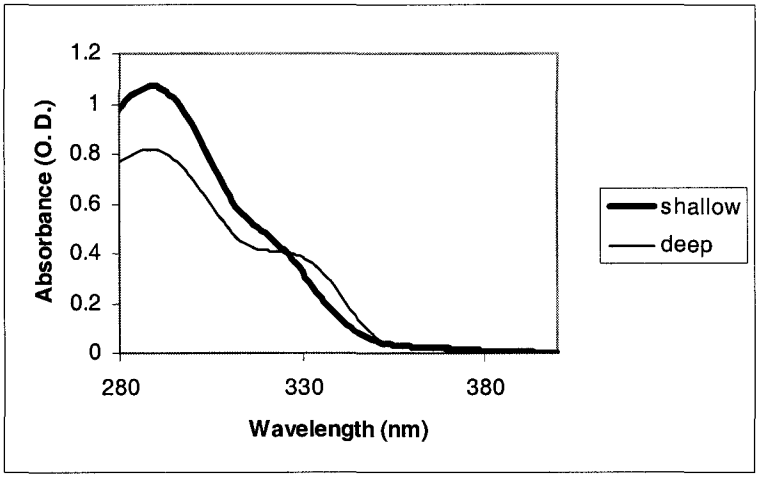

c

Figure 4.8. Absorbance spectra for shallow and deep individuals of each species. a) *Canthigaster jactator*, b) *Chaetodon multicinctus*, c) *Thalassoma duperrey*

Thalassoma duperrey had significantly lower center of absorbance values than either *C. multicinctus* or *C. jactator* (Figures 7b, 8a-c; General linear model, Species effect $F_{2,40} = 312.39$, $p < 0.0001$, Tukey post-hoc comparisons, $p < 0.05$); the latter two could not be distinguished from one another (pairwise comparisons, Tukey adjustment, $p > 0.05$).

DISCUSSION

UV absorbance of the three species' mucus consistently decreased with increasing depth of capture. As MAAs are acquired from the diet by fishes and other animals (Mason et al. 1998; Zamzow in review), this may be a simple expression of dietary availability with depth. Sunscreen concentrations in macroalgae, corals, and some other invertebrates have been shown to decrease with depth (Dunlap et al. 1986; Shick et al. 1992; Karentz et al. 1997; Dunlap and Shick 1998; Karsten et al. 1998b). However, experiments with coral reef fish have shown that fish fed a diet rich in MAAs differentially sequester compounds in the mucus according to their UV exposure regime (Zamzow in review), so the decreasing UV absorbance of mucus with depth may in fact be adaptive.

Size (and presumptive age) of fishes never had a significant effect on the integrated absorbance of the mucus. *Thalassoma duperrey* showed a non-significant positive correlation between mucus absorbance and size. The mucus of this species is significantly more absorbant in males than females (Zamzow in review), and as males are generally larger than females in this sex-changing species (Ross 1982), this may

account for the trend in the data. However, none of the fish in this experiment were sexed.

The spectrum of mucus absorbance shifted significantly toward the longer UV wavelengths with increasing depths for *T. duperrey*, and a similar trend was seen for *C. multicinctus*. In a recent review, Shick and Dunlap (2002) stated that attempts to correlate absorption maxima with the underwater spectrum have not been convincing. This may be the first convincing evidence for spectral shifting of UV-protective compounds with increasing depth. Apparently, this is achieved by the fishes sequestering proportionally more longer-wavelength compounds in the mucus with increasing depth (Figures 8b, 8c). In the case of *C. multicinctus*, the longer-wavelength shoulder of the absorbance curve (Figure 8b) may be indicative of the presence of palythene, which has been previously found in the ocular media of butterflyfishes (Dunlap et al. 1989). Palythene is found in the coral species that comprise the diet of *C. multicinctus*, and its concentration in coral tissues decreases with increasing depth (Kuffner et al. 1995). This supports the idea that differential sequestration of sunscreening compounds is occurring, because the proportions of compounds found in the mucus are not a mere reflection of their availability in the food supply. Further sampling of *T. duperrey* and *C. multicinctus* over a wider depth gradient would be valuable.

Overall, *C. multicinctus* had the highest mucus absorbance values. Whether this is due to dietary availability or some other factor is unknown. Fishes vary in mucus load, and *Chaetodon auriga* have less than half as much mucus (mg/cm2) as *T. duperrey* (Gorlick 1978). *C. multicinctus*, similarly, seem to have thinner mucus

coatings than either of the other two species, and it may be that higher concentrations of MAAs are necessary to provide a similar amount of sunscreen protection given the smaller mucus pathlength. The thickness of the mucus layer on the outside of a fish has proven surprisingly difficult to measure (Shephard 1994), and was not attempted in this study.

T. duperrey blocked shorter-wavelength radiation than did *C. jactator* or *C. multicinctus*. *T. duperrey* may be exposed to more damaging short-wavelength UV radiation due to its active lifestyle (fish in aquaria swim > 90% of the time, Zamzow unpubl.) and propensity for shallow habitats. *C. jactator* is often found under ledges and within the interstices of corals (pers. obs.), and *C. multicinctus* is rarely found shallower than 5m (D. Strang and A. Meyer, pers. comm.). UVB (300 nm) irradiance has been reduced by two orders of magnitude at 5m depths in Kaneohe Bay, Hawaii (Kuffner et al. 1995).

Despite differences in diet, behavior, life history and taxonomy, the three coral reef fish species investigated in this study all showed significant effects of depth of capture on integrated UV absorbance of the mucus. *Chaetodon multicinctus* and *Thalassoma duperrey* also shifted the spectrum of their mucus toward the longer wavelengths with depth. It would appear that these three very different coral reef fish species are able to acclimate their mucus sunscreen to their environment in similar manners despite differences in dietary availability of compounds.

CHAPTER 5:

TEMPERATE TIDEPOOL FISHES

Ultraviolet-absorbing compounds in the mucus of temperate Pacific tidepool sculpins: variation over local and geographic scales.

Manuscript

submitted to

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by
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ABSTRACT

Temperate tidepool fishes of the family Cottidae (Teleostei) display biogeographic distribution patterns that vary with latitude and elevation within the intertidal zone. Middle and high intertidal pool species show a pattern of "species replacement", with southern species that are replaced by northern species at discrete locations along the coast. Lower intertidal pools, on the other hand, are dominated by a single species, *Oligocottus snyderi*, that occurs over an extremely wide latitudinal range. As a consequence of both latitudinal and elevational patterns, these fishes experience variable amounts of ultraviolet (UV, 280-400 nm) irradiation both between and within species. The mucus of tidepool sculpins was analyzed by absorbance spectrophotometry to compare concentrations of UV-absorbing compounds in the mucus of the fishes with regard to geographic location or intertidal microhabitat. Mucus from northerly fishes absorbed significantly less UV than the mucus of southerly fishes. Differences were detectable over only 2 degrees change in latitude. Mucus from high intertidal pool fishes absorbed significantly more UV than mucus from middle or lower intertidal pool fishes at the same site. The mucus of all fishes surveyed contained UV-absorbing compounds with maximum absorbance in the short-wavelength UVA (320-400 nm). Compounds with maximal absorbance in the UVB (280-320 nm), which are common in tropical fishes, were not found in these temperate tidepool species.

INTRODUCTION

Sculpins (Cottidae: Teleostei) are an abundant and well-studied component of temperate Pacific tidepool communities (Morris 1962, Yoshiyama 1981, Yoshiyama et al. 1986, Yoshiyama et al. 1992). These fishes show species-specific distribution patterns, both vertically within the intertidal zone and along a latitudinal gradient. Within the intertidal zone, different species dominate high, mid-level, and low intertidal pools (Yoshiyama et al. 1986). The predominant fishes in high- and mid-intertidal pools show a latitudinal pattern of "geographical replacement" by closely related and ecologically similar species (Morris 1962, Yoshiyama et al. 1986). South of San Francisco Bay, *Clinocottus analis* is the most common fish in high intertidal pools, whereas to the north, *Oligocottus maculosus* occupies this niche. Similarly, in mid-intertidal pools, the southerly *C. recalvus* is replaced by the northerly (and morphologically nearly identical) *C. globiceps* between San Francisco Bay and Monterey Bay. However, *O. snyderi* is the predominant low intertidal species over a wide latitudinal range, from Alaska to Baja California (Eschmeyer et al. 1983).

Because of these variable distribution patterns, tidepool sculpins experience a wide range of incident ultraviolet radiation (UVR, 280-400nm), both between and within species. By virtue of their shallow water environment, these fish experience potentially maximal incident UV (barring behavioral avoidance). The damaging effects of UV radiation, particularly shorter-wavelength UVB (280-320 nm), on fishes include cataracts, corneal damage, and epithelial "sunburn" damage (Bullock 1982, Ahmed & Setlow 1993, Cullen & Monteith-McMaster 1993, Cullen et al. 1994). For fishes, sunburn can be fatal, but fish species (and even strains of a species)

are differentially tolerant of UV exposure (Blazer et al. 1997, Armstrong et al. 2002). The epithelial mucus of coral reef fishes (n>200 spp.) contains varying amounts of several "sunscreen" compounds, mycosporine-like amino acids (MAAs) and gadusol, that absorb both UVB and UVA (320-400 nm) radiation (Zamzow & Losey 2002). The strength of these sunscreens changes according to the UV exposure of the fish (Zamzow & Losey 2002), and they are sequestered from the diet (Zamzow in review, Mason et al. 1998). The diets of Pacific tidepool fishes are well documented (Grossman 1986), and are known to contain MAAs (Banaszak & Trench 1995, Shick & Dunlap 2002).

Incident UVR varies widely with both latitude (Gleason et al. 1993, Madronich et al. 1998) and depth in the water column (Jerlov 1976). Additionally, due to the nature of the tidal cycle, fishes in high intertidal pools spend more time exposed to high UV levels than fishes in low intertidal pools (Ricketts et al. 1992). This study investigated whether the UV absorbance of the epithelial mucus of tidepool sculpins varied with latitude, vertical location within the intertidal zone, or both. I hypothesized that fishes from higher latitudes would possess less sunscreen in the mucus than did fishes from lower latitudes, and fish from higher tidepools would possess more sunscreen than those from lower tidepools.

MATERIALS AND METHODS

Fishes were collected with dip nets at low tide from three sites along the Pacific Coast of North America (Table 5.1). The sites were Soberanes Point, California (36°27'N, 121°56'W; July 10-12, 2002); Arena Cove, California

(38°55'N, 123°43'W; July 23-25, 2002); and Sitka, Alaska (57°02'N, 135°21'W; August 7-11, 2002). The first two sites were chosen from Yoshiyama (1986), and were expected to represent the southern species complex, and an overlap zone between southern and northern species, respectively. The third site was chosen from Eschmeyer (1983) as the extreme northern range of *Oligocottus snyderi*, and was expected to elucidate any subtle changes that might occur with latitude, as well as representing the northern species complex. Sites were sampled beginning in the south, in order to bias the results against any possible cumulative effect of summertime UV exposure. The Arena Cove site was expected to be a zone of overlap for direct comparison of the mucus of *Clinocottus analis* and *O. maculosus* (Yoshiyama et al. 1986), however, no *C. analis* were present at the time of sampling (approximately 25 man-hours of search time).

Table 5.1: Cottid species used in general linear model of integrated UV absorbance.

Site	Date	Zone	Species	n
Soberanes Point	July 10-12, 2002	High	*Clinocottus analis*	8
		Mid	*Clinocottus recalvus*	10
		Low	*Oligocottus snyderi*	9
Arena Cove	July 23-25, 2002	High	*Oligocottus maculosus*	10
		Mid	*Clinocottus globiceps*	10
		Low	*Oligocottus snyderi*	11
Sitka	August 7-11, 2002	High	*Oligocottus maculosus*	10
		Mid	*Clinocottus globiceps*	2
			Clinocottus embryum	5
		Low	*Oligocottus snyderi*	8

The relative height of tidepools within the intertidal zone was characterized as low, mid, or high, modified from Yoshiyama 1981. For this study, mid-intertidal pools were defined as Yoshiyama's "high-offshore" and "high-intermediate" pool

categories, whereas high intertidal pools were his "high-nearshore" pool category (Yoshiyama 1981). All three sites consisted of wave-exposed rocky benches, and the types of tidepools present were quite similar. Low intertidal pools were of moderate to deep depths (~0.25 to 1.5m) and contained the most macroalgal cover, mid tidepools were generally deep (~0.75-1.5m) potholes or gorges with less macroalgal cover than low pools, and high tidepools had large surface-area-to-volume ratios (< 1m depths and 1-3m across) and the least amount of macroalgal cover.

Fishes were held in buckets of aerated seawater prior to sampling for no more than three hours post-capture. One fish of each species was euthanized, and transmission of the ocular media was determined following Losey et al. (2000). After sampling, fishes were returned to their home tidepools, and any subsequent collections took place at least 100 m distant.

Spectrophotometric methods followed Zamzow and Losey (2002). Briefly, a mucus sample was taken from the dorsal flank of each fish, "squashed" to a depth of 0.25 mm, and absorbance of each sample was measured from 280-400 nm (5.1). For the purposes of data analysis, the variable "integrated absorbance" was used. Integrated absorbance consists of sum of the absorbance values (units = optical density, O.D.) at each wavelength integer from 280 to 400 nm. General linear model (GLM) analysis was performed with SAS systems, release 8.02. The initial GLM tested the effects of geographic site, relative intertidal elevation, standard length, species of fish, and interaction terms, on integrated absorbance. Non-significant independent variables ($p > 0.5$) were sequentially excluded, and the explanatory

variables in the final model were geographic site, intertidal elevation, and their interaction.

RESULTS

The mucus of both *Oligocottus spp.* and *Clinocottus spp.* absorbed UV, with the main absorbance peak located at ca. 330 nm. Unlike tropical tidepool fishes, there was no UVB-absorbing peak at ca. 294 nm (e.g. *Istiblennius lineatus* from Majuro Atoll, 7°09'N, 171°12'E, 13 June 2001, Fig 1). Integrated absorbance showed

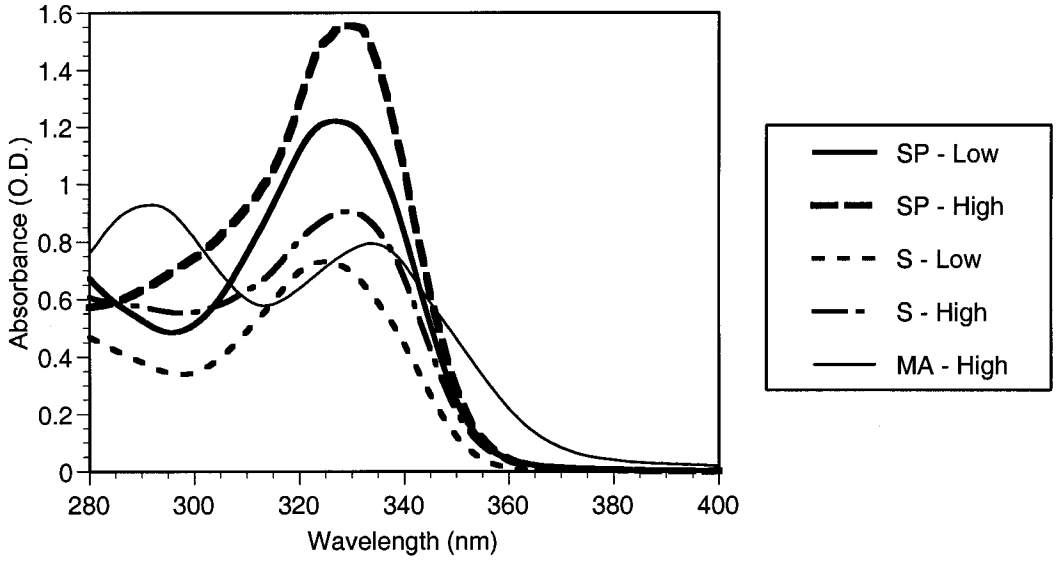

Figure 5.1: Example absorbance spectra of temperate cottid mucus, with a tropical blenniid species shown for comparison. Legend: SP = Soberanes Point, S = Sitka, MA = Majuro Atoll. Low pool spectra are *Oligocottus snyderi* mucus, and high tidepool species are *Clinocottus analis* (south), and *Oligocottus maculosus* (north). *Istiblennius lineatus*, a tropical high tidepool species, is shown for comparison.

significant effects of both site and relative height within the intertidal zone (Figure 5.2, ANOVA: Site $F(2,70) = 46.44$, $p < 0.0001$; Height $F(2, 70) = 9.41$, $p < 0.001$).

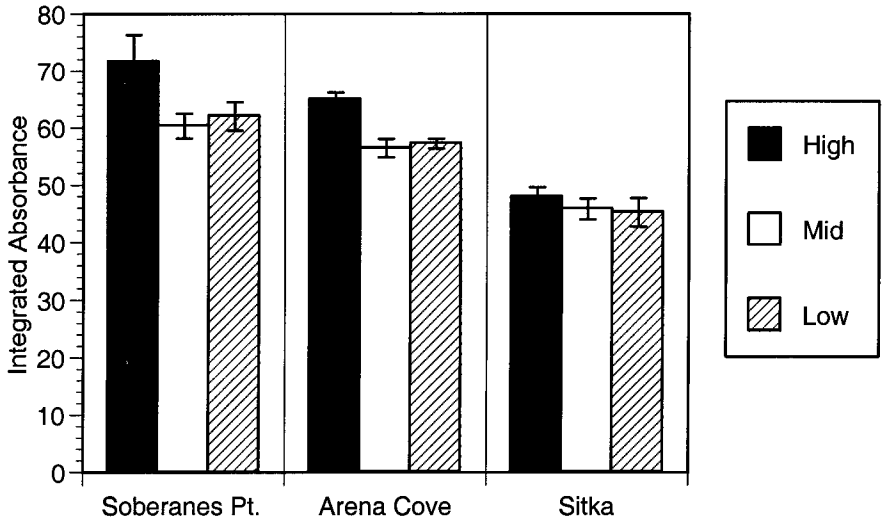

Figure 5.2: Integrated absorbance for *Clinocottus* and *Oligocottus* species from high, mid, and low intertidal pools at each site. Low tidepool data for all three sites are from *O. snyderi* mucus. Species sampled at Soberanes Point were *C. analis* (high tidepools) and *C. recalvus* (mid tidepools). At Arena Cove and Sitka, species sampled were *O. maculosus* (high tidepools) and *C. globiceps* (mid tidepools).

Fishes from high intertidal pools had significantly greater UV absorbance than those from low or mid intertidal pools (LSM comparison, Tukey post-hoc adjustment, Mid: $p < 0.001$, Low: $p<0.01$), but the mucus of low and mid intertidal pool fish could not be distinguished from one another (LSM, Tukey, $p = 0.94$). Fish from Soberanes Point had significantly higher mucus absorbance than fish from Arena Cove (LSM, Tukey, $p=0.02$) or Sitka (LSM, Tukey, $p<0.0001$), and fish from Arena Cove had significantly higher integrated UV absorbance than fish from Sitka (LSM, Tukey, $p<0.0001$). There was no interaction between relative height and geographic location. The ocular lens of all species surveyed blocked UVR from reaching the retina (Table 5.2).

Table 5.2: Cottid species sampled for ocular media transmission. * For *A. lateralis*, *C. globiceps* and *O snyderi*, corneal absorbance resulted in higher T50 values for whole eye transmission.

Species	Site	Lens T50 (nm)
Artedius lateralis	Sitka	410*
Clinocottus analis	Soberanes Point	409
C. globiceps	Arena Cove	412*
C. recalvus	Soberanes Point	412
Cebidichthys violaceus	Soberanes Point	407
Enophrys bison	Sitka	402
Oligocottus maculosus	Arena Cove	408
O. snyderi	Soberanes Point	399*

Additional intertidal species were opportunistically surveyed for mucus transmission, and the non-cottids had very low integrated absorbance values (Table 5.3). Among the cottids, *Artedius spp* mucus had integrated absorbance values were similar to those found for *Oligocottus* and *Clinocottus* species, whereas the mucus of *Enophrys bison* demonstrated little UV absorbance.

Table 5.3: Additional tidepool species sampled for mucus and ocular media.

Site	Species (Family)	Integrated Absorbance (mean +/- s.e.)	N
Soberanes Point	*Cebidichthys violaceus* (Stichaeidae)	11.1	1
	Gobiesox maeandricus (Gobiesocidae)	25.5	1
	Xererpes fucorum (Pholidae)	10.9	1
Arena Cove	*Artedius lateralis* (Cottidae)	51.1 +/- 0.9	2
Sitka	*Artedius harringtoni* (Cottidae)	35.5	1
	Artedius lateralis (Cottidae)	40.7 +/- 1.8	8
	Enophrys bison (Cottidae)	15.4 +/- 1.2	3

DISCUSSION

The UV absorbance of *Oligocottus* and *Clinocottus* species' mucus was clearly reflective of their habitat, both along a latitudinal gradient and within the intertidal zone. Fishes from high intertidal pools had more sunscreen in the mucus than those from mid or lower intertidal pools at the same site (Figure 5.2). Fish in higher intertidal pools experience more potentially damaging ultraviolet radiation than those in lower intertidal pools, both due to increased tidal exposure and because high pools were shallow with sparse macroalgal cover. High tidepool fish may mitigate their exposure to this radiation through increased mucus absorbance. Both *O. maculosus* and *C. analis* commonly occur in open sandy areas of large high tidepools, whereas *O. snyderi* shows a preference for plant cover in low tidepools (Nakamura 1976, pers. obs.). These behavioral differences may contribute to the significant differences found in mucus absorbance between these species. Similarly, fishes from lower latitudes experience higher levels of UV radiation than those from higher latitudes (Gleason et al. 1993, Madronich et al. 1998), and may defend against this UV radiation with increased mucus absorbance.

UV-absorbing compounds in the mucus and ocular media of fishes are derived from the diet (Mason 1998; Zamzow in review). Judging from the ocular media transmission values, all the *Clinocottus* and *Oligocottus* species surveyed were capable of sequestering UV-absorbing compounds from their diet. However, intertidal fishes do not seem to be simply passively excreting dietary compounds in their mucus. The differences between high and low intertidal species within each site, for example, are apparently not due to differences in dietary availability of UV-

absorbing compounds. *O. maculosus* and *O. snyderi* both consume invertebrates, primarily gammarid amphipods (Grossman 1986). Presumably, within each site the dietary source of MAAs for these species is comparable, yet the UV absorbance of their mucus differs significantly. Furthermore, *Xererpes fucorum* is also known to feed primarily on gammarid amphipods (Grossman 1986), yet this species showed almost no sunscreen in the mucus (Table 5.3). This may be due to the fact that this species is generally found under rocks or in crevices and thus may behaviorally regulate its UV exposure (Eschmeyer et al. 1983). Similarly, *Cebidicthys violaceus* (an herbivore) and *Gobiesox maeandricus* (an invertebrate predator) often shelter under rocks and algae (Yoshiyama 1981, Martin & Bridges 1999), and these species also had low levels of sunscreen in the mucus.

UV absorbance of the mucus varies not only between species (Zamzow & Losey 2002), but also within species as a function of latitude where individuals from higher latitude sites have significantly less sunscreen in the mucus than those from lower latitudes. Surprisingly, significant differences in mucus absorbance were detected between sites separated by only two degrees of latitude. It is likely that the dietary availability of MAAs varies over a latitudinal gradient (Karsten et al. 1998). However, the aforementioned within-site differences in mucus absorbance suggest that differential sequestration or secretion of MAAs, not differential dietary availability, may result in the latitudinal patterns found in this study. Experiments with coral reef fish have shown that fish fed a diet rich in MAAs differentially sequester compounds in the mucus according to their UV exposure regime (Zamzow in review).

It is impossible to say with the data presented here whether the observed variability in different species' mucus absorbance is simply an adaptation to their habitat, or whether the ability to sequester large amounts of sunscreen may be a limiting factor determining the geographic distribution of sister species. Experimental manipulations might help to elucidate this question. Nonetheless, it does seem that tidepool sculpins are able to adapt to their UV environment, on both local and geographic scales. The lack of UVB-blocking compounds found in this study indicates that tidepool species may be susceptible to damage from increasing UVB due to anthropogenic ozone depletion (Gleason et al. 1993).

CHAPTER 6:

BEHAVIORAL RESPONSES

Effects of diet and ultraviolet exposure on the shade-seeking behavior of a Hawaiian coral reef fish

Manuscript

by

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INTRODUCTION

Ultraviolet radiation is damaging to aquatic animals (Hofer 2000). Many studies have explored the lethal effects of UV radiation on aquatic vertebrates, generally on embryonic (Hunter et al. 1979; Battini et al. 2000; Beland et al. 1999), larval (Hunter et al. 1981; 1982; Beland et al. 1999) or juvenile forms (Walters and Ward 1998; Novales-Flamarique and Harrower 1999). Relatively few studies have examined adult animals or the sub-lethal effects of UV radiation on aquatic animals.

Sublethal effects of stressors such as UV (280-400 nm) are often assessed by means of organismal physiology. Winckler (1996) showed that supplementary UVA (320-400 nm) radiation decreased both metabolic rates and oxygen consumption in growing convict cichlids. UVB (280-320 nm) radiation increases the ventilatory frequency of plaice larvae and juveniles, and UV-exposed individuals show decreased respiratory control (i.e., decreased ability to adapt to changing dissolved oxygen levels) as compared to controls (Freitag et al. 1998; Steeger et al. 2001). Physiological studies may also measure the sublethal effects of UV in terms of cyclobutane pyrimidine dimer (CPD) formation, superoxide dismutase activity, p53 expression, or via histological analysis (Nagl and Hofer 1997; Lesser et al. 2001; Armstrong et al. 2002).

Another method of assessment of the sublethal effects of UV is to monitor behavior. Studies with amphibians have shown that UV exposure can impair predator responses, increase activity rates, and cause erratic swimming behavior (Nagl and Hofer 1997; Blaustein et al. 2000; Kats et al. 2000). Sea urchins are known to seek shade or cover themselves with beads that provide shade when exposed to UVR

(Adams 2001; Verling et al. 2002). The one investigation that exists concerning UV-induced behavior in fishes found that newly-emerged alevins and 2-month old juvenile coho salmon avoid UV radiation when given a binary choice between photosynthetically active radiation (PAR, 400-700 nm) and PAR + UVA or PAR + UVA + UVB treatments (Kelly and Bothwell 2002). Some salmonids have been shown to possess UV visual sensitivity (Bowmaker and Kunz 1987; Hawryshyn et al. 1989; Kunz et al. 1994), and coho salmon likely possess UV cones (Beaudet et al. 1997). Salmonid fry are susceptible to UV-induced sunburn lesions (Bullock and Roberts 1981).

Adult fishes react to stressors in a number of ways, including (1) avoidance of the stressor, (2) modifications in feeding behavior, or (3) modifications in activity levels. Avoidance of potential stressors, such as chemicals or adverse water temperatures, has been well studied (reviewed in Beitinger 1990). Appetite suppression is a common stress response in fishes, and is a "sensitive indicator of a stressed fish" (Beitinger 1990). Activity level modifications shown by fish under stressful conditions generally take one of two forms: hyper- or hypo-activity. Brown trout show increased activity when exposed to moderate levels of pollution, but decreased activity when exposed to heavy pollution (Triebskorn et al. 1997). Rainbow trout individuals exposed to identical hypoxic conditions showed two behavioral strategies, "wait and see", or vigorous attempts at avoidance (van Raaij et al. 1996).

The purpose of this study was to investigate the potential effects of UV radiation on the behavior of adult coral reef fish. *Thalassoma duperrey* is a highly

active diurnal omnivore. The ocular lens of *T. duperrey* blocks all UV radiation from reaching the retina, therefore the fish cannot visually perceive UV radiation (Barry and Hawryshyn 1999; Losey et al. in press). Previously, we have shown that *T. duperrey* is able to modify the UV absorbance of its mucus in response to experimental exposure regimes (Zamzow and Losey 2002). In this study, I explored possible stress responses that *T. duperrey* might show in response to UV radiation: shade seeking behavior, swimming behavior, and weight change. Over the course of the three-week experiment, I found no effect of UV (neither presence/absence nor ambient dose) on weight change or swimming behavior. Shade seeking behavior decreased in weeks two and three, and there was a significant correlation with UV dose, regardless of UV treatment.

MATERIALS AND METHODS

Thirty-two Hawaiian saddleback wrasse, *Thalassoma duperrey*, were collected with hook and line from 1-3 m depths in Kaneohe Bay, and transported to the Hawaii Institute of Marine Biology (HIMB). Sixteen 1 x 0.4 x 0.25 m deep fiberglass outdoor tanks at HIMB were equipped with acrylic covers that either transmitted (50% transmission cutoff [T_{50}] = 293 nm) or blocked (T_{50} = 408 nm) UV radiation. Each tank contained a standardized shelter area that consisted of two 25 cm long sections of 6 cm diameter plastic pipe. Fish were randomly assigned to treatments while controlling for size, housed two to a tank, and fed one of two experimental diets daily. Fish in each tank differed from one another by at least 1 cm in length, and could thus be individually identified by an observer. Tanks were

cleaned every day in order to eliminate fouling organisms as a possible food source. Tanks were located adjacent to an Eppley Total Ultraviolet Radiometer, which monitored UV irradiance and recorded hourly and daily UV dosages (mJ/m^2) for the duration of the experiment.

Fish were either exposed to (UV+) or protected from (UV-) UV radiation, and half the fish in each UV treatment were provided a diet rich in MAAs (MAA+), while half received a nutritionally complete, but MAA-free diet (MAA-). Details of diet preparation are published elsewhere (DeKoven et al. 1992; Mason et al. 1998; Zamzow and Losey 2002). Mucus was sampled from each fish on the day of capture, and once per week for 2 weeks thereafter, following Zamzow and Losey (2002). Fish were weighed in seawater prior to mucus sampling. At the conclusion of the study, fish were euthanized by overdose of tricaine methanesulphonate and sexed. One eye was excised, and ocular transmission measured following Losey et al. (2000).

Fish behavior was observed three times daily for three weeks, excluding mucus sampling days (2 days/week). Observations took place between 9 and 10 a.m., 11:30 a.m. and 1:30 p.m., and 3 p.m. and 4 p.m. An observer in a tower took "on the dot" fixed-interval time point data every ten seconds, indicating whether, at that moment, each fish in the focal tank was swimming or resting, and whether the fish was in the shade (shelter area) or sun. Due to variable amounts of shade available along the sides of the tank in the morning and afternoon hours, only noon observations were used in shade vs. sun comparisons. The observer rotated through eight tanks, watching a different tank each ten seconds, until 12 observations of each fish had been made. The proportions of time spent in the shade, and time spent

swimming, could then be calculated for each observation session. As long as the sample interval is short relative to the average duration of the behavior pattern, then instantaneous sampling can produce a record that approximates continuous recording (Martin and Bateson 1993).

Data analyses were performed with SAS systems v 8.02. A two-way ANOVA tested for effects of UV treatment and diet treatment on the change in weight over the course of the experiment. Proportion of time spent swimming was used as the dependent variable in a repeated-measures linear model. Estimation and hypothesis testing in this model used likelihood-based methods, and a compound symmetric covariance structure was assumed, with Satterthwaite determination of degrees of freedom (PROC MIXED, SAS systems, version 8.2). Independent variables tested were UV treatment, diet treatment, UV dose at the time of observations (from the Eppley cell radiometer), fish weight, week of the experiment, time of day, and all pertinent interactions. Non-significant interaction terms were sequentially eliminated and the final model contained only the interaction between time of day and week. For the dependent variable of proportion of time spent in the sun, only noon observations were used, as morning and afternoon observations had variably shaded areas of the tank in addition to the standardized shelter area. A repeated measures linear model was performed on proportion of time in the sun, with independent variables as above with the exception of the time of day variable.

RESULTS

Field-caught fish weighed between 9.9g and 56.9g (mean +/- SE = 29.0 +/- 2.2g). All fish lost weight over the course of the experiment (Figure 1). In general, UV-exposed fish lost more weight than UV-protected fish, however this difference was not statistically significant (ANOVA; UV: $F_{1,28} = 3.16$, $p = 0.09$; Diet: $F_{1,28} = 1.39$, $p = 0.25$).

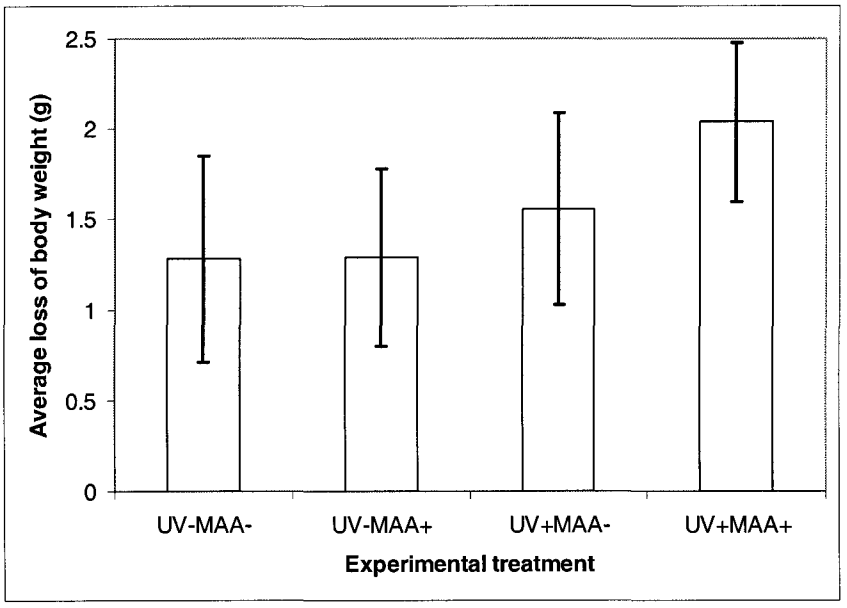

Figure 6.1: Loss of body weight shown by *Thalassoma duperrey* in each of the four experimental treatments

The repeated-measures linear model of proportion of time spent swimming showed significant effects of time of day and week, as well as a significant interaction between time of day and week (Figure 2; Time of Day: $F(2,1312) = 3.57$, $p=0.02$; Week: $F(2,1302) = 53.03$, $p < 0.0001$; Interaction $F(4,1302) = 6.57$, p ¸0.0001). In general, activity levels were higher at noon than at 9am or 3pm, but this relationship did not hold true for week two, hence the significant interaction term. Fish spent less

time swimming in week 1 than in weeks 2 or 3 (least squares means comparison, Tukey post hoc, $p < 0.0001$). There was no effect of fish weight, UV dose, UV treatment, or diet treatment on the proportion of time spent swimming (all $p > 0.1$).

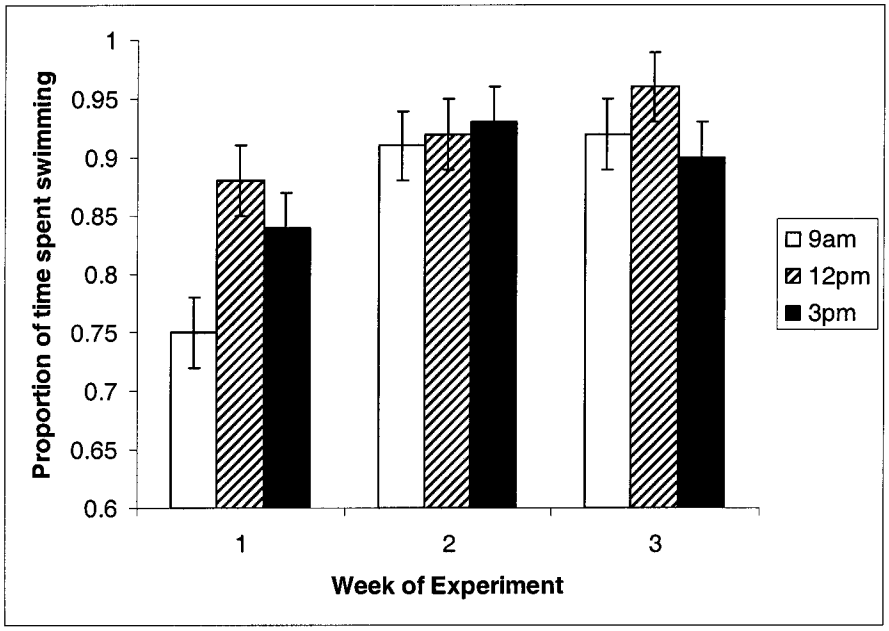

Figure 6.2. *Thalassoma duperrey* swimming behavior over time

The linear model analysis of proportion of time spent in the shade showed significant effects of UV dose, Week of experiment, and a significant UV treatment*Week of Experiment interaction (Figure 3; Dose $F(1,440) = 4.05$, $p < 0.05$; Week $F(2,440) = 18.77$, $p < 0.0001$; UV*Week $F(2,440) = 5.49$, $p < 0.01$). Fish spent less time in the shade in weeks two and three (LSM comparisons, Tukey post-hoc, $p<0.0001$). In week one, UV- fish spent more time in the shade than UV+ fish (Figure 3; LSM, Tukey, $p = 0.002$), but this relationship did not hold in weeks two or three, hence the significant UV*Week interaction. Surprisingly, there was no significant interaction between UV dose and UV treatment (Figure 4; $F(1,440) = 0.05$, $p = 0.82$).

There were no significant effects of fish weight, UV treatment, diet treatment, nor was there a diet*week interaction.

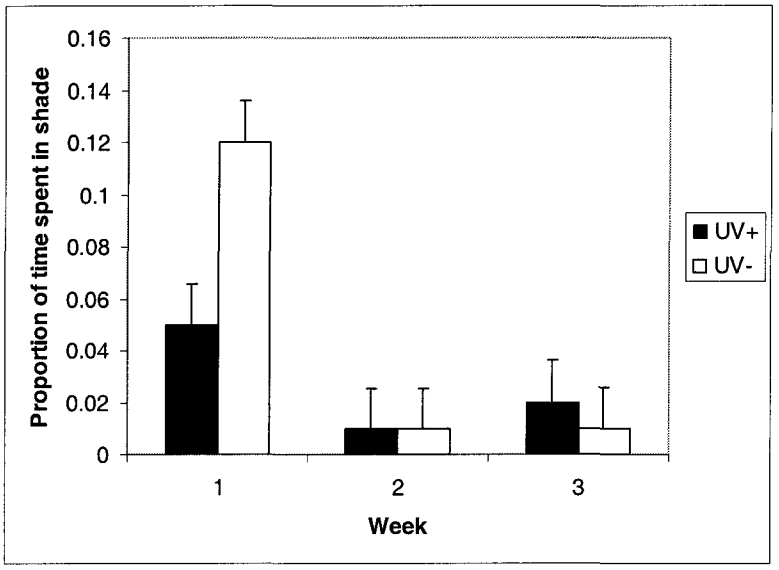

Figure 6.3. Proportion of time spent in the shade by *Thalassoma duperrey* in each experimental UV treatment.

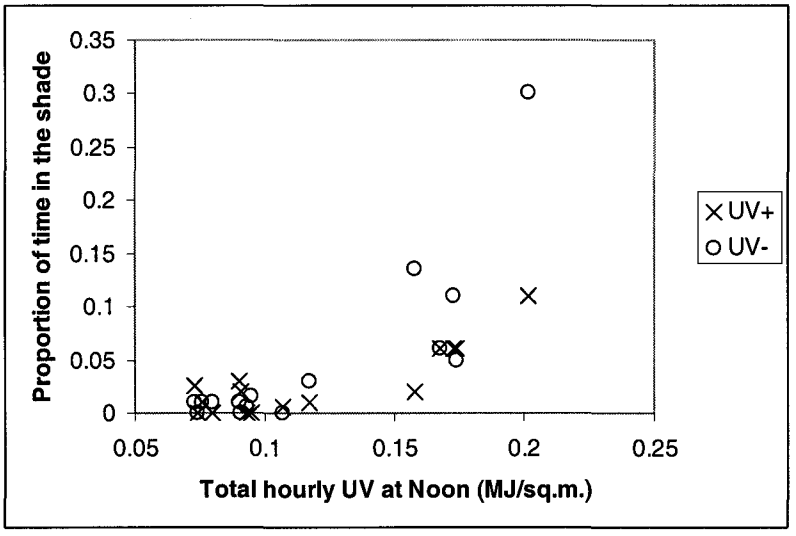

Figure 6.4. Proportion of time spent in the shade by *Thalassoma duperrey* vs. UV dose at noon for each experimental UV treatment.

DISCUSSION

All fish lost weight over the course of the experiment, indicating that they were probably stressed by captivity. There was a trend toward UV-exposed fish losing more weight, perhaps indicating an additional level of stress for these fishes. There was no effect of diet, so the provision of MAAs did not prevent weight loss.

Fish spent less time swimming in the first week of captivity, but there was no effect of UV or diet treatments on swimming behavior. Depressed swimming activity may have been due to the initial stress of capture and captivity, but it occurred across all treatments so no experimental effect was found. Fish tended to swim more at midday than in the morning or afternoon, but this may be an artifact as it did not hold true for week two of the experiment. Again, this trend with swimming behavior occurred across all treatments.

The proportion of time spent in the shade at noon decreased in weeks two and three of the experiment, and in the first week, fish in the UV+ treatment spent more time in the sun than UV- individuals. These results are the opposite of what would be expected if fish were able to somehow sense the damaging UV radiation (either directly, through cellular damage or sunburn, or perhaps via a pineal UV receptor, Shand and Foster 1999). It is possible in nature that fish seek protection from UV in deeper water, and were actively seeking escape from the UV, however we have no evidence to support this. A significant effect of ambient UV dose was found, with more shade-seeking found with higher UV levels, but this occurred across both UV treatments. Hence, it was not an effect of UV, per se, but perhaps was an effect of

higher overall irradiance, or higher water temperatures induced by the higher irradiance.

Overall, we were unable to correlate any aspect of the behavior of *Thalassoma duperrey* with experimental UV treatment, dietary treatment, or UV dose. This is, perhaps, unsurprising as *T. duperrey* cannot visually perceive UV. Previous studies have shown that *T. duperrey* accumulates UV-induced damage, particularly when exposed to UV and prevented from dietary sequestration of MAAs (Zamzow in review). Apparently this damage does not induce an adaptive response in terms of reducing UV exposure through shade-seeking. *Thalassoma duperrey* may show diel, temperature, or overall irradiance-induced behaviors, but definitive demonstration of an effect will require further experimentation.

CHAPTER 7:

SUMMARY AND CONCLUSIONS

This dissertation has detailed my discovery and investigation of sunscreening compounds found in marine fishes' epithelial mucus. UV-absorbing compounds (MAAs and gadusols) appear to be ubiquitous in the mucus of tropical fishes, and are also found in temperate tidepool fishes. My major findings are as follows:

Most diurnal coral reef fishes possess MAAs in the mucus, but the integrated absorbance and center of absorbance can be quite variable. Most species have more than one compound present in the mucus. Visual modeling indicates that a UV-sensitive fish such as *Dascyllus albisella* should be able to discern the difference seen in experimentally UV-exposed and UV-protected fish.

Experimental manipulations of UV (presence/absence) and diet (MAA-containing vs. MAA-devoid) demonstrated that *Thalassoma duperrey* acquires mucus sunscreen compounds from its diet. The amount of sunscreen sequestered is regulated by the UV-exposure regime. Notably, fish that are given MAAs in the diet but protected from UV do not sequester MAAs as do UV-exposed fish. This indicates a physiological cost to the sequestration of MAAs. These experiments also demonstrated that females sequester less sunscreen in the mucus than males, perhaps due to a conflict of interest between providing for epithelial protection vs. protecting the eggs that are spawned daily. Females are also damaged by UV to a greater extent than males.

A field study of three species of coral reef fish (*Canthigaster jactator*, *Chaetodon multicinctus* and *Thalassoma duperrey*) demonstrated that the (putative) concentration of MAAs in the mucus decreases with increasing depth of capture. Evidence for spectral shifting of the mucus absorbance toward longer wavelengths with increasing depth was seen for *Thalassoma duperrey* and possibly *Chaetodon multicinctus*. Fish size did not have an effect on integrated absorbance of the mucus. There was a significant correlation between increasing fish size and shorter-wavelength mucus absorbance for *Thalassoma duperrey*, and a trend toward the opposite effect in *C. multicinctus*. *Thalassoma duperrey* had shorter-wavelength blocking mucus than the other two species, and *C. multicinctus* had higher integrated absorbance values than the other two species.

Temperate tidepool sculpins were found to possess UV-absorbing compounds that decreased in strength with both latitude and elevation within the intertidal zone. Fish from high pools had significantly higher concentrations of sunscreen than fish from low or mid-intertidal pools. Both southern (more equatorial) species and southern individuals of wide-ranging species showed significantly higher sunscreen concentrations than northerly species and individuals. Three non-cottid species (*Cebidichthys violaceus, Xererpes fucorum* and *Gobiesox maeandricus*) that are generally found under rocks or in crevices were found to have very low MAA concentrations in the mucus as compared with the cottids.

Many hours were spent in behavioral observations of *T. duperrey*, but I was unable to find any significant behavioral effect of UV (presence/absence or ambient dose) or diet treatments. There was a trend toward differential weight loss for UV-

exposed fishes, and fish spent less time swimming and more time in the shade during the first week of the experiment. A significant effect of ambient UV dose on shade-seeking behavior occurred across all UV treatments, indicating that the effect stemmed from some other, unmeasured, significant factor. A pattern of swimming activity peaking during the noon hour was noted in two of three weeks of the experiment. This was the most frustrating chapter of my dissertation, and perhaps explains my apparent metamorphosis (degeneration?) from animal behaviorist to physiological ecologist.

Future directions:

A number of future directions are possible in the field of mucus sunscreen, in fact the possibilities are vast. A few topics that might be examined follow.

- One might test a visually UV-sensitive fish in the same fashion as the behavioral experiments with *T. duperrey* (Chapter 6). Juvenile salmonids have been shown to avoid UV, so there's no reason to expect the same might not hold true for a UV-sensitive coral reef species.
- One could repeat my UV x Diet experiment (Chapter 3) while monitoring fecal MAA concentration in order to determine what happens to the dietary sunscreen that isn't sequestered in the mucus by fish in the MAA+ UV- treatment. Are the compounds are being sequestered from the diet and stored or used elsewhere, or are they being excreted in the feces?

- One could perform a comparative study of water environments with different optical properties (green vs. blue vs. tannin rich, etc.) and see if there are spectral correlates in the resident fishes' mucus sunscreen compounds.
- One might test mucus for some really deep water or high latitude fishes. If UV-absorbing compounds are present in the mucus of deep sea fishes, for example, they might not be as adaptive as I have been lead to believe through my research.
- One might look at mucus from other taxa – amphibians, molluscs, etc. I was just told that hippopotomi excrete a pinkish mucus – it would be fun to look at that, or to look at the mucus exuded from the eyes of marine mammals. Really, the possibilities are limitless.

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