The role of multixenobiotic transporters in predatory marine molluscs as counter-defense mechanisms against dietary allelochemicals

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Abstract

Multixenobiotic transporters have been extensively studied for their ability to modulate the disposition and toxicity of pharmacological agents, yet their influence in regulating the levels of dietary toxins within marine consumers has only recently been explored. This study presents functional and molecular evidence for multixenobiotic transporter-mediated efflux activity and expression in the generalist gastropod *Cyphoma gibbosum,* and the specialist nudibranch *Tritonia hamnerorum,* obligate predators of chemically defended gorgonian corals. Immunochemical analysis revealed that proteins with homology to permeability glycoprotein (P-gp) were highly expressed in *T. hamnerorum* whole animal homogenates and localized to the apical tips of the gut epithelium, a location consistent with a role in protection against ingested prey toxins. *In vivo* dye assays with specific inhibitors of efflux transporters demonstrated the activity of P-gp and multidrug resistance-associated protein (MRP) families of ABC transporters in *T. hamnerorum*. In addition, we identified eight partial cDNA sequences encoding two ABCB and two ABCC proteins from each molluscan species. Digestive gland transcripts of *C. gibbosum* MRP-1, which have homology to vertebrate glutathione-conjugate transporters, were constitutively expressed regardless of gorgonian diet. This constitutive expression may reflect the ubiquitous presence of high affinity substrates for *C. gibbosum* glutathione transferases in gorgonian tissues likely necessitating export by MRPs. Our results suggest that differences in multixenobiotic transporter expression patterns and activity in molluscan predators may stem from the divergent foraging strategies of each consumer.

Key words

ABC-transporter, allelochemical, calcein-am, gorgonian, MK571, MRP, P-gp, verapamil

1. Introduction

Soft-bodied benthic organisms produce a vast number of structurally diverse secondary metabolites, many of which function as feeding deterrents in marine systems (Hay et al. 1988; Harvell et al. 1989; Paul 1992; Hay 1996; Stachowicz 2001). For marine consumers, the consequences of ingesting chemically defended prey can be quite severe (Targett et al. 2001), yet specialized consumers that solely feed on toxic prey can apparently tolerate these dietary compounds, and in some cases, even concentrate the defensive compounds for their own protection (Cimino et al. 1985; Cronin 2001). Few studies have explored the physiological targets of these compounds in generalist consumers or the mechanisms by which more specialized predators are able to cope with their toxic prey (Sotka et al. 2009).

The activity of multixenobiotic resistance transporters (MXRs) provides one mechanism by which consumers protect themselves from toxin-laden prey (Epel 1998). MXR proteins may actively export allelochemicals out of cells or facilitate the sequestration of toxins within specialized cells or organelles, effectively compartmentalizing them away from vulnerable protein and DNA targets (Sorensen et al. 2006). Many of the proteins involved in the transport of xenobiotics across membranes belong to the ATP Binding Cassette (ABC) family. Several members of the ABCB (P-glycoprotein; P-gp or MDR) and ABCC (multidrug resistance-associated protein or MRP) subfamilies function as highly promiscuous transporters, capable of trafficking a diverse array of moderately hydrophobic xenobiotics across cell membranes (Bodo et al. 2003). Together, the overexpression of both P-gp and MRP in tumor cells has long been known to mediate the ATP-dependent efflux of anticancer agents, conferring resistance to natural product chemotherapeutic compounds (Deeley et al. 2006; Sarkadi et al. 2006). Only

recently has it been suggested that ABC transporters are responsible for regulating the absorption of allelochemicals in the guts of consumers, and may therefore have a significant influence on the foraging patterns and ultimately diet choice of these organisms (Sorensen et al. 2006; Sotka et al. 2008).

The ubiquity of MXRs in aquatic organisms has been confirmed by immunological cross-reactivity studies, *in vivo* functional assays, competitive inhibition substrate binding assays (Bard 2000; Eufemia et al. 2000; Scherer et al. 2008; Lüders et al. 2009), and more recently by molecular evidence (Goldstone et al. 2006; Goldstone 2008; Sturm et al. 2009; Venn et al. 2009). The distribution of MXRs in tissues involved in absorption, secretion and barrier functions in aquatic invertebrates (Bard 2000) suggests that they may play a role in the prevention of dietary xenobiotic absorption. Furthermore, several pharmacological-based studies have also identified marine natural products from algae, sponges, tunicates, sea hares, gorgonians and marine bacteria that may be substrates for MXR proteins (Suganuma et al. 1988; Chambers et al. 1993; Williams et al. 1993; Aherne et al. 1996; Quesada et al. 1996; Schroder et al. 1998; Tanaka et al. 2002; Shi et al. 2007; Tanabe et al. 2007; Barthomeuf et al. 2008; Henrich et al. 2009), suggesting that the pool of potential substrates in marine ecosystems may be extensive. Given the myriad allelochemically-rich prey and hosts in marine communities, the constitutive or inducible expression of MXRs may serve as a protective counter-response in marine consumers by reducing dietary toxin absorption.

Studies from both human pharmacology (Marchetti et al. 2007) and aquatic systems (Contardo-Jara et al. 2008) (Amé et al. 2009) reveal that natural products from both terrestrial and marine

92 sources can induce the expression and activity of MXRs. If inducers of MXR activity are
93 present in sufficient concentration in the diet of a consumer, ingestion of compounds could result
94 in the enhanced efflux of co-ingested allelochemicals and possibly promote feeding. However,
95 recent evidence also suggests that the unpalatability of some diets may be linked, in part, to the
96 presence of potent MXR inhibitors (Smital et al. 2004) that are produced by the host/prey to
97 directly interfere in efflux activity. These inhibitory compounds could act as "potency
98 enhancers" by blocking transport activity, therefore resulting in increased accumulation of
99 additional noxious allelochemicals (Sorensen et al. 2006). This inhibitory strategy may be
100 employed by chemically defended invasive species to thwart their consumption (Smital et al.
101 1996; Schroder et al. 1998; Smital et al. 2004) by naïve consumers who may lack the adequate
102 molecular architecture to cope with the invasive's chemistry. These findings emphasize the need
103 to explore whether marine consumers that are regularly exposed to a diversity of toxic
104 allelochemicals in their diet may have evolved greater tolerance of chemical defenses if they
105 maintain high levels of ABC transporter protein and/or activity in vulnerable tissues.
106
107 The objective of this study was to characterize the MXR proteins potentially involved in
108 resistance to dietary allelochemicals in two species of tropical gastropods that feed exclusively
109 on allelochemically defended gorgonian corals. A combination of molecular, immunological
110 and functional approaches were used to examine the expression and activity of molluscan ABC
111 transporters in *Tritonia hamnerorum*, a specialist nudibranch that feeds on a single genus of
112 gorgonian, and *Cyphoma gibbosum*, a generalist gastropod that includes multiple gorgonian
113 families in its diet. Evidence from chemical ecology studies in terrestrial systems suggests that
114 generalists, as a result of their chemically diverse diets, have evolved a greater diversity of

115 catalytically versatile xenobiotic resistance mechanisms as compared to specialists that are
116 exposed to a reduced spectrum of allelochemicals due to their narrow foraging range (Li et al.
117 2004; Sorensen et al. 2006; Whalen et al. 2010b). This study presents the initial characterization
118 of MXRs likely responsible for xenobiotic resistance in these two molluscs as part of an effort to
119 obtain a more complete understanding of how generalists and specialists cope with their
120 allelochemical diet(s) (Whalen et al. 2010a; Whalen et al. 2010b).
121
122 **2. Material and Methods**
123 *2.1 Animal collection*
124 In 2004, over 200 adult *Tritonia hamnerorum*, ranging in size from 3 mm to 13 mm, were
125 collected from shallow reefs (< 10m) (Big Point – 23°47.383'N, 76°8.113'W; North Normans –
126 23°47.383'N, 76°8.264'W) surrounding the Perry Institute of Marine Science (PIMS), Lee
127 Stocking Island, Exuma Cays, Bahamas. The purple sea fan, *Gorgonia ventalina*, was the only
128 species of octocoral observed to serve as host for *T. hamnerorum* at our study site. The density
129 of *T. hamnerorum* on individual sea fans ranged from two to over 500 individuals per sea fan.
130 Nudibranchs were collected by removing the portion of sea fan housing them with scissors and
131 transporting both the gorgonian and nudibranchs back to wet laboratory facilities provided by
132 PIMS where they were maintained in flowing filtered seawater until further use. Nudibranchs
133 used for RNA and protein isolation were removed from their host gorgonian, pooled, flash frozen
134 in liquid nitrogen and kept at -80°C until processing.
135
136 In 2006, a total of 141 adult *Cyphoma gibbosum* (ca 2-3 cm length) were collected from five
137 shallow reefs (< 20m) (Big Point – 23°47.383'N, 76°8.113'W; North Normans – 23°47.383'N,

76°8.264'W; Rainbow Gardens – 23°47.792'N, 76°8.787'W; Shark Rock – 23°45.075'N, 76°7.475'W; Sugar Blue Holes – 23°41.910'N, 76°0.23'W) surrounding PIMS. Snails were immediately transported to web laboratory facilities provided by PIMS, where a series of feeding assays were conducted with seven gorgonian species (*Briareum asbestinum, Eunicea mammosa, Gorgonia ventalina, Pseudopterogorgia acerosa, Pseudopterogorgia americana, Pseudopterogorgia elisabethae, Plexaura homomalla*) observed to serve as hosts for *C. gibbosum* in the field. A detailed description of the feeding assay is reported in (Whalen 2008).

2.2 RNA isolation and RT-PCR cloning

Total RNA was isolated from a pool of whole *T. hamnerorum* (267.9 mg; n ~ 40 individuals) using the RNeasy Maxi Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. In addition, a series of feeding assays conducted in 2004 at PIMS with 15 adult *C. gibbosum* feeding on four gorgonian species (*Briareum asbestinum, Gorgonia ventalina, Pseudopterogorgia acerosa, Pseudopterogorgia americana)* provided the material for the initial cloning of ABC transporter cDNA fragments. Upon completion of these feeding assays, *C. gibbosum* digestive glands were immediately dissected and stored in RNALater® at -80°C (n = 15 digestive glands) until further processing. Total RNA was isolated from the pooled *C. gibbosum* digestive glands using RNA STAT-60 (Tel-Test B, Inc., Friendswood, TX) according to the manufacturer's protocol. Poly(A)+ RNA from both molluscan species was then purified using the MicroPoly(A)Purist mRNA purification kit (Ambion, Austin, TX) according to the manufacturer's instructions. First-strand cDNA was reverse transcribed from 2 μg poly(A)+ RNA using OmniScript reverse transcriptase (OmniScript RT kit, Qiagen) with random hexamer primers.

Degenerate primers for MRP were a generous gift from David Epel and Amro Hamdoun, Hopkins Marine Station and were designed against the conserved Walker A/B domains (Allikmets et al. 1998; Dean et al. 2001) (Supplementary Table S1). PCR and nested PCR were performed using AmpliTaq Gold DNA polymerase (Applied Biosystems) under the following conditions: 94^{o}C for 10 min; 35 cycles of 94^{o}C for 15 sec, 52^{o}C for 30 sec; 72^{o}C for 7 min and with degenerate primers pairs (MRP_F/MRP_R and nestMRP_F/nestMRP_R). PCR products were visualized on agarose gels, gel purified (Gene Clean II, Bio 101, Inc.), ligated into pGEM-T Easy© plasmid vector (Promega, Madison, WI), and transformed into JM109 cells (Promega). PCR products were sequenced in both directions using an ABI 3730XL capillary sequencer at the Keck Facility located at the Josephine Bay Paul Center for Comparative Molecular Biology & Evolution at the Marine Biological Laboratory (Woods Hole, MA). A minimum of twelve clones were sequenced for each PCR fragment. Clones were grouped based on nucleotide sequence with Sequencher 4.6 (Gene Codes Corporation) and a consensus sequence was generated and then examined by NCBI/GenBank BLASTX for gene identification (Altschul et al. 1997). BLASTX searches identified two partial sequences from *C. gibbosum* and two partial sequences from *T. hamnerorum* as having homology to MRP transporters; these were designated as CgMRP isoform 1, CgMRP isoform 2, ThMRP isoform 1 and ThMRP isoform 2.

2.3 Rapid amplification of cDNA ends (RACE)

Initial P-gp cDNA fragments were amplified from both gastropod species by RACE using degenerate primers designed to the nucleotide binding domain (Supplementary Table S1) and adaptor-ligated ds cDNA libraries that were constructed as described below (see Supplemental

184 File S1 for additional information). BLASTX searches identified two partial sequences from *C.*
185 *gibbosum* and two partial sequences from *T. hamnerorum* as having homology to P-gp
186 transporters; these were designated as CgPgp isoform 1, CgPgp isoform 2, ThPgp isoform 1 and
187 ThPgp isoform 2.
188
189 In order to obtain additional 5' and 3' sequence for molluscan ABC transporter cDNAs, gene
190 specific primers were designed to initial cDNA fragments for RACE. One microgram of
191 poly(A)+ RNA was isolated for both molluscan species as described above and used to create an
192 adaptor-ligated double-stranded cDNA library synthesized using the Marathon cDNA
193 Amplification Kit (BD Biosciences, Palo Alto, CA) according to the manufacturer's instructions.
194 All primers were obtained from Sigma Genosys (St. Louis, MO) and PCR amplification
195 conditions are described in Supplemental File S1. PCR products obtained from *C. gibbosum* and
196 *T. hamnerorum* RACE reactions were ligated into pGEM-T Easy© plasmid vector (Promega,
197 Madison, WI), transformed into JM109 cells (Promega), sequenced in both directions using an
198 ABI 3730XL capillary sequencer at the Keck Facility located at the Josephine Bay Paul Center
199 for Comparative Molecular Biology & Evolution at the Marine Biological Laboratory (Woods
200 Hole, MA), and analyzed as described below.
201
202 *2.4 Sequence alignments and phylogenetic analysis*
203 Clones were initially grouped based on nucleotide sequence identity (>80%) with Sequencher
204 4.6 and a consensus sequence was generated and examined by NCBI/GenBank BLASTX for
205 gene identification (Altschul et al. 1997). To place the ABC transporter sequences from
206 *Cyphoma* and *Tritonia* within a phylogenetic context, we pursued two lines of phylogenetic

207 approaches. We first assessed the relative position of gastropod sequences in relation to the full
208 suite of human ABC transporters (i.e., subfamilies ABCA-ABCG). Multiple alignments of
209 molluscan deduced amino acid sequences were performed using ClustalW (Thompson et al.
210 1994). Human ABC gene subfamilies were aligned using Muscle v3.7 (Edgar 2004) . In order
211 to maintain the proper relationships between the incompletely sequenced ABC domains, *C.*
212 *gibbosum* and *T. hamnerorum* P-gp and MRP sequences were aligned to each other separately
213 and then to the human ABCB (containing P-gp) and ABCC (containing MRP) families using the
214 profile-profile alignment settings of Muscle. Alignments were automatically masked using the
215 alignment quality scores of Muscle; (cut-off score of 20). Maximum likelihood (ML)
216 phylogenetic analyses were performed with RAxML (v7.0.0; (Stamatakis 2006). Two types of
217 analyses were performed: default rapid hill-climbing ML searches, starting from multiple
218 different randomized maximum parsimony (MP) trees; or 100 replicates of rapid MP
219 bootstrapping to find optimal start trees followed by rapid hill-climbing ML optimization. The
220 PROTMIXWAG model of amino acid substitution was used in all analyses; it uses a category
221 model of the WAG amino acid substitution matrix (Whelan et al. 2001) during the hill-climbing
222 ML search but a gamma distribution of substitution rates for a final tree optimization (Stamatakis
223 2006).
224
225 In the second approach, we assessed the ABCB and ABCC phylogeny from gastropods and
226 several other invertebrates (the nematode *Caenorhabditis elegans*, the arthropod *Drosophila*
227 *melanogaster,* the bivalves *Brachidontes pharaonis* and *Mytilus californianus*) and *Homo*
228 *sapiens.* The putative nucleotide binding domains (hereafter Domains One and Two) were
229 determined using the Conserved Domain Database algorithm on GenBank (Marchler-Bauer et al.

2007). The library *SEQINR* (Charif et al. 2007) within R (http://www.r-project.org) was used to download amino acid sequences from ProtSwiss (http://www.uniprot.org) and extract the approximately 215-220 amino acids that constitute each domain. Domains were aligned independently using ClustalX and then alignments were concatenated by hand. The most appropriate model of protein evolution as determined using ProtTest 2.4 (Abascal et al. 2005); based on AIC scores) was LG+I+G (Le et al. 2008), but because this recently described model is not implemented in most phylogenetic programs, we used the next most fit model (WAG+I+G) (Whelan et al. 2001). A maximum likelihood phylogeny was generated using PHYML v2.4.4 (Guindon et al. 2003) and supported with 1000 bootstrap replicates. A Bayesian phylogeny was generated using MrBayes (Ronquist et al. 2003) with two independent runs of $2x10^7$ generations each (sampled every 1,000 generations) and a burn-in of 20,000 generations. In both ML and Bayesian analyses, the gamma distribution of substitution was estimated using four categories (alpha=2.0), and the proportion of invariant sites was 0.2. Phylogenies were visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree/) and manipulated using Adobe Illustrator®.

2.5 Real-time quantitative RT-PCR and statistical analysis

MRP isoform 1 transcript expression levels in *C. gibbosum* digestive glands were quantified by real-time quantitative PCR using the iCycler MyiQ Real-Time PCR Detection System (Bio-Rad). Digestive gland total RNA from *C. gibbosum* participating in the 2006 feeding assays was purified and DNAse treated using the RNeasy Maxi Kit and RNase-free DNAse Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Poly(A)+ RNA was isolated using the MicroPoly(A) Purist mRNA purification kit according to the manufacturer's instructions. DNAse-treated poly(A)+ RNA (0.2 μg) isolated was used to synthesize cDNA using a blend of

oligo (dT) and random hexamers as described by the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Quantitative RT-PCR reactions contained 12.5 μL 2x SYBR Green Supermix reagent (Bio-Rad), 10 ng cDNA, and 100 nM of each gene-specific primer (136_1_F1/136_1_R1, Supplementary Table S1) in a final volume of 25 μL. The PCR conditions were as follows: 95^{o}C for 3min; 40 cycles of 95^{o}C for 15 sec, 64^{o}C for 1 min. PCR product specificity from each primer pair was confirmed by melt curve analysis to ensure that only a single product was amplified. Initially, a representative group of ten poly(A)+ RNA samples was used to perform control cDNA synthesis experiments without reverse transcriptase to check for contamination by genomic DNA. For each RNA sample, relative transcript abundance was calculated from a standard curve that was generated for each qPCR primer set by serially diluting plasmids containing the fragment to be amplified. Each sample and standard was run in duplicate and the expression of *C. gibbosum* β-actin was used to control for differences in cDNA synthesis among samples.

To examine whether *Cyphoma* MRP isoform 1 transcript expression differed between snails feeding on a control diet versus a gorgonian diet, a two way analysis of variance (ANOVA) was used with Diet (control vs. gorgonian diet) as a fixed factor and Reef (snail origin) as a random factor. The MRP-1 gene was considered a dependent variable. In addition, a one-way ANOVA was used to examine the variability in MRP-1 expression among reefs, where Reef was considered a random factor. This test was used to investigate reef-specific variation in transcript levels in time-zero snails, and to determine if any such variation persisted in snails collected from these same reefs after being fed a control diet for four days. P-values were corrected for

275 the two-way ANOVA analysis using by Bonferroni adjustment (Sankoh et al. 1997). Data
276 analysis was performed using SYSTAT® version 11 (Systat Software, Inc., San Jose, CA).
277
278 *2.6 Western blot for ABC-transporters*
279 Crude homogenates were prepared by homogenizing two *C. gibbosum* digestive glands and
280 whole *T. hamnerorum* in 10 mM potassium chloride, 1.5 mM magnesium chloride, 10 mM Tris-
281 HCl, (pH 7.4), containing 2 mM PMSF and 5% (by vol.) SDS at 4^{o}C. In addition, livers from
282 *Fundulus heteroclitus* collected from New Bedford Harbor, MA, were also homogenized as
283 described and used as a positive control (Bard et al. 2002). The crude homogenate was
284 centrifuged at 14,000 x g for 2 min at 4^{o}C, and the supernatant was stored at -80^{o}C until analysis.
285 Protein concentrations were determined using the bicinchoninic acid (BCA) assay (Smith et al.
286 1985). Crude tissue homogenates were then diluted to 20 μg total protein with standard SDS-
287 PAGE sample buffer (50 mM Tris, pH 6.8, 2% SDS, 10% sucrose, 0.2% β-mercaptoethanol,
288 0.001% bromphenol blue), denatured at 100^{o}C for 3 min, and loaded onto a 7.5% Tris-glycine
289 gel using a 4% stacking gel. Prestained molecular markers were purchased from Amersham
290 (Full-range Rainbow MW Markers, GE Healthcare, Buckinghamshire, UK). Following
291 electrophoresis, proteins were transferred onto a 0.45 μm nitrocellulose membrane (Schleicher &
292 Schuell, Keene, NH) at 15 V for 54 min. The membrane was blocked in 5% (wt/vol) non-fat
293 milk in Tris buffered saline-Tween 0.1% (TBST) for 1 hr and then hybridized overnight with
294 rocking at 4^{o}C in TBST containing 0.4 μg/mL of the monoclonal antibody C219 (Signet
295 Laboratories, Dedham, MA). The membrane was subsequently washed (3 x 10 min) with TBST
296 and incubated for 1 hr. with horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson

297 ImmunoResearch Inc., West Grove, PA) diluted 1:7000 in TBST. Detection of HRP activity was
298 by enhanced chemoluminescence (ECL) onto Hyperfilm ECL (Amersham).
299
300 *2.7 Immunohistochemical analysis*
301 Live nudibranchs and snails were transferred to a 50 mL beaker containing filtered seawater and
302 were relaxed by adding a few crystals of magnesium sulfate until they no longer recoiled to the
303 touch. Digestive glands and mantle tissues from *C. gibbosum* (n = 4) and whole *T. hamnerorum*
304 individuals (n = 2) were fixed for 2 hours at 4^{o}C in a buffered seawater solution of 2%
305 paraformaldehyde, 2.5% glutaraldehyde (Karnovsky's Fixative) obtained from Electron
306 Microscopy Sciences (Hatfield, PA). Tissue samples were then transferred to PBS and kept at
307 4^{o}C till embedding. Paraffin-embedded samples were sectioned at 5 μm and mounted on
308 Superfrost Plus (Fisher Scientific, St. Louis, MO) microscope slides, deparaffined, and then
309 hydrated with normal mouse serum in 10 mM phosphate buffered saline (pH 7.5). Sections were
310 incubated for 1 hr. at room temperature with the mAb C219 diluted (2 μg/mL) in Primary
311 Antibody Diluent (Signet). Antibody binding was visualized with a commercial avidin-biotin-
312 peroxidase kit using diaminobenzidine tetrahydrochlroide (DAB) as the chromogen (Vectastain®
313 Elite ABC Kit, Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's
314 instructions. Specific staining was evaluated by light microscopy by comparison of mAB C219
315 stained and stained serial sections with normal mouse serum.
316
317 *2.8 In-vivo dye transport assays*
318 MK571 was purchased from Cayman Chemical (Ann Arbor, MI). Calcein-acetoxymethylester
319 (C-AM) was purchased from Axxora (San Diego, CA). Calcein was purchased from Invitrogen

(Carlsbad, CA). Verapamil was purchased from MP Biomedicals (Solon, OH). Concentrated stocks of MK571 and C-AM were prepared in DMSO and were sufficiently concentrated so that the DMSO volume did not exceed 0.5% of the experimental volume.

ABC transporter activity was measured by the accumulation of calcein in whole *T. hamnerorum* individuals. Calcein-AM (C-AM) is a non-fluorescent substrate for both MRP and P-gp. Any C-AM entering the cell, if not transported out immediately by either ABC transporter, is rapidly hydrolyzed by intracellular esterases to form calcein, a fluorescent membrane-impermeable molecule (Essodaigui et al. 1998). Therefore, exposure to inhibitors of ABC transporter activity results in high calcein accumulation and increased fluorescence, while reduced intracellular accumulation of calcein and fluorescence is observed when transporter activity is increased.

Nudibranchs maintained in the laboratory were removed from their host, *G. ventalina*, and allowed to depurate for four hours in a beaker of sterile-filtered seawater with aeration. Five similarly sized *T. hamnerorum* (~ 7 mm in length) were placed in each well of a 24-well plate and incubated for two hours in 3 mL of 500 nM C-AM resuspended in sterile-filtered seawater. Incubations were performed in the presence or absence of two inhibitors at two concentrations. MK571 specifically inhibits MRP transport activity, while verapamil inhibits P-gp transport activity. Nudibranchs were then rapidly washed two times with sterile-filtered seawater and homogenized for 30 seconds using an electric drill fitted with a Teflon pestle in a 2 mL Wheaton glass homogenizer containing 125 µL of ethanol. The homogenate (~ 175 µL) was transferred to a 1.5 mL tube and centrifuged at room temperature for 7 min at 6.1 relative centrifugal force (RCF). Eighty-five microliters of supernatant was transferred to a 96-well black plate containing

343 a clear bottom to minimize light scattering. The level of calcein in the resulting supernatant was
344 measured fluorimetrically (λ_{ex} = 485 nm, λ_{em} = 530 nm) using a Cytofluor 2300 plate reader
345 (Millipore). The ratio of intracellular calcein fluorescence in the absence and presence of
346 MK571 or verapamil was used as a measure of the activity of *T. hamnerorum* MRP and P-gp
347 transporters. Statistical analysis was performed using a two sample *t* test of the means with a
348 significance level of 5%. *In vivo* dye assays were unable to be performed with *C. gibbosum*
349 tissues due to the highly variable natural auto-fluorescence of the digestive gland in the absence
350 of C-AM.
351
352 **3. Results**
353 *3.1 Identification and phylogenetic analysis of ABC transporters*
354 To define the range of ABC transporter genes expressed in molluscan tissues, a RT-PCR cloning
355 approach with pooled mRNA samples and degenerate oligonucleotide primers was used initially
356 to identify P-gp and MRP fragments with sequence corresponding to the conserved nucleotide
357 binding domain of ABC transporter genes. BLASTX (NCBI) comparison of translated cDNAs
358 indicated homology to mammalian ABCB and ABCC proteins. Initial gene fragment sizes were
359 as follows: *C. gibbosum* - CgMRP isoform 1 and CgMRP isoform 2, 168 bp; *C. gibbosum* -
360 CgPgp isoform 1 and CgPgp isoform 2, 216 bp; *T. hamnerorum* - ThMRP isoform 1 and ThMRP
361 isoform 2, 170 bp; *T. hamnerorum* - ThPgp isoform 1 and ThPgp isoform 2, 71 and 182 bp
362 respectively. Specific oligonucleotide primers designed to these initial fragments were used in 5'
363 and 3' RACE reactions to obtain the eight partial MXR sequences ranging in length from 390 to
364 4647 bp (Table 1).
365

366 Deduced amino acid alignments of the gastropod MRPs and P-gps generated from nucleotide
367 consensus sequences are shown in Figures S1 and S2 in Supplemental material. ThMRP isoform
368 1 amino acid alignments with human ABCC sequences revealed a nearly full-length coding
369 sequence of 1549 amino acids. A partial sequence encoding 951 amino acids, including the
370 putative start codon and first two transmembrane spanning domain/nucleotide binding domain,
371 was obtained for CgMRP isoform 1. Polypeptide lengths of 184 and 765 amino acids were
372 obtained for CgMRP isoform 2 and ThMRP isoform 2, respectively, both of which aligned to the
373 first nucleotide binding domain (Figure S1, Supplemental material). An alignment of gastropod
374 P-gp amino acid sequences with human ABCB1 revealed that both *Tritonia* P-gp sequences align
375 with the first nucleotide binding domain, while *Cyphoma* P-gp sequences cover the second half
376 of the protein, including the stop codon (Figure S2, Supplemental material). In addition,
377 hydrophobicity plots (Kyte et al. 1982) of *C. gibbosum* and *T. hamnerorum* ABC transporters
378 agreed well with human ABCB1 and ABCC3 models (Figure S3, S4 in Supplemental material),
379 increasing our confidence in the assignment of molluscan transmembrane and nucleotide binding
380 domains.
381
382 Phylogenetic analysis of the deduced amino acid sequences revealed that the gastropod ABC
383 transporters belong to the ABCB and ABCC subfamilies (Figure 1). We constructed a
384 phylogeny of ABCB and ABCC subfamilies in order to better define the relationships among
385 proteins from humans, gastropods and a variety of other invertebrates (Figure 2). There is strong
386 Bayesian and ML support (1.0 and 99%, respectively) for the monophyly of these two
387 subfamilies, and sequences within subfamilies are more similar to each other (Mean ± S.D.

388 pairwise similarity of ABCB subfamily = 48 ± 8%; ABCC subfamily = 54 ± 7%) than sequences
389 of the other subfamily (Mean ± S.D. pairwise similiarity = 29 ± 3%).
390
391 Within the ABCB subfamily, *Tritonia* Pgp isoform 1 and *Cyphoma* Pgp isoform 2 are embedded
392 within a well supported clade (0.99 Bayesian posterior probability) that includes sequences from
393 human (ABCB2, ABCB3, ABCB8, ABCB9 and ABCB10) and *Mytilus californianus* ABCB-
394 like protein. *Tritonia* Pgp isoform 2 and *Cyphoma* Pgp isoform 1 cluster with each with other
395 (0.91 Bayesian posterior probability) and sequences from human (ABCB5, ABCB11, ABCB1,
396 and ABCB4) and the bivalve *Brachidontes pharaonis* (1.0 Bayesian posterior probability).
397
398 Within the ABCC subfamily, two *Tritonia* (ThMRP isoform 1 and ThMRP isoform 2) and a
399 *Cyphoma* (CgMRP isoform 1) sequences cluster significantly with human (ABCC1, ABCC2,
400 ABCC3 and ABCC6), *Drosophila* (CG6214) and *Mytilus californianus* sequences (1.0 and 48%
401 by Bayesian and ML analysis, respectively). *Cyphoma* isoform 2 is embedded within a clade
402 with human sequences (ABCC5, ABCC11, and ABCC12).
403
404 *3.2 Quantitative RT-PCR analysis of Cyphoma ABCC (MRP-1) expression*
405 Quantitative RT-PCR analysis was used to investigate the constitutive and inducible expression
406 of MRP isoform 1 transcripts in *Cyphoma* after dietary exposure to several gorgonian species
407 with varying allelochemical profiles. *Cyphoma* MRP isoform 1 was selected for expression
408 analysis because it is found within a well-supported clade that contains both human MRP1 and
409 MRP2 (ABCC1 and ABCC2, respectively), known for their ability to transport a range of
410 xenobiotics and phase II metabolites, including glutathione-conjugated substrates (Keppler et al.

411 1997; Nies et al. 2007; Zimmermann et al. 2008). This is particularly relevant to the present
412 study because *Cyphoma* digestive gland tissues constitutively express high levels of the
413 glutathione-conjugating enzyme, glutathione *S*-transferase, likely necessitated by the presence of
414 numerous potent inhibitors and/or substrates in its gorgonian diets (Whalen et al. 2008; Whalen
415 et al. 2010b).
416
417 Digestive gland CgMRP isoform 1 transcripts were measured by quantitative RT-PCR from
418 adult *Cyphoma gibbosum* allowed to feed ad libitum for four days on one of seven gorgonian
419 diets or a control diet lacking gorgonian chemistry. MRP isoform 1 transcripts were
420 constitutively expressed in *Cyphoma* digestive gland and the expression was not affected by
421 gorgonian diet (Supplemental Table S3, Figure 3). MRP isoform 1 transcript expression was
422 also measured in time-zero individuals to determine if collection location had any influence on
423 gene expression. MRP isoform 1 expression was found to significantly differ in time-zero snails
424 as a function of reef location (ANOVA, $F_{4,26} = 2.74$, $p = 0.05$, Figure 4 and Table S4 in
425 Supplemental material), but did not significantly differ among snails allowed to feed on a control
426 diet for four days (ANOVA, $F_{4,28} = 0.637$, $p = 0.640$).
427
428 *3.3 Expression and localization of ABCB1 (P-gp)*
429 Immunochemical detection of P-gp-like enzymes in molluscan tissue homogenates with the
430 monoclonal antibody C219 revealed the presence of immunoreactive bands only for *Tritonia*
431 homogenates. The antibody recognized a distinct band of expected size, 170 kDa (Juliano et al.
432 1976), and two larger diffuse bands, > 200 kDa, possibility representing differentially
433 glycosylated isoforms of P-gp (Schinkel et al. 1993) or the presence of multiple proteins in

434 *Tritonia* (Figure 5). However, similar immunoreactivity was absent for *Cyphoma* digestive
435 gland homogenates. Based on deduced amino acid sequences, the lack of reactivity in *Cyphoma*
436 homogenates is somewhat surprising considering that at least one of the *Cyphoma* proteins
437 (CgPgp isoform 1) contains the epitope VQEALD, among those recognized by C219. Although
438 C219 recognizes this epitope containing Glu (E), it does so less strongly than the epitope
439 containing Ala (A) (VQAALD) (Georges et al. 1990). The partial *Tritonia* Pgp isoform 1
440 sequence contains the Ala at this position (VQAALD), which may explain the difference in
441 reactivity between molluscan species. Alternatively, or in addition, there may be differences in
442 P-gp expression that contribute to the differential recognition by C219 in these immunoblot
443 studies.
444
445 The cellular localization of P-glycoprotein was examined in fixed, paraffin-embedded molluscan
446 tissue sections probed with mAb C219. P-glycoprotein expression was detected in the apical tips
447 of the ciliated columnar epithelia cells lining the lumen of the midgut in *T. hamnerorum* (Figure
448 6). In the non-distended midgut, immunohistochemical staining helps to accentuate the folded
449 nature of this heavily ciliated epithelia (Millott 1937; Morse 1968). Faint staining was also noted
450 in goblet-like cells found on the nudibranch's epidermis, which may have a role in mucus
451 secretion (Figure 6A). In contrast to the staining in *T. hamnerorum* tissues, immunoreactive
452 protein was not detected in *C. gibbosum* mantle or digestive gland tissues. Negative control
453 sections of *T. hamnerorum* (Figure 6E) or *C. gibbosum* (data not shown) tissues stained with
454 normal mouse serum displayed no immunolabelling.
455
456 *3.4 In vivo calcein efflux transport assay*

In vivo examination of transporter activity in *Tritonia* was assessed using the calcein efflux assay. The calcein assay is a functional diagnostic method developed to quantify MXR-mediated efflux activity in cells and tissues. Non-fluorescent calcein-AM rapidly traverses cell membranes where it can be converted to fluorescent calcein by non-specific esterases or extruded by P-gp and MRP before intracellular conversion to the non-MXR substrate. High levels of MXR transport activity can prevent the accumulation of free calcein in the cell, resulting in minimal fluorescent calcein accumulation. Transporter-specific MXR-mediated efflux can be measured by the use of specific MRP (MK571) and P-gp (verapamil) inhibitors. If transport activity is minimal, the transport inhibitor will have limited or no effect resulting in little change in cell fluorescence. However, if transport activity is significant, the presence of an inhibitor will increase calcein accumulation, as indicated by intracellular fluorescence, compared to incubations without the inhibitor present.

Changes in fluorescence intensity of calcein in *Tritonia* tissues incubated in the presence of 500 nM extracellular C-AM with and without varying concentrations of inhibitors are shown in Figure 7. The addition of verapamil or MK571 to the seawater containing *Tritonia* significantly increased calcein accumulation compared to DMSO controls. These results indicate that MXR-mediated efflux activity prevents calcein accumulation in *Tritonia* tissues and that inhibiting this activity causes calcein-AM to be retained and hydrolyzed to calcein. Calcein accumulation varied depending on the inhibitor used and its concentration. Increasing verapamil concentration to 25 μM did not cause a measurable increase in calcein accumulation compared to 5 μM of verapamil, suggesting that the lower concentration of verapamil is sufficient to block the majority of P-gp transporters. In contrast, 30 μM MK571 resulted in a further 57% increase in

480 calcein accumulation compared to that caused by 5 μM MK571, and the calcein accumulation
481 was greater than the maximal level obtained in the presence of verapamil. This difference in the
482 absolute level of calcein accumulation suggests that MRP transporters are either more highly
483 expressed or more efficient at exporting C-AM in *Tritonia* tissues.
484
485 **4. Discussion**
486 Our knowledge of the function of ABC transporters in marine invertebrates is mostly limited to
487 the response of these proteins under anthropogenic stress conditions (Kurelec 1992; Minier et al.
488 1993; Smital et al. 2000; Smital et al. 2003; Kingtong et al. 2007). However, for marine
489 consumers that regularly feed on prey/hosts laden with natural toxins, efflux transporters may
490 play a critical role in regulating the absorption of these allelochemicals, thereby influencing diet
491 selection. This study is the first to examine the expression and activity of ABC transporters from
492 two predatory molluscs that feed solely on chemically defended prey.
493
494 *Molluscan P-glycoproteins*
495 Using a combination of molecular approaches, four partial cDNA sequences of P-gps related to
496 multixenobiotic transporters in vertebrates were identified from the digestive gland of *Cyphoma*
497 *gibbosum* (CgPgp isoform 1, CgPgp isoform 2) and whole *Tritonia hamnerorum* (ThPgp isoform
498 1, ThPgp isoform 2). ThPgp isoform 2 and CgPgp isoform 1 clusters with human ABCB1,
499 ABCB4, ABCB5 and ABCB11 genes. ABCB1 (MDR1 or Pgp1) is possibly the most well
500 studied of all the multixenobiotic transporters and is known for its promiscuous transport of
501 hydrophobic substrates, including xenobiotics, lipids, sterols, and chemotherapeutic agents
502 derived from natural products (Choudhuri et al. 2006). Human ABCB4 (MDR3) and ABCB11

503 are both expressed in the liver and are involved in the secretion of bile acids (Gerloff et al. 1998;
504 Oude Elferink et al. 2007), while ABCB5 is hypothesized to function as a drug resistance
505 mediator, similar to ABCB1, and is primarily expressed in the epidermis (Frank et al. 2005).
506 Acquisition of complete ABCB sequences would help to better define the relationships of the
507 molluscan P-gps with particular ABCB members. Nevertheless, the phylogenetic analysis
508 presented here clearly groups these molluscan transporters with those ABCB members known to
509 mediate xenobiotic resistance.
510
511 *Tritonia* Pgp isoform 1 and *Cyphoma* Pgp isoform 2 appear to cluster with human half
512 transporters ABCB2(TAP1), ABCB3(TAP2), ABCB8(M-ABC1), ABCB9 and ABCB10(M-
513 ABC2) genes. Human ABCB2 and ABCB3 encode half transporters that form a heterodimer
514 and transport peptides derived from proteasomal degradation from the cytosol into the
515 endoplasmic reticulum for loading onto major histocompatibility complex (MHC) class I
516 molecules (Sturm et al. 2009). Human ABCB9 is homodimeric and shares structural similarities
517 to ABC2/3, however its function has yet to be defined, while human mitochondrial transporters
518 ABCB8 and ABCB10 function in iron metabolism and transport of Fe/S protein precursors. It is
519 therefore possible that these gastropod isoforms are also half transporters; however, since
520 invertebrates lack the mammalian adaptive immune response, the functional role of these
521 molluscan transporters is not so obvious.
522
523 Western blot analysis of molluscan tissues with the mAb C219, which recognizes both human
524 MDR1 (ABCB1) and MDR3 (ABCB4) proteins (Van den Elsen et al. 1999), suggested that
525 homologs of human MDR proteins are expressed in *Tritonia* tissues but not in *Cyphoma*

526 digestive gland. The apparent disparity in P-gp expression between the two predatory molluscs
527 may reflect differences in their foraging behavior. A previous study showed that specialist
528 herbivores that consumed a diet rich in allelochemicals had a higher intestinal P-gp capacity in
529 comparison to sympatric generalists that were unable to tolerate the toxin-laden diet (Green et al.
530 2004). These results suggest that dietary toxins could be handled differently depending on
531 consumer experience.
532
533 Deduced amino acid sequences from two partial *Cyphoma* P-gps indicate that at least one of the
534 forms (CgPgp isoform 1) contains the correct epitope (VQEALD) in its nucleotide binding
535 domain and therefore would be recognized by C219 if expressed in the digestive gland. It is
536 possible that CgPgp isoform 1 is expressed in tissues other than the digestive gland; however,
537 immunohistochemical analysis of whole *Cyphoma* tissue sections with C219 did not detect any
538 immunoreactive protein. Based on sequence data, multiple forms of P-gp are expressed in
539 *Cyphoma* digestive gland, yet their detection may be limited by the reactivity of the antibody
540 used here, suggesting that western blots should be interpreted with caution. In contrast, *Tritonia*
541 tissue homogenates displayed a distinct band at 170 kDa, in agreement with human MDR
542 proteins (Choudhuri et al. 2006) and two diffuse bands at > 200 kDa. Similar multiple banding
543 patterns (i.e., 170 kDa, and > 200 kDa banding) have also been observed in the gill tissue of
544 mussels *Mytilus californianus*, *M. galloprovincialis*, and *M. edulis* and oysters *Crassostrea gigas*
545 and *C. virginica* (i.e., > 200 kDa) (reviewed in Bard 2000). Protein bands > 200 kDa may
546 represent post-translational modifications or differential glycosylation states, not uncommon
547 among mammalian P-glycoproteins (Schinkel et al. 1993).
548

549 Immunohistochemical staining of *Tritonia* tissues by C219 indicated that P-gp proteins were
550 localized to the apical epithelial of the midgut and to a lesser extent in the epidermis. The
551 location of P-gp in *Tritonia* digestive tissues is in agreement with the localization of mammalian
552 ABCB1 on the apical (or luminal) surface of polarized epithelia of many tissues, such as the
553 gastrointestinal tract, kidney proximal tubules and biliary heptocytes (Klein et al. 1999). Based
554 on the location of mammalian ABCB1 in barrier tissues coupled with its function as a
555 unidirectional transporter of a range of toxic substrates, it is likely that the physiological role of
556 ABCB1 and its orthologs is to protect cells and ultimately organisms against toxic compounds
557 (Schinkel et al. 1994; Schinkel et al. 1997). The two tissues types observed to exhibit the
558 greatest P-gp expression in *Tritonia*, the gut epithelia and the epidermis, correspond to those
559 tissues that would be exposed to the greatest concentration of gorgonian allelochemicals, due to
560 the fact that *Tritonia* both resides on and consumes its gorgonian host.
561
562 Interestingly, this nudibranch is able to selectively sequester a furano-germacrene feeding
563 deterrent from its gorgonian host, *Gorgonia ventalina*, and concentrate this chemical defense
564 four-fold (dry mass basis) relative to the gorgonian (Cronin et al. 1995). While the exact
565 mechanism of sequestration in nudibranch tissues is unknown, recent studies have demonstrated
566 that mammalian MXR transporters can reduce the intracellular concentration of toxins by
567 actively sequestering them in subcellular compartments, away from vulnerable cellular targets
568 (Van Luyn et al. 1998; Molinari et al. 2002; Rajagopal et al. 2003; Ifergan et al. 2005) or within
569 certain organs (Jonker et al. 2005). Furthermore, MXR members can have different activity
570 profiles depending on membrane location (i.e., plasma membrane versus lysosomal membrane),
571 which may be linked to the lipid environment or post-translational modification of the

572 transporter that can occur within subcellular compartments (Rajagopal et al. 2003). This
573 intracellular sequestration phenomenon has also been observed in mussel blood cells, whereby
574 the intracellular accumulation of the fluorescent P-gp substrate Rhodamine B in lysosomes could
575 be reversed if incubated with verapamil (Svensson et al. 2003). In addition, MXR-mediated
576 subcellular localization is suspected to be responsible for the sequestration of the marine toxin,
577 okadaic acid in the mussel *Mytilus edulis* (Svensson et al. 2003). Transporters have also been
578 suggested as a mechanism by which herbivorous insects can sequester unmetabolized host plant
579 toxins in diverticular pouches, defensive glands or hemolymph (Sorensen et al. 2006). Chemical
580 analysis of nudibranch tissues indicated that diet-derived allelochemicals are selectively
581 accumulated in the mantle border and in mucus secretions (Pawlik et al. 1988; Garcia-Gomez et
582 al. 1990; Fontana et al. 1994; Avila et al. 1997). In *Tritonia*, the precise tissue location of toxin
583 sequestration is unknown; however, P-gp immunoreactive protein was detected on this
584 nudibranch's epidermis, a location consistent with the site of toxin accumulation in other
585 nudibranch species. Further work is needed to determine whether ABCB1-like proteins within
586 the dermis of nudibranchs participate in toxin accumulation; nevertheless, the presence of ABC
587 transporters in these specialist consumers may not only function as a protective mechanism
588 against cytotoxicity, but may have an added advantage of providing these consumers with their
589 own chemical protection.
590
591 The *in vivo* activity of MXR transporters was investigated in *Tritonia* using ABC subfamily
592 specific transport inhibitors capable of blocking the P-gp- and MRP-mediated efflux of C-AM.
593 Both inhibitors resulted in increased dye accumulation in *Tritonia* tissues, which is consistent
594 with previous competitive dye transport assays reporting MXR-mediated transport activity in a

595 variety of marine invertebrates (grass shrimp (Finley et al. 1998), worm (Toomey et al. 1993),
596 sponge (Muller et al. 1996), oyster (Keppler 1997), mussel (McFadzen et al. 2000), sea urchin
597 (Hamdoun et al. 2004)). Varying inhibitor concentration did not increase P-gp-mediated calcein
598 accumulation, but did cause a substantial impact on MRP-mediated calcein accumulation. The
599 greater level of calcein accumulation in *Tritonia* exposed to MRP inhibitor suggests that MRP-
600 mediated efflux may contribute more to xenobiotic resistance than P-gp in *Tritonia*.
601
602 *Molluscan multixenobiotic resistance-associated proteins*
603 While both subfamilies of transporters confer multixenobiotic resistance, the substrate
604 selectivities of P-gp and MRP differ markedly. P-gp transport neutral or mildly positive
605 lipophilic compounds, while MRP substrates are lipophilic anions capable of transporting a
606 range of substrates including glutathione (GSH), glucuronide, or sulfate conjugates of phase II
607 detoxification reactions (Kruh et al. 2003). Phylogenetic analysis of molluscan MRP sequences
608 obtained here indicate that CgMRP isoform 1, ThMRP isoform 1 and ThMRP isoform 2 fall
609 within a well-supported clade containing the mammalian ABCC1, ABCC2, ABCC3 and ABCC6
610 proteins, known for their ability to transport a variety of glutathione conjugates including natural
611 product cancer drugs and prostaglandins (Evers et al. 1997; de Waart et al. 2006). The
612 Caribbean gorgonian *Plexaura homomalla* is regularly consumed by *Cyphoma* despite the high
613 concentration of prostaglandin A_2 esters (Gerhart 1986), which function as potent feeding
614 deterrents in this coral (Gerhart 1984; Pawlik et al. 1989). Recent evidence suggests that
615 *Cyphoma* may be able to tolerate dietary prostaglandins and other lipophilic gorgonian
616 compounds by conjugating them with GSH, catalyzed by glutathione *S*-transferases (GSTs).
617 GSTs are highly expressed in the digestive gland of this predator, and a screening of gorgonian

618 lipophilic extracts suggests that all gorgonian diets may contain substrates for *Cyphoma* GSTs
619 (Whalen et al. 2010b). Therefore, the constitutive expression of MRP isoform 1 in the digestive
620 gland of *Cyphoma* may facilitate the efficient biliary excretion of putative glutathione-conjugates
621 of prostaglandins and other lipophilic compounds (Evers et al. 1997; Paumi et al. 2003; de Waart
622 et al. 2006). MRP isoform 1 expression was also detected in the snails feeding on control diets
623 lacking gorgonian allelochemicals, suggesting that these transporters may also be responsible for
624 the export of physiological substrates, in addition to their roles in multixenobiotic resistance. In
625 contrast, MRP isoform 1 expression significantly varied in time-zero snails as a function of reef
626 location. While it is difficult to conclusively pin point the cause of this reef-specific variation,
627 the length of snail foraging time on a single colony or the presence of additional gorgonian
628 species not tested in our feeding assays may have had an effect on MRP isoform 1 expression.
629
630 Phylogenetic analysis revealed that *Cyphoma* MRP isoform 2 is significantly divergent from the
631 other molluscan MRPs identified in this study and falls into the clade containing human ABCC5,
632 ABCC11 and ABCC12. All three of these transporters are at the early stages of investigation
633 and their physiological functions are not well understood. However, studies indicate that they
634 mediate the transport of antiretroviral nucleosides and lipophilic glutathione-conjugates
635 (Wijnholds et al. 2000; Kruh et al. 2007).
636
637 **Conclusions**
638 Mounting evidence suggests that ABC transporters likely regulate the absorption and subsequent
639 distribution of natural toxins in marine organisms (Toomey et al. 1993; Toomey et al. 1996;
640 Keppler et al. 2001; Eufemia et al. 2002). The present work describes the first efforts to identify

641 MXR genes that may protect marine invertebrates that have adapted to exclusively feed on
642 allelochemically-rich prey. Messenger RNA encoding MXR transporters is expressed in both
643 generalist and specialist molluscan species and these transporters share sequence and
644 organizational structure similar to MXRs of distantly related organisms, indicating the likelihood
645 of shared function as natural product transporters between mammalian and molluscan orthologs.
646 A second piece of evidence supporting the role of MXR proteins as dietary allelochemical efflux
647 pumps comes from the results of immunohistochemical analyses in *Tritonia* tissues, where P-gp
648 expression was highest in the tissues most vulnerable to allelochemical exposure. Additionally,
649 *in vivo* studies of MXR activity using ABC subfamily-specific inhibitors provided added support
650 for the presence and function of MXR transporters in *Tritonia*. However, for organisms where *in*
651 *vivo* dye assays may not be feasible, real-time qPCR provided a highly robust and sensitive
652 method for quantifying MXR isoform-specific expression. Constitutive expression of selected
653 promiscuous MXR transporters may be advantageous to a generalist predator like *Cyphoma* that
654 maintains a chemically diverse diet. Support for this hypothesis may come from screening
655 gorgonian extracts in competitive substrate inhibition assays with transfected cell lines or
656 isolated membrane vesicles overexpressing molluscan MXRs of interest. This initial screening
657 approach would facilitate comparisons of substrate breadth between MXRs from generalist and
658 specialist consumers and allow one to begin to test predictions about whether generalist MXRs
659 possess greater structural and functional flexibility in their substrate specificity compared to
660 specialists (Li et al. 2004).
661
662 The elucidation of the molecular mechanisms underlying consumer tolerance is a prerequisite to
663 understanding the foraging behavior of marine consumers. This study provides a much needed

first step in identifying putative environmental chemical stress genes capable of ameliorating allelochemical-induced toxicity. It is likely that both MRP and P-gp play significant roles in natural toxicant efflux in marine consumers. Future work aimed at elucidating those marine natural products capable of interacting with substrate-binding sites on MXRs will provide valuable insight into the evolution of ABC transporter-mediated consumer counter-defense mechanisms.

Abbreviations

ATP Binding Cassette (ABC); Calcein-AM (C-AM); Multidrug resistance-associated protein (MRP); P-glycoprotein (P-gp).

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The work described in this article has carried out in accordance with the EC Directive 86/609/EEC for animal experiments.

The authors have declared that no competing interests exist.

687 **Figure Captions**

688 **Figure 1. Phylogenetic relationships of *Cyphoma*, *Tritonia* and human ABC transporters**.
689 Maximum likelihood (ML) trees were constructed as described in the Methods. Out of a
690 possible 3450 positions, 1821 positions (or 52.8%) were used to construct the final tree.
691 Molluscan sequences are highlighted in red. Values at branch points represent ML bootstrap
692 values calculated with 100 replications. Triangles represent portions of the tree that were
693 collapsed due to poor resolution of the taxa within each clade as evidenced by bootstrap analysis.
694 GenBank sequences in the tree include: Human ABCA proteins (ABCA1, **NP_005493.2**;
695 ABCA2, **NP_001597.2**; ABCA3, **NP001080.2**; ABCA4, **NP_000341.2**; ABCA5, **NP_061142.2**;
696 ABCA6, **NP_525023.2**; ABCA7, **NP_061985.2**; ABCA8, **NP_009099.1**; ABCA9,
697 **NP_525022.2**; ABCA10, **NP_525021.3**; ABCA12, **NP_775099.2**), human ABCB proteins
698 (ABCB1, **NP_000918.2**; ABCB2, **NP_000584.2**; ABCB3, **NP_000535.3**; ABCB4,
699 **NP_000434.1**; ABCB5, **NP_848654.3**; ABCB6, **NP_005680.1**; ABCB7, **NP_004290.2**;
700 ABCB8, **NP_009119.2**; ABCB9, **NP_982269.1**; ABCB10, **NP_036221.1**; ABCB11,
701 **NP_003733.2**), human ABCC proteins (ABCC1, **NP_004987.2**; ABCC2, **NP_000383.1**;
702 ABCC3, **NP_003777.2**; ABCC4, **NP_005836.2**; ABCC5, **NP_005679.2**; ABCC6,
703 **NP_001162.3**; ABCC8, **NP_000343.2**; ABCC9 a/b, **NP_005682.2**, **NP_064693.2**; ABCC10,
704 **NP_258261.2**; ABCC11, **NP_115972.2**; ABCC12, **NP_150229.2**; ABCC13, **EAX10058.1**),
705 human ABCD (ABCD1, **NP_000024.2**; ABCD2, **NP_005155.1**; ABCD3, **NP_002849.1**,
706 ABCD4, **NP_005041.1**), human ABCE (ABCE1, **NP_002931.2**), human ABCF (ABCF1,
707 **NP_001020262.1**; ABCF2, **NP_009120.1**; ABCF3, **NP_060828.2**), human ABCG (ABCG1,
708 **NP_997057.1**; ABCG2, **NP_004818.2**; ABCG4, **NP_071452.2**; ABCG5, **NP_071881.1**;
709 ABCG8, **NP_071882.1**), *Cyphoma gibbosum* MRP isoform 1 (**EU487192**), MRP isoform 2

710 (**EU487193**), Pgp isoform 1 (**EU487190**), Pgp isoform 2 (**EU487191**), *Tritonia hamnerorum*
711 MRP isoform 1 (**EU487194**), MRP isoform 2 (**EU487195**), Pgp isoform 1 (**EU487196**), Pgp
712 isoform 2 (**EU487197**).
713
714 **Figure 2. Phylogenetic relationships of *Cyphoma* and *Tritonia* ABC proteins with human**
715 **and invertebrate ABCB and ABCC subfamilies.** The Bayesian phylogeny is presented with
716 posterior probability and ML bootstrap support indicated for each branch (e.g., Bayesian/ML).
717 ML bootstrap support greater or equal to 70% is indicated by an asterisk (*), less than 70% is
718 indicated by a dash (-). Gastropod sequences are highlighted in red. GenBank sequences
719 included in the tree can be found in Supplemental File S2.
720
721 **Figure 3. Mean MRP isoform 1 transcript expression among *C. gibbosum* individuals**
722 **feeding on a gorgonian or control diet for four days**. Bars represent the mean transcript
723 expression (± SE) of snails feeding on *B. asbestinum* (n = 13), *E. mammosa* (n = 12), *G.*
724 *ventalina* (n = 13), *P. acerosa* (n =10), *P. americana* (n = 12), *P. elisabethae* (n = 6), *P.*
725 *homomalla* (n = 11) or a control diet (n = 33). The relative number of transcripts per 0.2 μg of
726 poly(A)+ RNA was calculated from the standard curve and normalized by a β-actin correction
727 factor. Results of a two-way ANOVA indicate no differences in MRP isoform 1 expression in
728 snails feeding on different diets.
729
730 **Figure 4. Mean MRP isoform 1 transcript expression among time-zero and four day**
731 **control diet fed *C. gibbosum* collected from five reefs.** (A) Time-zero snails; mean MRP
732 isoform 1 expression (± SE) in snails (n = 31 snails) collected from five reefs and immediately

733 dissected to preserve reef-specific gene expression signals. (B) Control snails; mean MRP
734 isoform1 expression (± SE) in snails (n = 33 snails) collected from five reefs and fed a control
735 diet (e.g., alginic acid + squid powder) for four days. Values in parentheses indicate the number
736 of replicate snails examined per reef. The relative number of transcripts per 0.2 μg of poly(A)+
737 RNA was calculated from the standard curve and normalized by a β-actin correction factor.
738
739 **Figure 5. Detection of P-glycoprotein proteins in molluscan tissue homogenates.** (A)
740 Western blot was probed with anti-P-glycoprotein mAb C219 which reacted with one sharp band
741 at 170 kDa and two diffuse bands at > 170 kDa in *T. hamnerorum* whole cell lysates. No bands
742 were detected in *C. gibbosum* digestive gland lysates. (B) *Fundulus heteroclitus* liver cell lysates
743 were used as a positive control.
744
745 **Figure 6. Immunohistochemical analysis of *T. hamnerorum* tissues.** Tangential sections
746 through *T. hamnerorum* probed with mAb C219. Orange-brown staining indicates
747 immunoreactive protein. (A) Black arrows indicate intense staining by C219; grey arrow
748 indicates lighter staining by C219 along the epidermis; *bm*. buccal mass; *mg*. midgut; *oe*.
749 esophagus; *ra*. radula; (50x). (B) Magnification (100x) of esophagus and midgut. (C) Further
750 magnification (200x) of the midgut ciliated columnar epithelia; cilia indicated by white arrow.
751 (D, E) C219 probed tissue section (D) and unlabeled serial section (E) depicting P-glycoprotein
752 localization to the apical tips of the midgut epithelium (indicated by black arrows) (200x).
753
754 **Figure 7. Change in intracellular fluorescence in *T. hamnerorum* incubated with MRP**
755 **(MK571) and P-gp (verapamil) inhibitors compared to untreated controls.** Bars represent
756 mean fluorescence (± SE) of *T. hamnerorum* tissue homogenates from four or eight replicate

wells. Number of replicate wells for DMSO control (grey bars) and inhibitor exposed (black bars) are indicated in the parentheses. All inhibitor exposures resulted in a significant increase in calcein accumulation. MK571 at 30 μM exposure resulted in the greatest inhibition of transport activity in *T. hamnerorum*. **P*=0.001, ***P*<0.001

Tables

Table 1. Summary of MXR cDNAs from *Cyphoma gibbosum* and *Tritonia hamnerorum*

				Length of cloned fragment		
Gene subfamily	Species	Tissue	Gene designation	Nucleotide (bp)	Amino acid (a.a.)	GenBank Accession no.
ABCB	*Cyphoma gibbosum*	Digestive gland	Pgp isoform 1	1755	584	**EU487190**
			Pgp isoform 2	1029	342	**EU487191**
	Tritonia hamnerorum	Whole animal	Pgp isoform 1	390	130	**EU487196**
			Pgp isoform 2	410	136	**EU487197**
ABCC	*Cyphoma gibbosum*	Digestive gland	MRP isoform 1	2853	951	**EU487192**
			MRP isoform 2	554	184	**EU487193**
	Tritonia hamnerorum	Whole animal	MRP isoform 1	4647	1549	**EU487194**
			MRP isoform 2	2297	765	**EU487195**

Supplementary Material

Table Descriptions

Supplemental Table S1. Primers used in the present study

Supplemental Table S2. Results of a two-way ANOVA to investigate differences in MRP-1 gene expression in *C. gibbosum* feeding on control versus gorgonian diets

Supplemental Table S3. Results of a one-way ANOVA to investigate reef-dependent MRP-1 gene expression in time-zero and four day control fed *C. gibbosum*

Figure Descriptions

Supplemental Figure S1. Alignment of deduced amino acid sequences of *Cyphoma* and *Tritonia* MRP proteins

Supplemental Figure S2. Alignment of deduced amino acid sequences of *Cyphoma* and *Tritonia* P-gp proteins with Human ABCB1

Supplemental Figure S3. Comparison of molluscan and human P-gp hydropathy profiles

Supplemental Figure S4. Comparison of molluscan and human MRP hydropathy profiles

Additional Files

Supplemental File S1. Description of Rapid Amplification of cDNA Ends (RACE)

Supplemental File S2. List of GenBank accession no. for Figure 2.

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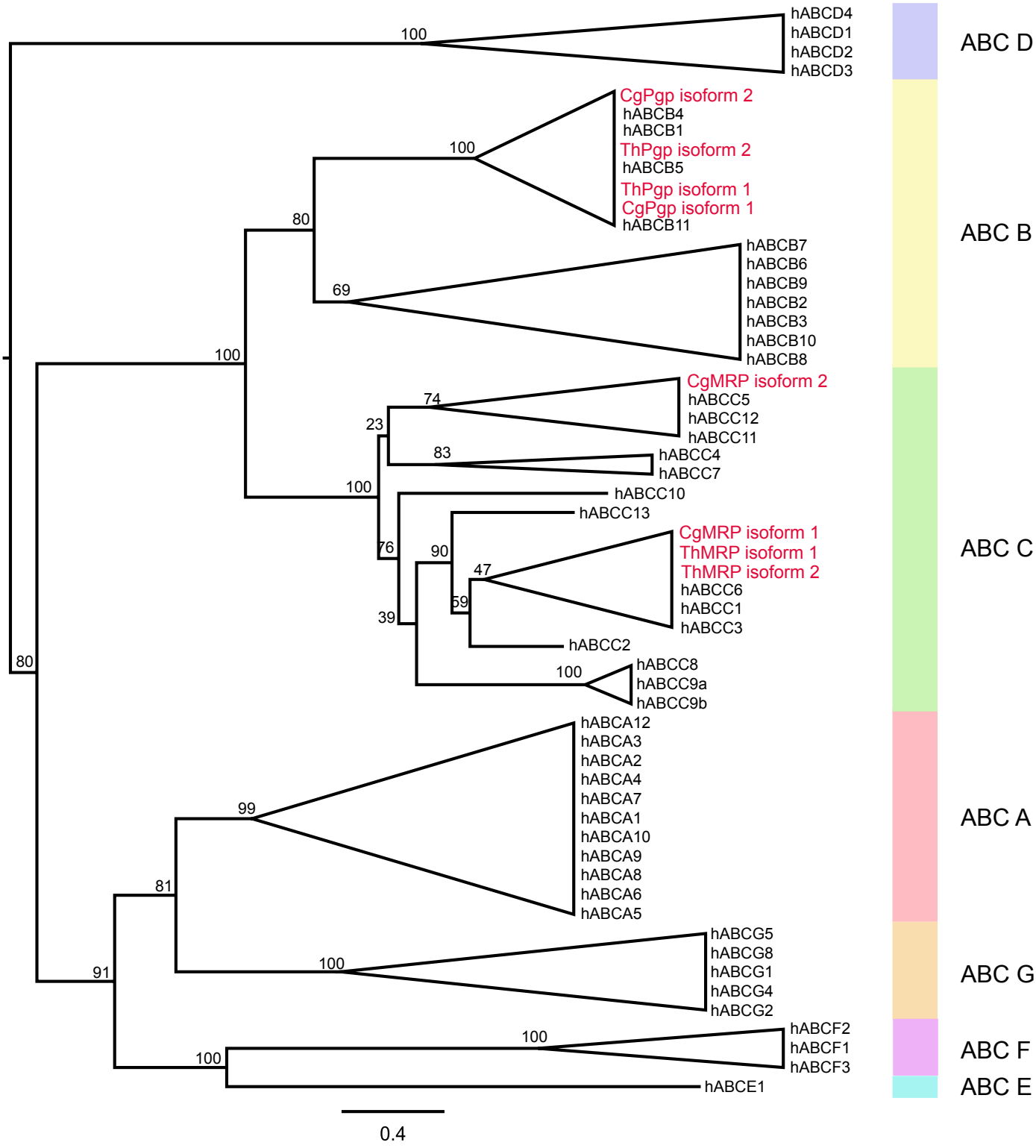
1099

Figure 1. In color online only



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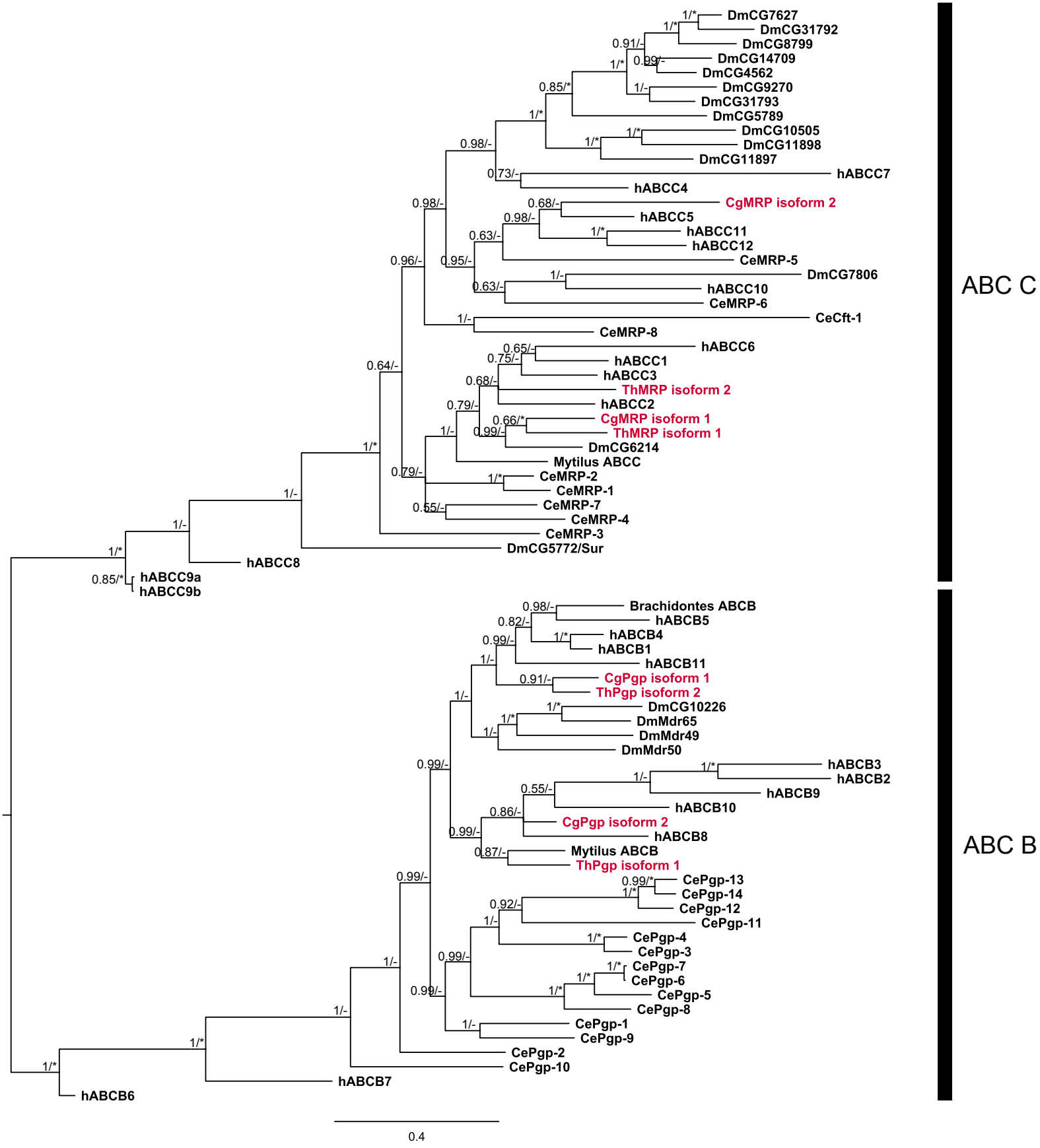

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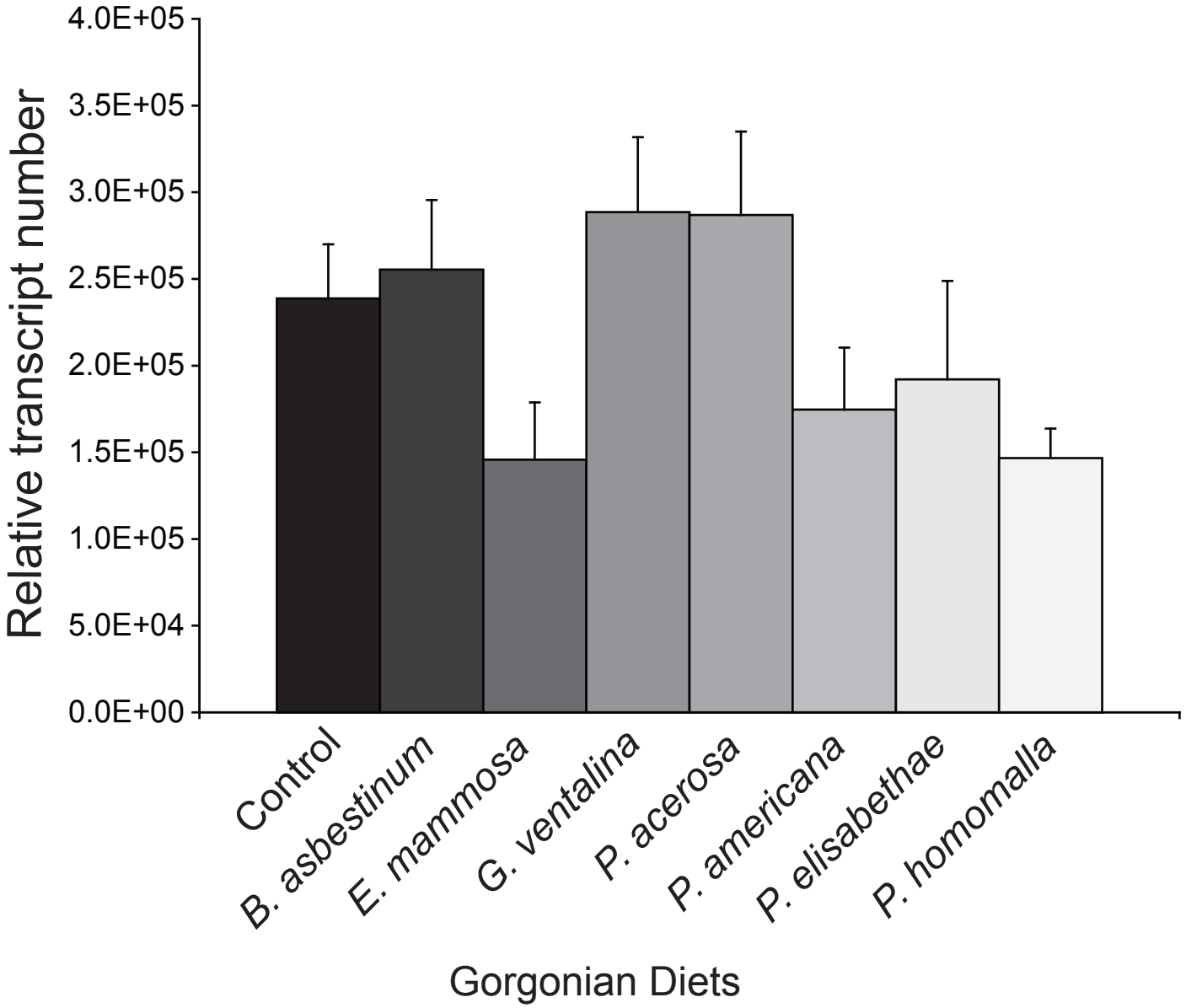
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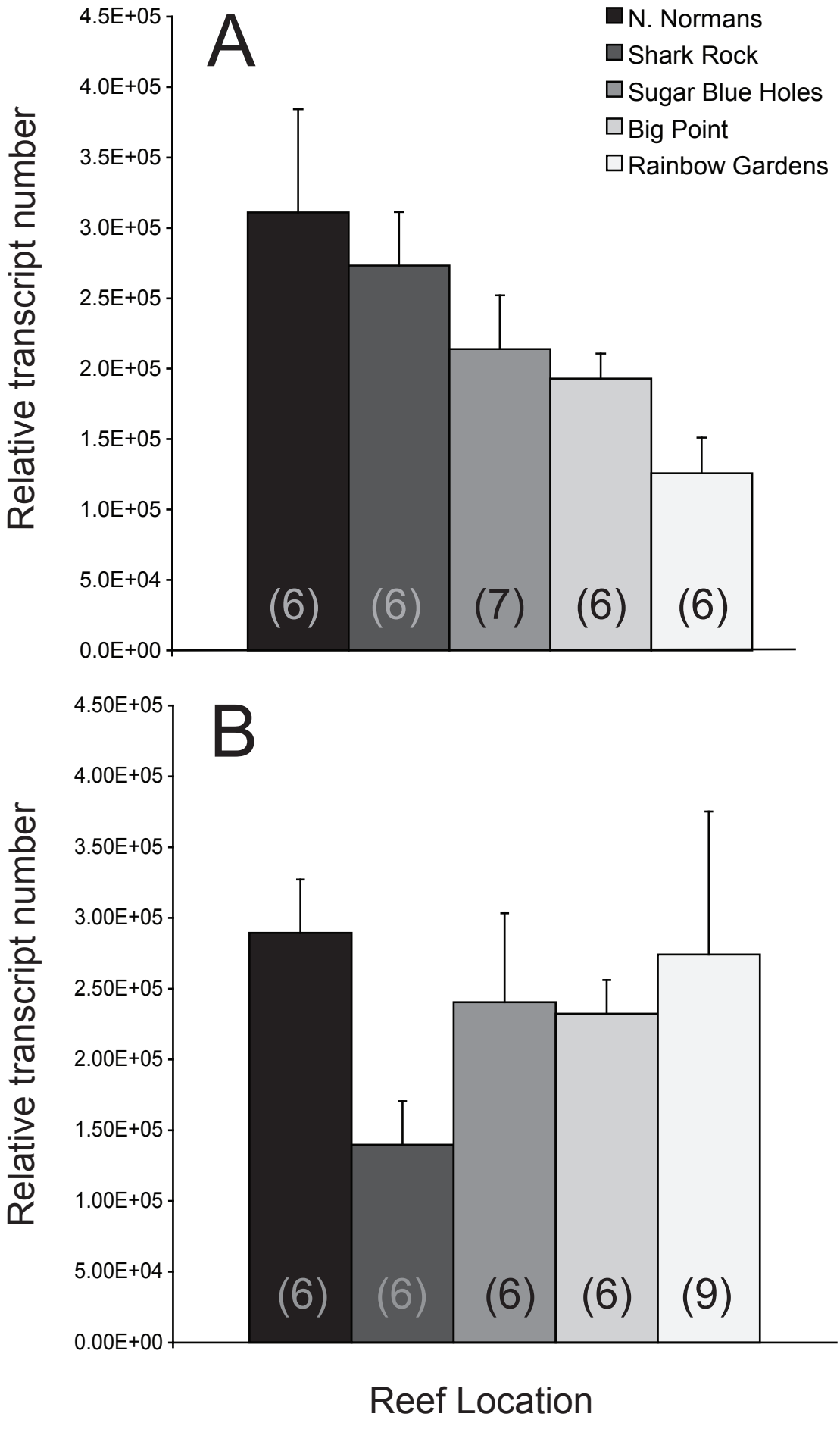
P. acerosa
P. americana
P. elisabethae
P. homomalla
Gorgonian Diets

Figure 4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Reef Location

Figure 5

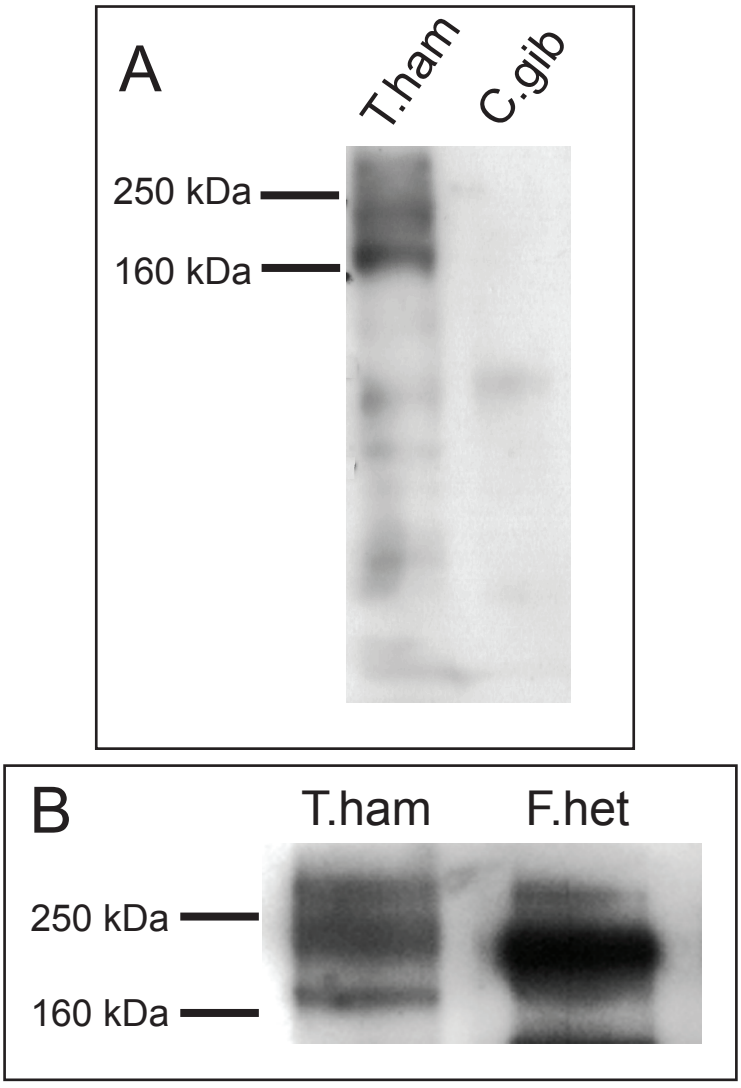


Figure 6. In color in print and online

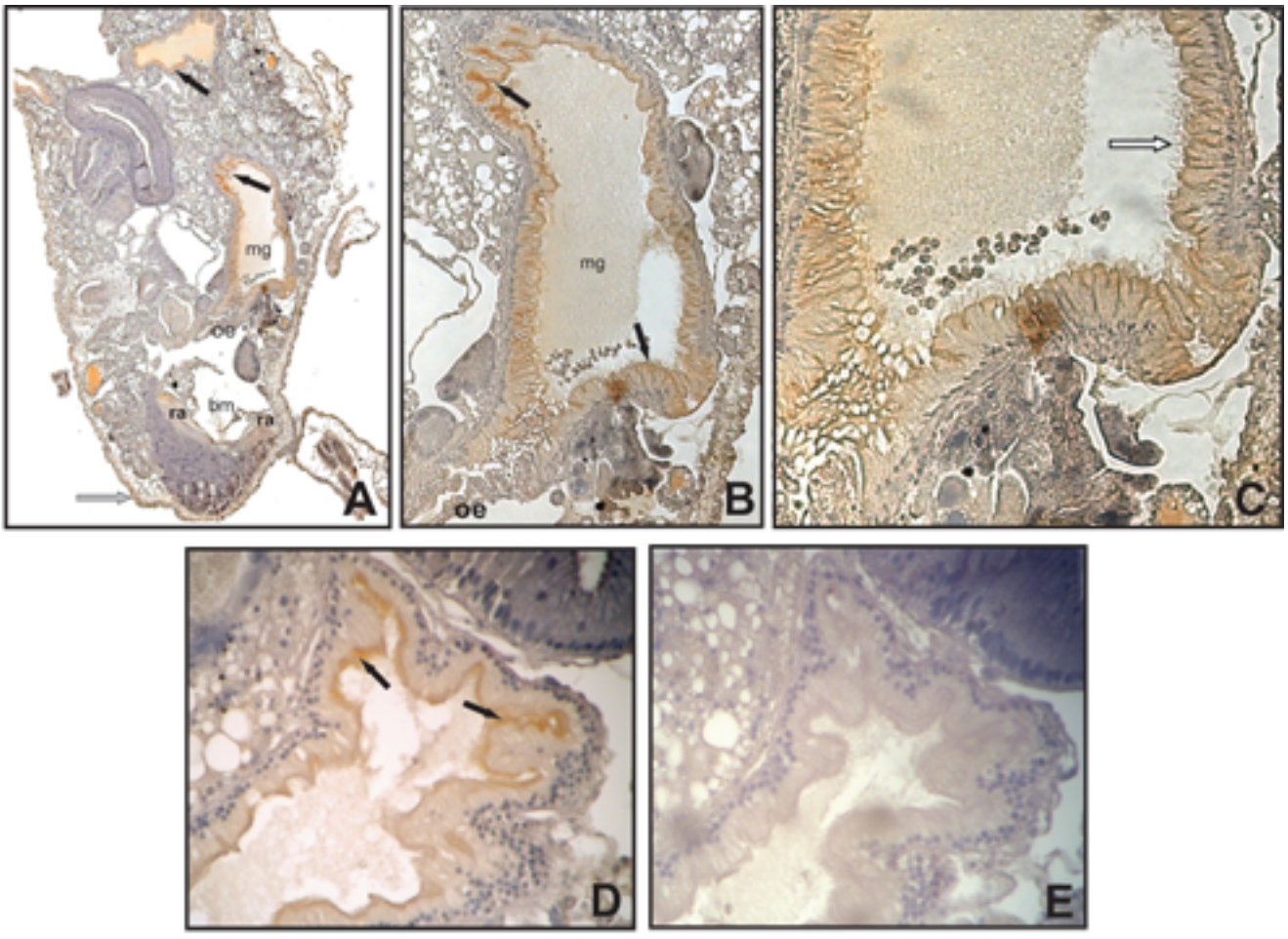
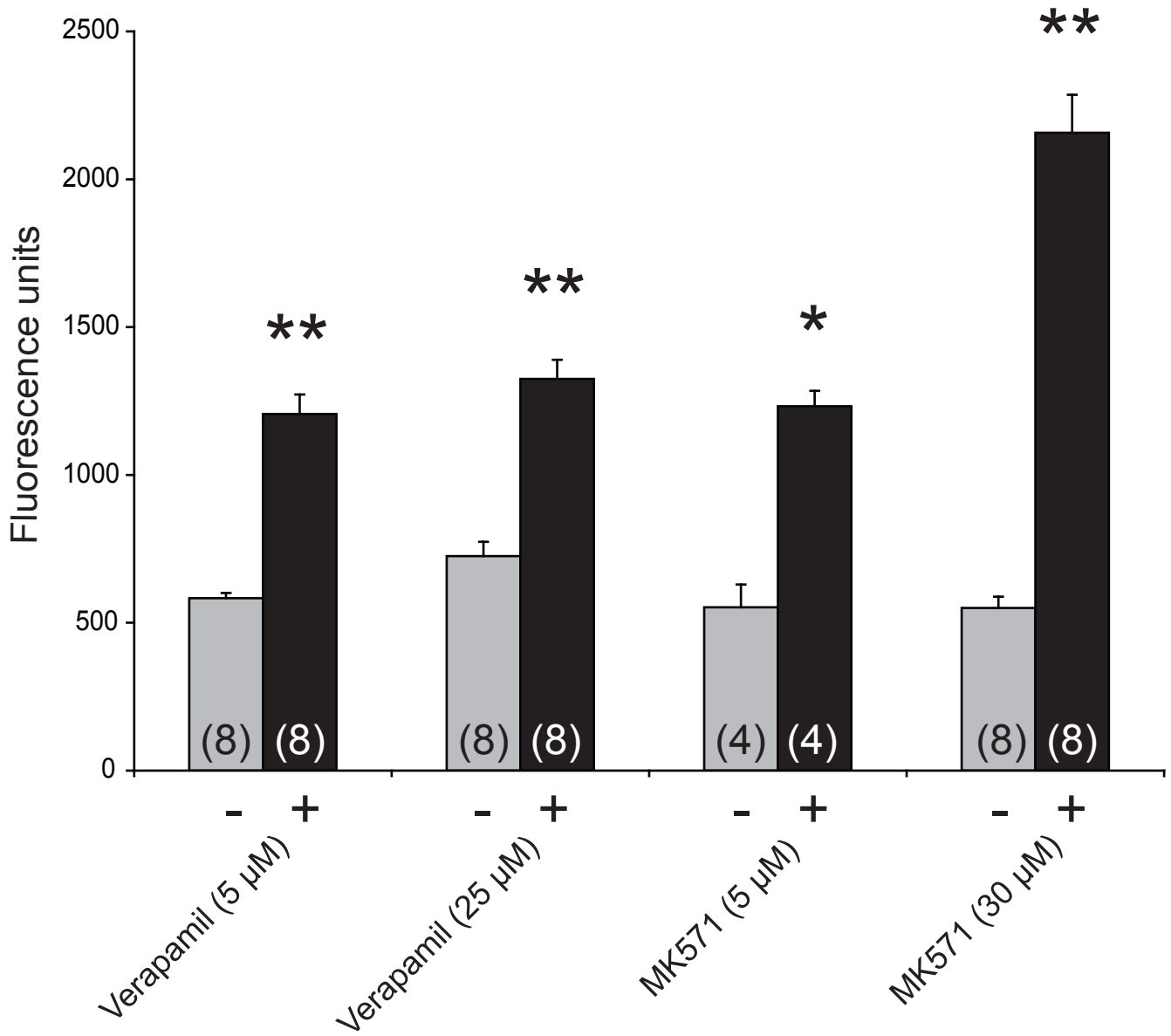


Figure 7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(8)
(8)
-
+
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-
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-
+
Verapamil (5 µM)
Verapamil (25 µM)
MK571 (5 µM)
MK571 (30 µM)

Supplemental Table S1. Primers used in the present study

Gene	Primers	Direction	Sequences (5' to 3')
Degenerate Primers			
MRP	MRP_F	Forward	CTD GTD GCD GTD GTD GG
	MRP_R	Reverse	RCT NAV NGC NSW NAR NGG NTC RCT
	nest_MRP_F	Forward	CGG GAT CCA GRG ARA AYA THC TNT TTG G
	nest_MRP_R	Reverse	CGG AAT TCN TCR TCH AGN AGR TAD ATR TC
P-gp	Pgp_Bbox_F	Forward	GGI GGI CAG AAR CAR MGI ATI GC
	Pgp_Cdomain_F	Forward	GAY GAR GCI ACI TCI GCI CTI G
Gene-specific Primers **(*Cyphoma gibbosum* = Cg; *Tritonia hamnerorum* = Th)**			
CgMRP isoform1	MRP-R1	Reverse	CCG GGC TAA ACT CAC TCT CTG TTT CTG G
	CgMRP-1_R8	Reverse	CAT CCA TGA CCT GAG AAG ATC TGG
	CgMRP-1_R9	Reverse	GGT TCA CAA ACT GCA ACA GGT CG
	CgMRP-1_F8	Forward	CCA GAA ACA GAG AGT GAG TTT AGC C
	CgMRP-1_F9	Forward	GAA TGG CAC CGT CAT CGT ACA GAG
	CgMRP-1_F10	Forward	ATT GAC GCC TGT GCC CTT CGC AC
CgMRP isoform 2	CgMRP-2_F6	Forward	GAG ACA TTT GGA GCT GGG GAC CAG
	CgMRP-2_F7	Forward	GTG TCA GCA TGC TGT CTG GAC AAA G
CgPgp isoform 1	P-gp_R4	Reverse	CCT CTG TCA CCA CAC CAT GTC G
CgPgpisoform 2	P-gp_R5	Reverse	GTT GGC CAC AAA GCC AGC GTA GGC
	P-gp_R6	Reverse	CTC CAA CAC CTG ACC GTT CTC C
ThMRP isoform 1	MRP_F4	Forward	CAA TCG GAT ATT GAC ATT CTC CCT GGC
	MRP_F5	Forward	GCT GTG TAC AGT GAC CAA GAC
	MRP_R4	Reverse	CAG GGA GAA TGT CAA TAT CCG ATT GG
	MRP_R5	Reverse	GTC TTG GTC ACT GTA CAC AGC
	ThMRP-1_F6	Forward	AAA TGA CGT CCG CTG CTG GTG TCC
	ThMRP-1_F7	Forward	AGG CAT TTG GCT GAC GGA GTG GAC
	ThMRP-1_R6	Reverse	AAA CAT GGC CAC GCC TAG TTG GTC
	ThMRP-1_R7	Reverse	GGA AGT CTC ATC GCC AAT GCT GG
ThMRP isoform 2	MRP_F2	Forward	GGT CCA GAT CTG CAA ATG CTG
	MRP_F3	Forward	GCA AAT GCT GCC AGA TGG TGA TC
	MRP_R2	Reverse	CAG CAT TTG CAG ATC TGG ACC
	MRP_R3	Reverse	GAT CAC CAT CTG GCA GCA TTT GC
	ThMRP-2_R9	Reverse	GGA CTT GAC CAA ATT GCC CAG CC
	ThMRP-2_R8	Reverse	TGA CCC GTG AAG TGA AGC GCT ATC
	ThMRP-2_F10	Forward	GTG GAA GGG AGT GTT GTC ATG AGG
	ThMRP-2_F11	Forward	GAA TCA ACG TGA CGA TTC CAG ACC
Adaptor primers	RACE_1_F		AAT ACG ACT CAC TAT AGG
	AP1		CCA TCC TAA TAC GAC TCA CTA TAG GGC
	AP2		ACT CAC TAT AGG GCT CGA GCG GC
qPCR primers			
CgMRP isoform1	136_1_F1	Forward	ACG AGG AGC TGA TGT GTC ACG ATG G
	136_1_R1	Reverse	GTC CTC CTC ATC CAC ATC TTC ATC G

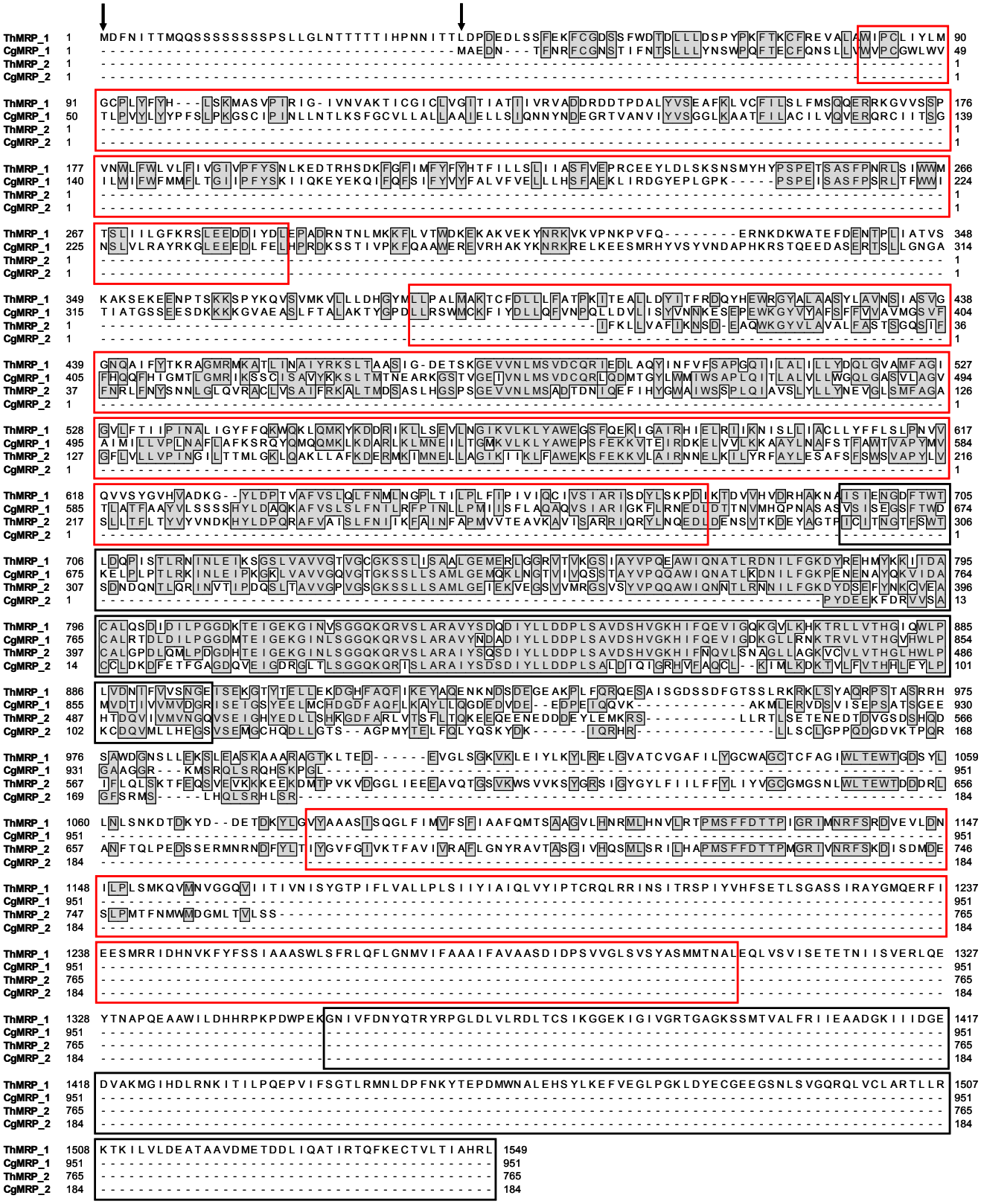
Supplemental Table S2. Results of a two-way ANOVA to investigate differences in MRP-1 gene expression in *C. gibbosum* feeding on control versus gorgonian diets.

Factors	df	MS	*F*	p
Control vs. *B. asbestinum*				
Diet	1	5.3×10^9	0.797	0.422
Reef	4	1.4×10^{10}	0.423	0.791
Diet x Reef	4	6.6×10^9	0.204	0.935
Error	36	3.2×10^{10}		
Control vs. *E. mammosa*				
Diet	1	5.4×10^9	3.22	0.147
Reef	4	3.2×10^9	0.106	0.980
Diet x Reef	4	1.7×10^{10}	0.555	0.697
Error	35	3.0×10^{10}		
Control vs. *G. ventalina*				
Diet	1	5.9×10^{10}	5.91	0.072
Reef	4	1.4×10^{10}	0.432	0.785
Diet x Reef	4	1.0×10^{10}	0.313	0.867
Error	35	3.2×10^{10}		
Control vs. *P. acerosa*				
Diet	1	2.0×10^{10}	0.948	0.385
Reef	4	5.5×10^{10}	1.87	0.139
Diet x Reef	4	2.1×10^{10}	0.723	0.582
Error	33	3.0×10^{10}		
Control vs. *P. americana*				
Diet	1	1.9×10^{10}	1.01	0.371
Reef	4	2.5×10^{10}	0.852	0.502
Diet x Reef	4	1.9×10^{10}	0.651	0.629
Error	35	2.9×10^{10}		
Control vs. *P. elisabethae*				
Diet	1	1.7×10^{10}	5.02	0.154
Reef	2	1.8×10^{10}	1.30	0.297
Diet x Reef	2	3.5×10^{10}	0.249	0.782
Error	18	1.4×10^{10}		
Control vs. *P. homomalla*				
Diet	1	6.4×10^{10}	9.66	0.036
Reef	4	4.5×10^9	0.157	0.958
Diet x Reef	4	6.6×10^9	0.230	0.920
Error	34	2.9×10^{10}		

A $p \leq 0.007$ should be accepted as significant (Bonferroni adjusted).

Supplemental Table S3. Results of a one-way ANOVA to investigate reef-dependent MRP-1 gene expression in time-zero and four day control fed *C. gibbosum*. Reefs considered random factors.

Factors	df	MS	F	p
Reef (Experiment 1)				
Control diet	4, 28	2.1×10^{10}	0.637	0.640
Reef (Experiment 2)				
Time-zero group	4, 26	3.1×10^{10}	2.74	**0.050**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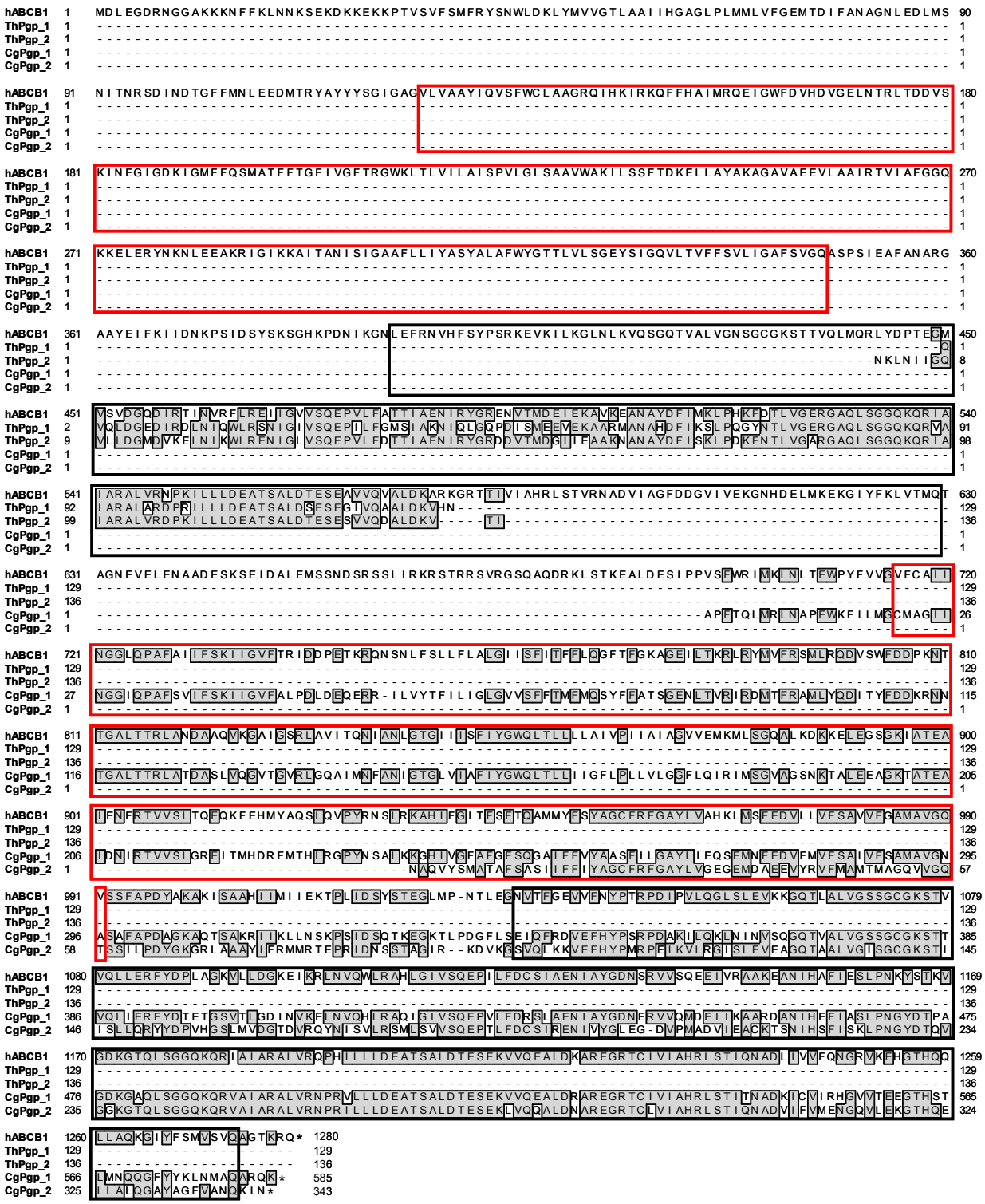
CgMRP_1
ThMRP_2
CgMRP_2

Supplemental Figure S1. Alignment of deduced amino acid sequences of *Cyphoma* and *Tritonia* MRP proteins. Molluscan deduced amino acids sequences were aligned using ClustalX. Identical residues in at least two sequences are outlined and shaded in grey. Black and red boxes represent the putative nucleotide binding domains and hydrophobic transmembrane domains, respectively. Domains were predicted using the algorithm of Kyte and Doolittle (1982) (window of 13 amino acids) and with NCBI Conserved Domain Database searches (Marchler-Bauer 2007). Predicted start codons for *Cyphoma* MRP isoform 1 and *Tritonia* MRP isoform 1 are indicated by a downward arrow above the alignment. A dash (-) indicates missing or gapped residues. GenBank sequences included in the alignment: *Cyphoma* MRP isoform 1 (CgMRP_1; ACA53359.1); *Cyphoma* MRP isoform 2 (CgMRP_2; ACA53360.1); *Tritonia* MRP isoform 1 (ThMRP_1; ACA53361.1); *Tritonia* MRP isoform 2 (ThMRP_2; ACA53362.1).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ThPgp_2 1 1
CgPgp_1 1 1
CgPgp_2 1 1
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CgPgp_2 1 1
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CgPgp_2 1 1
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CgPgp_2 146 ISLLQRYYDPVHGSLMVDGTDVRQYNISVLRSMLSVVSQEPTLFDCSTRENIVYGLEG-DVPMADVIEACKTSNIHSFISKLPNGYDTQV 234
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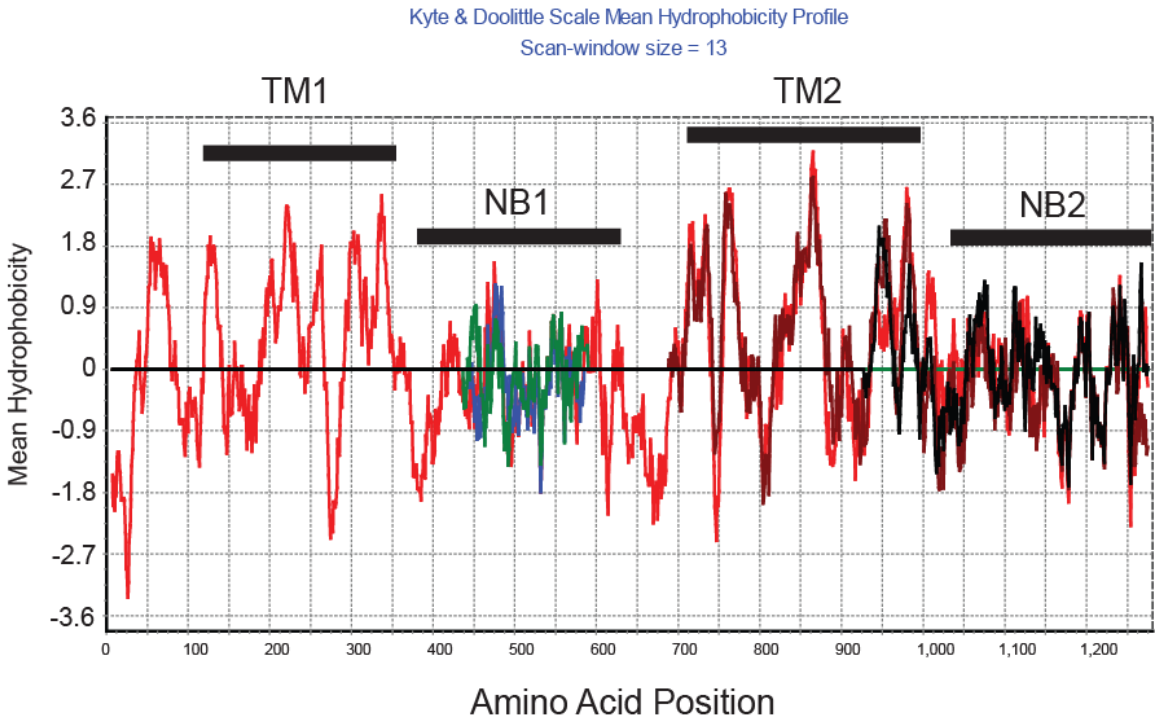
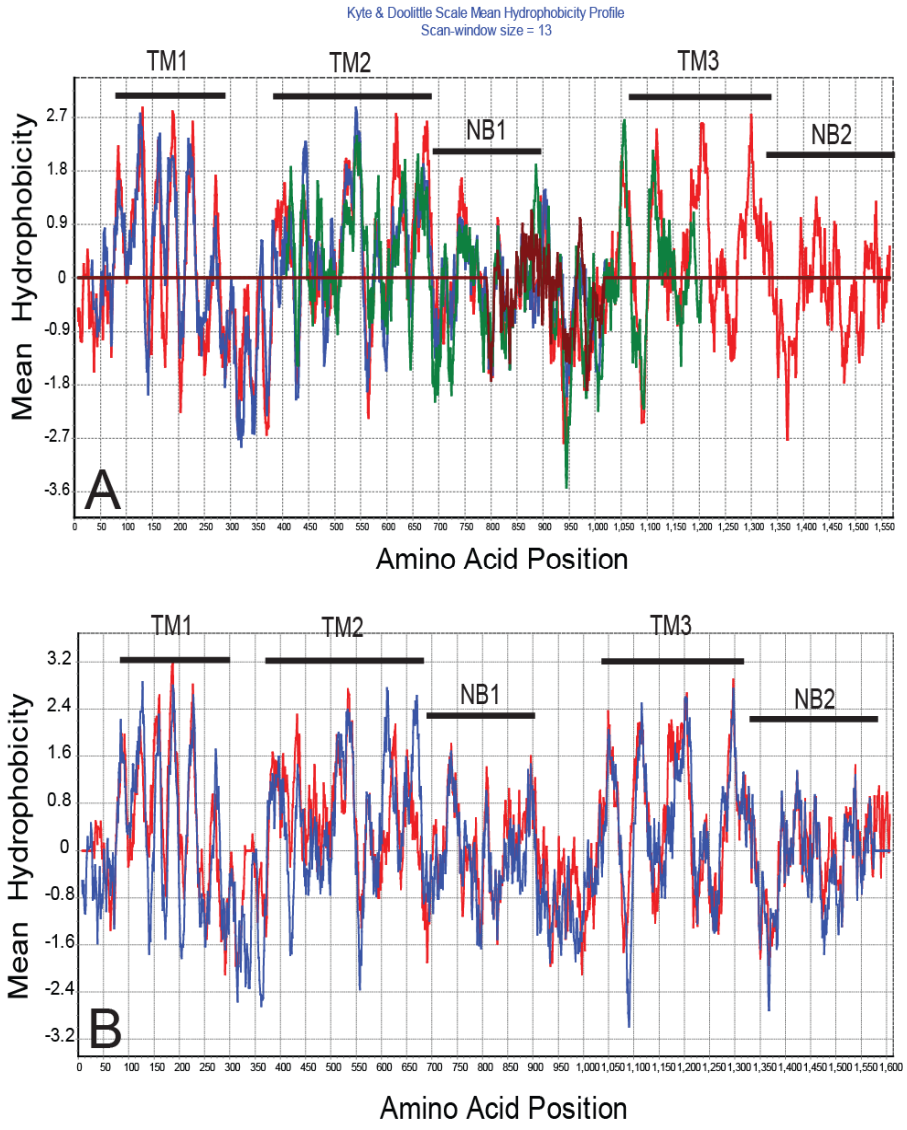
CgPgp_2 325 LLALQGAYAGFVANQKIN* 343

Supplemental Figure S2. Alignment of deduced amino acid sequences of *Cyphoma* and *Tritonia* P-gp proteins with Human ABCB1. Molluscan deduced amino acid sequences and human protein sequence were aligned using ClustalX. Identical residues are outlined and shaded in grey. Black boxes represent the putative nucleotide binding domains and red boxes represent the putative hydrophobic transmembrane domains. Transmembrane and nucleotide binding domains were predicted using the algorithm of Kyte and Doolittle (Kyte et al. 1982) (window of 13 amino acids) and with NCBI Conserved Domain Database searches (Marchler-Bauer et al. 2007). An asterisk indicates a stop codon and a dash (-) indicates missing or gapped residues. GenBank sequences included in the alignment: human ABCB1 (NP_000918.2), *C. gibbosum* Pgp isoform 1 (CgPgp_1; ACA53357.1), *C. gibbosum* Pgp isoform 2 (CgPgp_2; ACA53358.1), *T. hamnerorum* Pgp isoform 1 (ThPgp_1; ACA53363.1), *T. hamnerorum* Pgp isoform 2 (ThPgp_2; ACA53364.1).

Marchler-Bauer, A., Anderson, J., Derbyshire, M., DeWeese-Scott C, Gonzales NR, Gwadz M, Hao L, He S, Hurwitz DI, Jackson JD, Ke Z, Krylov D, Lanczycki CJ, Liebert CA, Liu C, Lu F, Lu S, Marchler GH, Mullokandov M, Song JS, Thanki N, Yamashita RA, Yin JJ, Zhang D, SH., B., 2007. CDD: a conserved domain database for interactive domain family analysis. Nucleic Acids Research 35, 237-240.



Supplemental Figure S3. Comparison of molluscan and human P-gp hydropathy profiles. Deduced amino acid sequences from *Cyphoma* and *Tritonia* P-glycoprotein profiles were aligned to human ABCB1 using ClustalX. The human ABCB1 plot is indicated by the red line and is overlaid by *Cyphoma* Pgp isoforom 1 (maroon line), *Cyphoma* Pgp isoform 2 (black line), *Tritonia* Pgp isoform 1 (blue line), and *Tritonia* Pgp isoform 2 (green line). Black bars above the plots indicate positions of the two transmembrane regions (TM1, TM2) and nucleotide binding domains (NB1, NB2) in ABCB proteins. Values above and below the horizontal line indicate hydrophobic and hydrophilic regions, respectively. Hydrophobicity plots were generated using the Kyte-Doolittle algorithm (Kyte et al. 1982) with a window of 13 residues by BioEdit v7.0.5.2. GenBank sequences included in the alignment: human ABCB1 (NP_000918.2), *C. gibbosum* Pgp isoform 1 (ACA53357.1), *C. gibbosum* Pgp isoform 2 (ACA53358.1), *T. hamnerorum* Pgp isoform 1 (ACA53363.1), *T. hamnerorum* Pgp isoform 2 (ACA53364.1).



Supplemental Figure S4. Comparison of molluscan and human MRP hydropathy profiles. (**A**) Deduced amino acid sequences from *Cyphoma* and *Tritonia* MRP profiles were aligned using ClustalX. *Tritonia* MRP isoform 1 plot is indicated by the red line and the *Cyphoma* MRP isoform 1 (blue line), *Tritonia* MRP isoform 2 (green line), *Cyphoma* MRP isoform 2 (maroon line) are layered on top. (**B**) Deduced amino acid sequence from *Tritonia* MRP isoform 1 (blue line) aligned with human ABCC3 (red line) using ClustalX. Black bars above the plots indicate positions of the three transmembrane regions (TM1, TM2, TM3) and nucleotide binding domains (NB1, NB2) in ABCC proteins. Values above and below the horizontal line indicate hydrophobic and hydrophilic regions, respectively. Hydrophobicity plots were generated using the Kyte-Doolittle algorithm (Kyte et al. 1982) with a window of 13 residues by BioEdit v7.0.5.2. GenBank sequences included in the alignment: human ABCC3 (NP_003777.2), *C. gibbosum* MRP isoform 1 (ACA53359.1), *C. gibbosum* MRP isoform 2 (ACA53360.1), *T. hamnerorum* MRP isoform 1 (ACA53361.1), *T. hamnerorum* MRP isoform 2 (ACA53362.1).

Kyte, J., Doolittle, R.F., 1982. A simple method for displaying the hydropathic character of a protein. Journal of Molecular Biology 157, 105-132.

Supplemental File S1. Description of Rapid Amplification of cDNA Ends (RACE)

Cyphoma RACE-PCR.
One microgram of poly(A)+RNA isolated from pooled *C. gibbosum* digestive glands, as described above, was primed with modified oligo (dT) primers and used to create an adaptor-ligated, double-stranded (ds) cDNA library using the Marathon cDNA Amplification Kit (BD Biosciences, Palo Alto, CA) according to the manufacturer's instructions. Gene specific primers were designed to both MRP cDNA fragments (CgMRP isoform 1 and CgMRP isoform 2) (Supplementary Table S1) and were used in combination with adaptor-specific primers in nested 5' and 3' RACE reactions. Amplification of *C. gibbosum* MRP specific PCR products was carried out according to the Advantage 2 PCR Enzyme Kit (Clontech, Mountain View, CA) and cycling parameters were as follows: 94^{o}C for 30sec; 5 cycles of 94^{o}C for 5 sec, 72^{o}C for 2.5 min; 5 cycles of 94^{o}C for 5 sec, 70^{o}C for 2.5 min; 25 cycles of 94^{o}C for 5 sec, 68^{o}C for 2.5 min; 68^{o}C for 5 min with the following specific primers pairs (MRP-R1/RACE_1_F); or 94^{o}C for 30sec; 5 cycles of 94^{o}C for 5 sec, 72^{o}C for 4 min; 5 cycles of 94^{o}C for 5 sec, 70^{o}C for 4 min; 25 cycles of 94^{o}C for 5 sec, 68^{o}C for 4 min with the following specific primer pairs (CgMRP-1_F9/AP1 then CgMRP-1_F10/AP2; Cg_MRP-1_F10/AP1 then CgMRP-1_F8/AP2; CgMRP-1_R9/AP1 then CgMRP-1_R8/AP2; CgMRP-2_F7/AP1 then CgMRP-2_F6/AP2).

The *C. gibbosum* adaptor-ligated ds cDNA library was also used to amplify partial P-gp cDNA sequences by RACE using degenerate primers designed to the conserved portion of the nucleotide binding domains (Supplementary Table S1). Nested-RACE was performed with the following degenerate primers pairs (Pgp_Bbox_F/RACE_1_F then Pgp_Cdomain_F/RACE_1_F) (Supplementary Table S1) using the Advantage 2 PCR Enzyme Kit with the following cycling parameters: 94^{o}C for 30sec; 30 cycles for 94^{o}C for 5 sec, 55^{o}C for 30 sec, 68^{o}C for 2 min. BLASTX searches identified two partial sequences having homology to P-gp and these were designated as CgPgp isoform 1 and CgPgp isoform 2. Gene specific primers were designed to CgPgp isoform 1 and CgPgp isoform 2 fragments and used in combination with adaptor-specific primers in nested 5' RACE reactions. PCR products were amplified with Advantage 2 polymerase with the following cycling parameters: 94^{o}C for 30sec; 5 cycles for 94^{o}C for 5 sec, 72^{o}C for 3 min; 5 cycles for 94^{o}C for 5 sec, 70^{o}C for 3 min; 25 cycles for 94^{o}C for 5 sec, 68^{o}C for 3 min with the following specific primer pairs (Pgp_R4/AP1; Pgp_R5/AP1 then Pgp_R6/AP2) (Supplementary Table S1).

Tritonia RACE PCR.
One microgram of poly(A)+RNA, isolated as described above, was used to create an adaptor-ligated ds cDNA library using the Marathon cDNA Amplification Kit according to the manufacturer's instructions. Gene specific primers were designed to both MRP cDNA fragments (ThMRP isoform 1 and ThMRP isoform 2) (Supplementary Table S1) and were used in combination with adaptor-specific primers in nested 5' and 3' RACE reactions. *T. hamnerorum* MRP specific PCR products were amplified using the

Advantage 2 PCR Enzyme Kit; cycling parameters were as follows: 94°C for 30sec; 5 cycles for 94°C for 5 sec, 72°C for 3 min; 5 cycles for 94°C for 5 sec, 70°C for 3 min; 25 cycles for 94°C for 5 sec, 68°C for 3 min with the following specific primer pairs (MRP_F4/AP1 then MRP_F5/AP2; MRP_R5/AP1 then MRP_R4/AP2; ThMRP-1_R6/AP1 then ThMRP-1_R7/AP2; ThMRP-1_F7/AP1 then ThMRP-1_F6/AP1; MRP_R3/AP1 then MRP_R2/AP2; MRP_F2/AP1 then MRP_F3/AP2; ThMRP-2_R9/AP1 then ThMRP-2_R8/AP2; ThMRP-2_F11/AP1 then MRP_F3/AP2; ThMRP-2_F10/AP1 then MRP_F3/AP2).

The *T. hamnerorum* adaptor-ligated ds cDNA library was also used to amplify partial P-gp cDNA sequences by RACE using a combination of degenerate primers designed to the conserved nucleotide binding region and specific adaptor primers (Supplementary Table S1). Nested-RACE was performed with degenerate primer pairs (Pgp_Bbox_F/RACE_1_F then Pgp_Cdomain_F/RACE_1_F) using the Advantage 2 PCR Enzyme Kit with the following cycling parameters: 94°C for 30sec; 5 cycles for 94°C for 5 sec, 61°C for 30 sec, 68°C for 2 min; 5 cycles for 94°C for 5 sec, 59°C for 30 sec, 68°C for 2 min; 20 cycles for 94°C for 5 sec, 57°C for 30 sec, 68°C for 2 min. BLASTX searches identified the two partial sequences as having homology to P-gp; these were designated as ThPgp isoform 1 and ThPgp isoform 2. Gene specific primers were designed to ThPgp isoform 1 and ThPgp isoform 2 fragments and used in combination with adaptor-specific primers in nested RACE reactions. PCR products were amplified with Advantage 2 polymerase with the following cycling parameters: 94°C for 30sec; 5 cycles for 94°C for 5 sec, 72°C for 3 min; 5 cycles for 94°C for 5 sec, 70°C for 3 min; 25 cycles for 94°C for 5 sec, 68°C for 3 min with the following specific primer pairs (ThPgp-1_R8/AP1 then ThPgp-1_R7/AP2; ThPgp-1_R8/AP1 then ThPgp-1_R7; ThPgp-2_R10/AP1 then ThPgp-2_R9/AP2).

Supplemental File S2. List of GenBank accession no. for Figure 2

GenBank sequences in the tree include: *Brachidontes pharaonis* ABCB (**CAI99869**); *Caenorhabditis elegans* ABCB proteins (CePgp-1, **CAB01232**; CePgp-2, **AAB52482**; CePgp-3 **CAA91467**; CePgp-4, **CAA91463**; CePgp-5, **CAA94202**; CePgp-6, **CAA94220**; CePgp-7, **CAA94219**; CePgp-8, **CAA94203**; CePgp-9, **CAB03973**; CePgp-10, **AAC48149**; CePgp-11, **CAA88940**; CePgp-12, **CAA91799**; CePgp-13, **CAA91800**; CePgp-14, **CAA91801**), *Caenorhabditis elegans* ABCC proteins (CeMrp-1, **AAD31550**; CeMrp-2, **AAB07022**; CeMrp-3, **CAA92148**; CeMrp-4, **CAB02667**; CeMrp-5, **CAB54225**; CeMrp-6, **AAA82317**; CeMrp-7, **CAA21622**; CeMrp-8, **CAA22110**), *Drosophila melanogaster* ABCB proteins (DmCG10226, **AAF50670**; DmMdr65, **NP_476831**; DmMdr49, **NP_523724**; DmMdr50, **NP_523740**), *Drosophilia melanogaster* ABCC proteins (DmCG5772/Sur, **NP_477472**; DmCG10505, **AAF46706**; DmCG11898, **AAF56870**; DmCG11897, **AAF56869**; DmCG7627, **AAF52648**; DmCG31792, **NP_724148**; DmCG8799, **AAF58947**; DmCG9270, **AAF53950**; DmCG31793, **NP_609930**; DmCG14709, **AAF54656**; DmCG4562, **AAF55707**; DmCG5789, **AAF56312**; DmCG7806, **AAF52639**), *Homo sapiens* ABCB proteins (ABCB1, **NP_000918.2**; ABCB2, **NP_000584.2**; ABCB3, **NP_000535.3**; ABCB4, **NP_000434.1**; ABCB5, **NP_848654.3**; ABCB6, **NP_005680.1**; ABCB7, **NP_004290.2**; ABCB8, **NP_009119.2**; ABCB9**, NP_982269.1**; ABCB10, **NP_036221.1**; ABCB11, **NP_003733.2**), *Homo sapiens* ABCC proteins (ABCC1, **NP_004987.2**; ABCC2, **NP_000383.1**; ABCC3, **NP_003777.2**; ABCC4, **NP_005836.2**; ABCC5, **NP_005679.2**; ABCC6, **NP_001162.3**; ABCC7, **NP_00483.3**; ABCC8, **NP_000343.2**; ABCC9 a/b, **NP_005682.2**, **NP_064693.2**; ABCC10, **NP_258261.2**; ABCC11, **NP_115972.2**; ABCC12, **NP_150229.2**), *Mytilus californianus* ABCB (**ABS83556.1**) and ABCC (**ABS83557**), *Cyphoma gibbosum* ABCB proteins (CgPgp isoform 1, **ACA53357.1**; CgPgp isoform 2, **ACA53358.1)**; *Cyphoma gibbosum* ABCC proteins (CgMRP isoform 1 **ACA53359.1**; CgMRP isoform 2, **ACA53360.1**), *Tritonia hamnerorum* ABCB proteins (ThPgp isoform 1, **ACA53363.1**; ThPgp isoform 2, **ACA53364.1**), *Tritonia hamnerorum* ABCC proteins (ThMRP isoform 1, **ACA53361.1**; ThMRP isoform 2, **ACA53362.1**).