Molecular interactions of environmental chemicals with tuna and honeybee xenobiotic defense transporter P-glycoprotein: Using ligand-binding site conservation to predict chemical bioaccumulation

By

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Abstract

Environmental chemicals can affect the world's food quality and quantity in multiple ways. There is increasing evidence for chemical contamination of food and water, such as pesticide residues on fruits or polyfluoroalkyl substances (PFAS) in drinking water. Some of these chemicals can persist in the environment and ultimately bioaccumulate through organism of the terrestrial and aquatic food chains, including fish and other seafood. Other chemicals, such as crop pesticides, can cause collateral or unintended toxicities to non-target organism, including pollinator insects, that are essential workforces for agricultural industries. Yet, the mechanism of chemical bioaccumulation in marine top predators or the (toxic) effects of unintended chemical co-exposures to pollinator insects are still poorly understood.

To counteract xenobiotic insults, all living organisms possess a sophisticated cellular defense system consisting of three major mechanisms, including xenobiotic sensors like the aryl hydrocarbon receptor (AhR), detoxifying enzymes like cytochrome P450 (CYP450), and efflux transporters like P-glycoprotein (P-gp). P-glycoprotein (aka MDR1 or ABCB1) is a Multidrug resistance (MDR) transporter ubiquitously expressed in biological barriers, including liver, kidneys, lungs, intestine, brain, and gills. Due to its poly-specific recognition and elimination of xenobiotics, the ABCB1 is considered a key determinant of drug and xenobiotic disposition in all organisms.

In this thesis, I seek to explore how environmental chemicals interact with the protective ABCB1 transporter of the apex predator yellowfin tuna and the European honeybee as an agriculturally important pollinator insect. The results of my thesis will provide new avenues to better predict pollutant bioaccumulation in commercial fish species as well as evaluate and mitigate potentially toxic mixture effects of crop pesticides and in-hive medicines to honeybees.

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Introduction

There are over 86,000 manufactured or processed chemicals in the chemical substances control inventory compiled by the Environmental Protection Agency as mandated by the Toxic Substance Control Act (US EPA 2015). These chemicals, transported by wind, water, or human activity, can enter the environment where they can contaminate important food and water sources. Some of those chemicals, including bisphenol A and several phthalates, have been regularly detected in humans and animal tissues, disrupting normal biochemical processes ("Fourth National Report on Human Exposure to Environmental Chemicals Update" 2021a; "Fourth National Report on Human Exposure to Environmental Chemicals Update" 2021b; "Fourth National Report on Human Exposure to Environmental Chemicals Update" 2021c; "Fourth National Report on Human Exposure to Environmental Chemicals Update" 2021d). At present, governing bodies like the EPA, FDA, and USDA have implemented rules and regulations regarding chemical use, quantity, and disposal. Furthermore, the FDA has guidelines for chemicals that impact the food supply, including guides on maximum levels of chemicals tolerable in human and animal food and what quantities of fish are safe to consume (Nutrition 2021; 2020). However, toxic accumulation of environmental chemicals in organisms can also indirectly affect human food supply. Pollinator insects, like honeybees, are vital to human food supply and perform most of the crop pollination in the US and across the globe. Yet, many agricultural pesticides, applied to protect the crops against disease and pests, have been recently shown to be highly toxic to bees and other pollinators. The need for sensible use of pesticides for optimal pest control and human and environmental protection has sparked EPA's integrated pest management practices (US EPA 2013b).

The fact that agricultural insecticides could also harm non-target pollinator insects was not surprising, since most pesticides act on well-conserved nerve targets (Casida 2009). Still, most

organisms have evolved to adapt to a variety of environmental and chemical stressors, particularly insects with short generation times. As first line of defense, these organisms typically upregulate a set of cellular defense systems to combat xenobiotics. These systems include xenobiotic receptors, like the aryl-hydrocarbon receptor, to recognize and trigger biological responses to the chemicals, metabolizing enzymes, like cytochrome P450s, to break down the chemicals, and drug efflux transporters, like P-glycoprotein, that govern the transfer of xenobiotics across barrier membranes and excretory organs. Drug efflux transporters are thought to play a key role in controlling which compounds enter and exit the body (Giacomini, et al 2010; Döring and Petzinger 2014; Nigam 2015). However, these transporter proteins can themselves be targets of toxic action. An emerging class of compounds, Transporter Interfering Chemicals (TICs), have been shown to hinder normal transporter function in vertebrates, including mouse, human and fish (Nicklisch et al. 2016a; Nicklisch and Hamdoun 2020b; Nicklisch et al. 2021). Exposure to TICs through diet or inhalation could lead to transporter dysfunction and intracellular accumulation of compounds that would normally be transported out of the cell or tissue compartment. Since drug efflux transporters of the ABC-type family are highly conserved across all kingdoms of life, TIC accumulation is likely to occur on all trophic levels. Thereby, organisms at the top of the food chain, such as humans and other apex predators, are more susceptible to high toxicant accumulation that bio-magnify up the food chain (Borgå, Gabrielsen, and Skaare 2001). However, detailed studies on the interaction of environmental chemicals with drug efflux transporters in top predators that are also a human food source are still elusive.

Another critical gap in knowledge exists with respect to the effects of environmental chemical mixtures on these xenobiotic efflux systems. Organisms are rarely exposed to only one compound and exposure to mixtures of compounds appears environmentally more relevant. To

date, only a handful of environmental chemicals have been tested for their interactions with selected drug efflux transporters, including P-glycoprotein. Understanding how mixtures of TICs and other environmental chemicals could promote synergistic effects on drug efflux transporter inhibition or activation will be a key step in the development of new predictive tools to better evaluate both chemical bioaccumulation potentials and cumulative toxicity.

Rationale

This project aims to elucidate the underlying mechanism(s) that determine which environmental chemicals, or mixtures thereof, could interfere with vertebrate and insect protective efflux transporter activity and cause toxicity. The project focuses on the membrane transporter P-glycoprotein (aka P-gp or ABCB1). ABCB1 has been well characterized in mammals where it has a major role in determining drug absorption and distribution in the body (Hoffmann and Kroemer 2004; A. B. Shapiro and Ling 1994; Sharom 2011). ABCB1 is located in barrier and excretory tissues such as the blood-brain-barrier, the blood-intestine-barrier, and the kidneys (Nicklisch and Hamdoun 2020a; Giacomini, et al. 2010). In addition to mammals, ABCB1 homologs can be found in insects, fish, mollusks and plants (Merzendorfer 2014; Irene Bosch and Croop 1998a; Bard et al. 2002; Y.-Y. Xu et al. 2014). Across species, ligand-binding sites on the protein are highly conserved, both functionally and biochemically (Nicklisch et al. 2021; Nicklisch and Hamdoun 2020a). Therefore, the inhibitory TIC effects observed in mammalian P-glycoprotein could be a common mechanism how environmental chemicals bioconcentrate and biomagnify across the food web. In Chapter 1, we sought to investigate the interactions of ten previously identified TICs with yellowfin tuna P-glycoprotein, an apex predator, and important commercial fish species. In Chapter 2, we aim to identify environmentally relevant pesticide and in-hive medicine mixtures and predict their effects on honeybee P-glycoprotein, a global crop pollinator.

To understand the implications of ABCB1 function on marine pollutant bioaccumulation, yellowfin tuna, *Thunnus albacares*, will be used as the study organism in the first Chapter. Yellowfin tuna is an important commercial fish species and human food source, especially for communities that get most of their protein intake from fish, such as Maldivians and Japanese (ISSF 2021; Yadav et al. 2020; Endo and Haraguchi 2010). In addition, the American Heart Association

recommends eating fish twice a week for pregnant and nursing women to ensure optimal omega-3 fatty acid intake. However, the FDA has placed a warning for pregnant women and children on high trophic level fish like tuna due to high mercury exposure ("Fish and Omega-3 Fatty Acids | American Heart Association" n.d.; Nutrition 2020). Yellowfin tuna are also ecologically important due to their global distribution and their role as an apex predator (Essington et al. 2002). Studies on chemical bioaccumulation in yellowfin tuna will help to inform the impact of marine pollutants on marine life and human health.

The second chapter of this thesis will explore possible synergistic effects of unintended pesticide and hive medicine co-exposures on ABCB1 from European honeybees, *Apis mellifera*. Honeybees are important to crop production and ecological health, but they are facing population decline. A leading theory for the decline is the high exposure to industrial pesticides (Farooqui 2013; Johnson et al. 2010; Magal, Webb, and Wu 2019; 2020). Agricultural chemicals, including pesticides, are regularly tested for off-target effects before being released to market ("Food and Pesticides | US EPA" 2021). However, there are currently no regulations in place requiring testing of pesticide mixtures, even though mixtures are much more environmentally relevant. Bees could be intentionally exposed to pesticide mixtures applied by farmers to combat several different ailments at once, like a fungal infection and an aphid infestation. In addition, unintentional exposures to chemical mixtures of both in-hive medications, like antibiotic and parricides, and field-applied pesticides can occur.

This thesis consists of two chapters that address the following hypotheses:

1) **Transporter Interfering Chemicals (TICs) inhibit vertebrate ABCB1 by binding to highly conserved ligand-binding sites and promote chemical bioaccumulation across the food web**
2) **Structure-function analysis of conserved ABCB1 ligand binding sites across pollinator and pest insects can predict pesticide accumulation potentials and lead to new avenues for the design of safer and more targeted pest management practices**

To evaluate the first hypothesis, we biochemically characterized ABCB1 from yellowfin tuna using combined ATPase activity and competitive dye efflux assays. These experiments determined chemical inhibition potentials and ligand-binding sites for tuna ABCB1 which we compared to similar binding sites in several vertebrates, including important commercial and aquaculture fish species. These comparisons showed that ligand-binding sites were highly conserved across fish. Thus, ABCB1 inhibition by TICs is likely to impair environmental chemical efflux and promote chemical bioaccumulation in a wide range of commercially important fish species raised or caught for human consumption.

To investigate the second hypothesis, we cloned ABCB1 from the European honeybee, *Apis mellifera*, a beneficial pollinator insect important to commercial crop production and ecological health. The honeybee ABCB1 gene sequence was compared to ABCB1 of other pest or disease-carrying insects *in silico* to determine whether binding site differences could be exploited to design more targeted insecticides and to inform better pest management practices. The results show that honeybee and other pollinator ABCB1 homologs show very low sequence similarity to model insect *Drosophila melanogaster*, common in-hive pests, or human disease vector insects.

This indicates a need for a new insect model system for beneficial pollinators when studying the effects of environmental chemical accumulation and toxicity in bees. In addition, it provides an opportunity for the design of more targeted pesticides that inhibit ABCB1 proteins in pests and disease vectors but are well recognized and eliminated by pollinator insect ABCB1.

In summary, the goal of this thesis is to provide comprehensive information on how a novel class of environmental chemicals, the Transporter-Interfering Chemicals or TICs, alone or as environmental mixtures interact with evolutionarily conserved defense proteins to promote toxic chemical bioaccumulation in humans and other organisms.

Note: This thesis follows the nomenclature put forth by the HUGO Gene Nomenclature Committee that states that ATP-Binding Cassette Subfamily B Member 1 (ABCB1) also goes by the aliases Multidrug Resistance Protein 1 (MDR1) and P-glycoprotein (P-gp) ("Gene Symbol Report | HUGO Gene Nomenclature Committee" 2021). *For sake of conformity, we will refer to the tuna and honeybee P-glycoprotein orthologs as Ta-ABCB1 and Am-ABCB1 throughout the thesis.*

Chapter 1: Structural and functional characterization of P-glycoproteins from *Thunnus albacares* and *Mus musculus* to identify the molecular mechanisms underlying chemical bioaccumulation across vertebrates

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Keywords

Yellowfin tuna, P-glycoprotein, ABC transporter, persistent organic pollutants, bioaccumulation, Transporter-interfering chemicals

Abstract

Marine pollutants bioaccumulate at high trophic levels of marine food webs and are transferred to humans through consumption of apex species. Yellowfin tuna (*Thunnus albacares*) are marine predators, and one of largest commercial fisheries in the world. Previous studies have shown that yellowfin tuna can accumulate high levels of persistent organic pollutants, including several that are Transporter Interfering Chemicals (TICs) which bind to human xenobiotic transporters and interfere with their function. Here, we examined the extent to which these same compounds interfere with activity of the yellowfin tuna (*Thunnus albacares*) ortholog of this transporter. To accomplish this goal we identified, expressed, and functionally assayed tuna ABCB1. The results demonstrated a common mode of vertebrate ABCB1 interaction with TICs that predicts effects across these species, based on high conservation of specific interacting residues. Importantly several TICs showed potent inhibition of *Ta*-ABCB1, such as the

organochlorine pesticides Endrin (EC_{50} = 1.2±0.2 μM) and Mirex (EC_{50} = 2.3±0.9 μM). However, unlike the effects observed on mouse ABCB1, low concentrations of the organochlorine pesticide TICs p,p'-DDT and its metabolite p,p'-DDD co-stimulated verapamil-induced *Ta*-ABCB1 ATPase activity possibly suggesting a low transport activity for these ligands in tuna. These results provide a mechanistic basis for understanding the potential vulnerability of tuna to co-exposure to diverse marine pollutants, including those that interfere with normal detoxification pathways.

Introduction

Yellowfin tuna are apex marine predators that inhabit tropical and subtropical waters around the world. This species accounts for the world's second largest tuna fishery with annual landings in excess of 1.25 million pounds (C. Pecoraro et al. 2017; Carlo Pecoraro et al. 2018). As apex predators they can accumulate high levels of marine pollutants, but the primary concern about these pollutants has been on the potential transfer to humans who eat tuna (Xie et al. 2020; Choy et al. 2009; Nicklisch et al. 2017a; 2017b; Pulster et al. 2020). Less understood are the potential impacts of these pollutants on tuna themselves. Tuna shares many of the pathways targeted by pollutants in mammals and thus are likely to have many similar effects from pollutant exposure. For example, both embryonic fish and mammals share cardiac ion channels sensitive to polyaromatic hydrocarbons found in air pollution and crude oil spills (Brette et al. 2014; Holme et al. 2019; Incardona, Collier, and Scholz 2004; Incardona et al. 2005; 2009; 2013; 2014; Marris et al. 2020). As such the pollutants carried in fish not only represent a hazard to the humans who consume them, but also to the fish themselves.

One of the cellular pathways on which many organic contaminants converge involves xenobiotic transporters (XTs). These conserved proteins evolved to protect against toxic foreign

molecules in diet and the environment. Among XTs, several ATP-binding cassette (ABC) transporters including ABCB1 (P-glycoprotein), ABCC1, and ABCG2, are ubiquitously expressed in biological barriers, including kidney, liver, brain and intestine, and act to limit the cellular entry and accumulation of diverse xenobiotics by binding and effluxing them (Nicklisch and Hamdoun 2020b; Dean, Hamon, and Chimini 2001; Giacomini, et al 2010). P-glycoprotein or ABCB1 is a major determinant of human drug disposition. As such it, and its orthologs, are structurally and functionally one of the best characterized xenobiotic efflux pumps (Ambudkar et al. 2003; Irene Bosch and Croop 1998a; R. Callaghan 2015; Morrissey et al. 2012; Palmeira et al. 2012) with currently over 350 known drug substrates (https://go.drugbank.com).

Persistent environmental chemicals which bioaccumulate in apex marine predators also bind with high affinity to these transporters, but rather than being effectively eliminated, they can inhibit the function of the transporter (Nicklisch et al. 2016b; Sreeramulu, Liu, and Sharom 2007; Xie et al. 2020). Of concern is that the interfering action of these chemicals on xenobiotic metabolism can limit the normal detoxification capacity of XTs. These Transporter-Interfering Chemicals (TICs) include environmentally ubiquitous compounds such as several persistent organic pollutants (POPs), including organochlorine pesticides, brominated flame retardants (brominated diphenyl ethers or BDEs) and polychlorinated biphenyls. We previously examined nine of these that were commonly detected in tuna (Nicklisch et al. 2017a). These included the brominated flame retardants BDE-47 and BDE-100, both of which showed high levels of accumulation between 1 and 3 ppb in yellowfin tuna in comparison to other BDE congener flame retardants (Nicklisch et al. 2017a).

The goal of this study was to understand the similarities and differences in TIC effects between tuna and murine ABCB1 – one of the major xenobiotic transporters in vertebrates. In our

previous study (Nicklisch et al. 2016b), we demonstrated that persistent environmental chemicals can intimately interact with the ligand binding domain of this protein. The co-crystal structure of mouse ABCB1a in complex with one of these two flame retardants, BDE-100, showed an intricate network of hydrophobic and electrostatic interactions of the pollutant deep within a ligand binding site of the transporter. Importantly, 87% of the residues across the full protein sequence and those interacting with the bound flame-retardant are conserved between mouse and human homologs, suggesting potential conservation of this site. However, given the promiscuity of ABCB1 for its ligands it remains uncertain whether TIC interactions can be extrapolated across species as divergent as fish and mice.

To accomplish this goal, we cloned and expressed functional *Ta*-ABCB1 and probed the purified, protein against the same TIC compounds found in tuna and shown to inhibit mouse and human ABCB1. The results demonstrated a common mode of vertebrate ABCB1 interaction with TICs that predicts effects across these species, based on high conservation of specific interacting residues, with two important implications. The first is that ubiquitous TICs such as persistent pollutants could act at multiple steps in the food chain in a sort of positive feedback loop to amplify inhibitory effects that lead to enhanced pollutant bioaccumulation. The second is that TICs could act to sensitize yellowfin tuna themselves to exposures in their environment. This could be of particular relevance for populations with high TIC exposure.

Materials and Methods

Chemicals: Cyclosporine A (CSA), verapamil (VER), Triton X-100, and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Calcein-AM (CAM) was purchased from Biotium (Hayward, CA). Except for verapamil (dissolved in H_2O), all stock solutions were

prepared in DMSO and diluted to final concentrations in filtered seawater. The final DMSO concentration in the ATPase and cell assays did not exceed 2% and 0.5%, respectively.

Animal and tissue handling: Mature yellowfin tuna (*Thunnus albacares*) organs were extracted from wild tuna caught off the coast of Louisiana in the Gulf of Mexico. The fish had an average length of 111-185cm (Nicklisch et al. 2017a). Samples were shock-frozen in dry ice and stored at -80 °C until total RNA extraction or homogenization. Purple sea urchins (*Strongylocentrotus purpuratus*) were collected and maintained in aquaria as detailed previously (Campanale and Hamdoun 2012).

Tuna tissue homogenization and immunoblotting: Approximately 100mg tuna tissue was dissected on dry ice. Tools were disinfected with 70% ethanol in between tissue preparations. A 1:9 (w/v) ratio of tuna tissue and 3x homogenization buffer (RIPA buffer, EDTA-free protease inhibitor tablets (Pierce EDTA-free) was transferred to 2 ml centrifuge tubes with 2.8 mm ceramic beads (Omni International) and twice homogenized in a bead mill (Fisherbrand Bead Mill 24) Samples were incubated on ice for 30 min prior to transferring the homogenate into 1.5mL centrifuge tubes and centrifuging at 15,000 x g for 2 min at 4 °C. The supernatant was stored at -80 °C until use. *Ta*-ABCB1 detection was performed using the C219 anti-P-glycoprotein antibody (van Den Elsen et al. 1999). The SDS mini-gels were hand-cast using standard reagents to create a 4% stacking and 7.5% resolving gel. Approximately 20 ug of total lysate protein was separated on a 7.5% SDS gel (Laemmli 1970). Gels were transferred to a 0.2 um PVDF membrane for 30 min at 100 V using a mini-PROTEAN II system (Biorad). Membranes were blocked for 1 hour in 5% BSA in TBST (20 mM Tris and 150 mM NaCl with 0.1% Tween 20, pH 7.6). Primary mouse C219 monoclonal

anti-P-glycoprotein (Invitrogen) was added in a 1:1000 dilution and incubated overnight (16 h at 4 °C). Membrane was washed 3 times for 10 min each in TBST then incubated for 1 hr at RT in secondary goat anti-mouse (BioRad) diluted at 1:10,000 in 5% BSA in TBST. Membrane was washed 3 times in TBST for 10 min each and then developed using Clarity Western ECL (BioRad). Images were taken in a BioRad ChemiDoc station using the ImageLab software v6.0.1.

Cloning and subcloning of *Ta-abcb1*: Primers were designed based on highly conserved regions among fish ABCB1 orthologs. The oligonucleotides were obtained from IDT (Coralville, Iowa, USA). Total RNA was isolated from approximately 30mg of liver tissue of wild yellowfin tuna from the Gulf of Mexico using an RNA II kit (Macherey-Nagel, Düren, Germany) and reverse transcribed using the RNA-to-cDNA kit according to the manufacturer's instructions (High-Capacity RNA-to-cDNA Kit, Applied Biosystems, Foster City, CA, USA). PCR was performed in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) using Phusion high fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA). 5'- and 3' ends of the gene were cloned from RACE-ready cDNA using the SMARTer RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA). The full-length sequence of the transporter (3894 bps) was cloned into the *Pichia* expression plasmid pPICZc (Invitrogen, Carlsbad, CA, USA) harboring a C-terminal 3C Protease site and C-terminal His_6-tag or into the pCS2+8 vector harboring an N-terminal mCherry tag (Gökirmak et al. 2012). The *Ta-abcb1* cDNA generated in this study is available through the Addgene (www.addgene.org) public repository.

Ta-abcb1 overexpression and purification: *Ta-ABCB1* was expressed and purified as described previously (Nicklisch et al. 2016b). Briefly, the gene was cloned into a pPICZc vector and

mutagenized to remove potential N-glycosylation sites (N101Q, N104Q, N109Q, and N116Q, using codon CAA in these positions). The deglycosylated construct was transformed into *P. pastoris* strain KM71H (ThermoFisher Scientific, Waltham, MA, USA) using a GenePulser Xcell electroporation system (BioRad). Resulting clones were grown in 10-L BioFlo 415 bioreactors (New Brunswick Scientific, Edison, NJ, USA) and induced for 16-18 hrs with a 2.5% flow rate of 50% MeOH. A typical 10-L growth would typically yield 20-40 mg of protein. Resulting cells were harvested by centrifugation and lysed at 40 KPSI by a single pass through a cell disruptor (TS-Series, Constant Systems, Daventry, Northants, UK). Cell debris was removed by centrifugation at 12,500xg, followed by membrane isolation at 38,400xg into lysis buffer (20 mM Tris pH 8.0, 100 mM NaCl, 15% glycerol). Membranes were solubilized and *Ta-ABCB1* was purified using a Ni–nitrilotriacetic acid Superflow resin (Qiagen) via fast protein liquid chromatography (AEKTA, GE Life Sciences). The protein was concentrated at 1,500xg (Millipore, Burlington, MA, USA), and a single, monomeric peak was isolated by size exclusion chromatography using a prep-grade Superdex 200 column (Fisher Scientific). The calculated molecular mass of *Ta*-ABCB1 (1297aa) is ~143.3kDa (http://web.expasy.org/protparam/). The total concentration of the purified protein was determined using the Micro BCA Protein Assay Kit (Pierce, Rockford, lL, USA). Protein purity was evaluated by combined 7.5% SDS PAGE and wet electroblotting on 0.45 mM PVDF using a primary mouse 6-His Epitope Tag monoclonal antibody (1:2000) and secondary goat anti-mouse IgG-HRP (1:5000) in 5% skim milk/TBST (0.1 M Tris-base, 150 mM NaCl, 0.05% Tween 20). The proteins were visualized using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, lL, USA).

MALDI mass spectrometry analysis: The molecular mass of the purified, recombinant *Ta*-ABCB1 was determined using Matrix-assisted laser desorption ionization (MALDI) mass spectrometry analysis with time-of-flight (TOF) detector. The analysis was performed on a Voyager Mass Spectrometer LBT2 (Applied Biosystems, San Jose, CA) with 1.2-meter ion path in the positive ion linear mode. As matrix solution, sinapinic acid in 50 % acetonitrile and 0.1 % trifluoroacetic acid (TFA) was used. Samples were diluted 1:20 with matrix solution and 1 µL was spotted onto the MALDI sample target plates and air-dried on the bench. Spectra were obtained in the mass range between 5,000 and 200,000 Da with 256 laser shots per spectrum. Internal calibration was performed using bovine serum albumin standard (Sigma-Aldrich, St. Louis, MO) with a calculated molecular mass of 66.5 kDa. All data was analyzed using Voyager Data Explorer 4.0.0.0 (Applied Biosystems) and plotted using OriginPro 2016 (Originlab, Northampton, MA).

ATPase activity of solubilized *Ta*-ABCB1: For the determination of *Ta*-ABCB1 ATPase activity we used a modified malachite green assay for detection of inorganic phosphate (Pi) release as described previously (Nicklisch et al. 2016b). Briefly, 2µg of purified and solubilized *Ta*-ABCB1 activated protein was added to a 96 well plate containing 60 µl of ATP-free reaction buffer (10 mM $MgSO_4$, 0.05% w/v DDM, 1mM TCEP, 0.1 mg/ml *E. coli* Polar Extract lipids in 50 mM Tris-Cl buffer pH 7.5) with serial dilutions of verapamil or cyclosporin A with 100 µM verapamil. Then 60 µl of ATP solution (5 mM Na-ATP, 10 mM $MgSO_4$, 0.05 % w/v DDM, 1 mM TCEP, 0.1 mg/ml *E. coli* Polar Extract lipids in 50 mM Tris-Cl buffer pH 7.5) was added, mixed and incubated for 5 min on ice. After incubation, the reaction mixtures in the 96 well PCR plate were transferred to a thermocycler and the reaction was kept for 5 min at 37°C before a 15 sec incubation at 80°C (heat inactivation). 30 µl of the ATPase reactions were transferred to a 96 well ELISA plate and

the liberated Pi was measured by adding 150 µl of an activated stock color development solution (17 mg malachite green in 3.75 mL MilliQ H_2O, 0.525 g ammonium molybdate tetrahydrate in 12.5 mL of 4N HCl, activated with 0.02% v/v Triton X-100) in each sample well. The absorbance of each sample was immediately measured at 600 nm in a microplate reader (Spectramax M2, Sunnyvale, CA, US). Control samples containing buffer and DMSO (cyclosporine A) or H_2O (verapamil) without any added ABCB1 protein were subtracted as background values. Inorganic phosphate standards (KH_2PO_4) from 0.125 to 2 nmol served as internal controls.

Ta-ABCB1 efflux activity assays in sea urchin embryos: Efflux activity was determined at ~16 hrs post fertilization (hpf) in embryos expressing *Ta*-ABCB1 protein with N-terminal fluorescent mCherry tag as previously described (Gökirmak et al. 2014). Embryos were incubated with CAM at a final concentration of 250 nM at 15°C for 90 minutes. Intracellular accumulation was measured using a Zeiss (Jena, Deutschland) LSM 700 laser scanning confocal microscope equipped with a 20X objective. 4.1 µm thick equatorial section images of 10-28 embryos from two separate experiments (2 different females) were collected for the transporter-drug pair.

Phylogenetic Analysis: Using the software CLC Main Workbench v21.0.2 (Qiagen N.V., Hilden, Germany), multi-sequence alignments and phylogenetic trees were created. Briefly, protein sequences for ABCB1 orthologs of vertebrate model organisms were first identified using the Kyoto Encyclopedia of Genes and Genomes (KEGG) reference pathway on ABC transporters. The reference protein sequences were then downloaded from the National Center for Biotechnology Information (NCBI) *Reference Sequence Database* (RefSeq). Additional orthologous vertebrate genes and proteins were identified using *NCBI Orthologs* search based on NCBI's Eukaryotic

Genome Annotation pipeline. Truncated proteins and additional protein isoforms (IF) or variants (e.g., X1, X2, etc.) with identical amino acid sequences were omitted from the phylogenetic analysis. All protein sequence alignments were performed using the integrated algorithm for progressive alignments with a gap open cost of 10 and a gap extension cost of 1. Gaps at the ends of each sequence were treated like gaps in any other place in the sequence. Distance-based tree construction was performed with neighbor joining method and Jukes-Cantor protein distance measure and based on 1,000 bootstrap iterations. Alternative tree construction methods using the unweighted pair group method with arithmetic mean (UPGMA) and the Kimura protein distance measure algorithm resulted in similar tree topologies and confirmed that distance measures were robust. Amino acid sequences in an alignment were additionally analyzed for percent sequence identity and differences using the *Pairwise Comparison* algorithm of CLC Main Workbench. Each comparison table displays the differences in alignment position in the upper comparison and the percentage of identical amino acid alignment positions in the lower comparison.

Transport Kinetic Data Analysis: ATPase activity data are given as means ±standard error of the mean (SEM) from triplicate measurements. To calculate EC_{50} values, the data were fitted to a Hill function: $y = v_1 + (v_2 - v_1) * x^n / (k^n + x^n)$, where v_1 and v_2 are the initial and final reaction velocities, respectively, n is the Hill coefficient or the cooperativity of the dependence on x, and k is the effective concentration (EC_{50}) that corresponds to 50% of maximal effect (i.e., inhibition or stimulation). All studies were performed in 3–5 independent experiments and representative experiments are shown. All calculations were performed using OriginPro 2016 software (Originlab, Northampton, MA). For the sea urchin embryo dye efflux experiments, the average efflux activity of each transporter was calculated by measuring the intracellular substrate

fluorescence intensity per pixel in microinjected embryos relative to control embryos using measurement module of the free image processing software Fiji (i.e., ImageJ).

Results

Cloning of *Ta-abcb1* from tuna liver

Table S1: List of primers for subcloning of *Ta-abcb1* into expression vectors. Shown are primers with complementary overhangs (bold and italics nucleotides) for cloning into expression vectors for Pichia ("pPICZc") and sea urchin embryos with either N-terminal ("NMC") or C-terminal ("CMC") mCherry tag in pCS2+8 vectors. Primers to clone the full-length *Ta-abcb1* gene from RACE-ready cDNA were designed to cover the open reading frame ("gene").

Primer Name	Length (bps)	Primer Sequence (5' --> 3')
NMC-TaABCB1-FW	44	***GCCATTAATTAAAGGCCGGCC***A ATG GAG GGA AAG GAA GAG ATG G
NMC-TaABCB1-REV	40	***GTTCTAGAGGCTCGAG*** TCA ATT CCT CTC GTG ACC CAT CTG
CMC-TaABCB1-FW	40	***TCCACTAGTGGCGCGCC***A ATG GAG GGA AAG GAA GAG ATG G
CMC-TaABCB1-REV	41	***GCTGGCCGGCCTTTAATTAA*** ATT CCT CTC GTG ACC CAT CTG
PPICZ-TaABCB1-FW	38	***TGA CGA TAA GTC TAG A*** ATG GAG GGA AAG GAA GAG ATG G
PPICZ-TaABCB1-REV	41	***TGG TGA GAA CCT CTG GTA CC*** ATT CCT CTC GTG ACC CAT CTG
Gene-TaABCB1-FW	22	ATG GAG GGA AAG GAA GAG ATG G
Gene-TaABCB1-REV	22	TCA ATT CCT CTC GTG ACC CAT C

Figure S1: Full length amino acid sequence alignment of *T.a.*-ABCB1 with two model fish species. Tuna ABCB1 shares 80% of its amino acid sequence with Mummichog (*F. heteroclitus*) ABCB1 (XP_035989740) and 74% with Zebrafish (*D. rerio*) ABCB4 (XP_005158095). Conserved amino acids are marked in blue. The characteristic structural motifs of the ABC transporter nucleotide binding domains are marked in green, including the Walker A, Q-loop, ABC Signature, Walker B, D-loop, and H-loop. The two epitopes recognized by the C219 antibody are marked in red (van Den Elsen et al. 1999).

A full-length *Ta-abcb1* gene (*Ta*-ABCB1) was cloned from liver samples with a full-length ORF of 3894 bp, resulting in a full-length protein of 1297 amino acids (Figure S1). To identify possible

isoforms of the gene, we screened 3-4 sets of additional liver sample cDNAs with gene-specific end-to-end primers (Table S1). An alignment of the *Ta-abcb1* gene sequences cloned from these four liver samples only showed three synonymous SNPs that did not alter the amino acid sequence of the *Ta*-ABCB1 protein. Structural motifs unique to the catalytic ABC domain were identified in both nucleotide binding domains (NBDs) of *Ta*-ABCB1 (Figure S1). Using TOPCONS (https://topcons.net/pred/) consensus prediction server, a topology analysis of *Ta*-ABCB1 amino acid sequence was performed and revealed 12 distinct transmembrane domains with cytoplasmic N- and C-termini, characteristic for other ABCB1 homologs (Figure S2). A protein-protein BLAST (BLASTP) analysis of the full-length *Ta*-ABCB1 amino acid sequence showed high sequence similarity with other fish ABCB1 orthologues (Table S2).

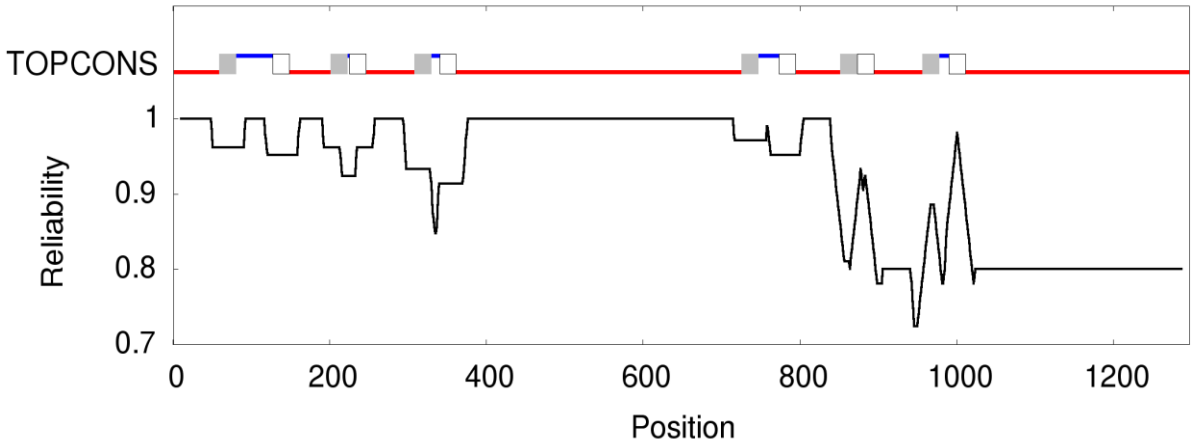


Figure S2: Predicted membrane topology of *Ta*-ABCB1 using TOPCONS (https://topcons.net). Shown are the consensus prediction of the overall membrane protein topology and the respective reliability score across the full-length amino acid sequence. Inside (red) or outside (blue) orientations of the predicted membrane spanning segments relative to the membrane are displayed. The predicted 12 TM helices are highlighted and go from outside to inside (grey) or inside to outside (white) of the membrane.

Table S2: Amino acid sequence similarities between *T.a.*-ABCB1 and other fish ABCB1 orthologues. The table lists of an NCBI BLASTP (protein-protein BLAST) of the *T. albacares* ABCB1 amino acid sequence. Proteins were identified using non-redundant (nr) protein sequences database filtered by bony fish (taxid: 7898) and were ranked according to >80% sequence identity. The results were filtered to remove low quality proteins, partial and hypothetical proteins. Only the isoform with the highest identity was listed for each orthologue. Species marked in grey are commercial fish, including common aquaculture species (Food and Agriculture Organization (FAO) 2020; National Marine Fisheries Service 2020; USDA 2019).

Description	Common Name	Query Cover	Identity (%)	Length (aa)	Accession
multidrug resistance protein 1 [Seriola lalandi dorsalis]	California yellowtail	99%	85.1	1293	XP_023256533.1
multidrug resistance protein 1 [Seriola dumerili]	Greater amberjack	99%	85.0	1293	XP_022621891.1
multidrug resistance protein 1-like [Echeneis naucrates]	Live sharksucker	88%	84.7	1133	XP_029359235.1
PREDICTED: multidrug resistance protein 1 [Lates calcarifer]	Barramundi perch	100%	83.9	1287	XP_018541648.1
multidrug resistance protein 1-like [Monopterus albus]	Swamp eel	99%	83.7	1293	XP_020462921.1
ATP-binding cassette, sub-family B (MDR/TAP), member 4 [Anabas testudineus]	Climbing perch	99%	83.6	1293	XP_026226619.1
ATP-binding cassette, sub-family B (MDR/TAP), member 4 [Acanthopagrus latus]	Yellowfin seabream	96%	83.2	1296	XP_036929042.1
ATP-dependent translocase ABCB1 [Myripristis murdjan]	Pinecone soldierfish	99%	83.0	1284	XP_029929374.1
multidrug resistance protein 1-like isoform X2 [Gouania willdenowi]	Blunt-snouted clingfish	97%	82.9	1143	XP_028326097.1
ATP-dependent translocase ABCB1 [Sparus aurata]	Gilthead seabream	96%	82.5	1297	XP_030250179.1
ATP-binding cassette, sub-family B (MDR/TAP), member 4 [Thalassophryne amazonica]	Thalassophryne amazonica	96%	82.5	1308	XP_034030717.1
PREDICTED: multidrug resistance protein 1-like isoform X3 [Hippocampus comes]	Tiger tail seahorse	96%	82.5	1282	XP_019747631.1
ATP-dependent translocase ABCB1-like isoform X4 [Salvelinus namaycush]	Lake trout	89%	82.1	1159	XP_038850936.1
ATP-dependent translocase ABCB1 isoform X2 [Oncorhynchus mykiss]	Rainbow trout	89%	82.0	1159	XP_036794815.1
multidrug resistance protein 1-like isoform X2 [Oncorhynchus nerka]	Sockeye salmon	89%	81.9	1158	XP_029544664.1
ATP-binding cassette, sub-family B (MDR/TAP), member 4 [Kryptolebias marmoratus]	Mangrove rivulus	97%	81.6	1300	XP_017280598.1
ATP-binding cassette, sub-family B (MDR/TAP), member 4 isoform X2 [Amphiprion ocellaris]	Clown anemonefish	99%	81.4	1292	XP_023145774.1
Multidrug resistance protein 1 [Channa argus]	Northern snakehead	99%	81.4	1250	KAF3691922.1
ATP-binding cassette, sub-family B (MDR/TAP), member 4 [Cyprinodon tularosa]	White sands pupfish	96%	81.3	1241	XP_038125251.1
multidrug resistance protein 1 [Parambassis ranga]	Indian glassy fish	99%	81.3	1284	XP_028280398.1
PREDICTED: multidrug resistance protein 1 [Stegastes partitus]	Bicolor damselfish	99%	81.1	1293	XP_008297780.1
ATP-binding cassette, sub-family B (MDR/TAP), member 4 isoform X3 [Syngnathus acus]	Greater pipefish	98%	81.1	1276	XP_037130091.1
ATP-binding cassette, sub-family B (MDR/TAP), member 4 isoform X3 [Gymnodraco acuticeps]	Ploughfish	90%	81.1	1173	XP_034084200.1
ATP-binding cassette, sub-family B (MDR/TAP), member 4 [Epinephelus lanceolatus]	Giant grouper	99%	81.0	1286	XP_033496509.1
ATP-binding cassette, sub-family B (MDR/TAP), member 4 [Morone saxatilis]	Striped sea-bass	99%	81.0	1286	XP_035527748.1
P-glycoprotein Abcb1 [Trematomus bernacchii]	Emerald rockcod	90%	81.0	1173	ACX30417.1
ATP-binding cassette, sub-family B (MDR/TAP), member 4 [Scophthalmus maximus]	Turbot	99%	80.9	1292	XP_035482908.1
Multidrug resistance protein 1 [Oryzias melastigma]	Indian medaka	95%	80.9	1234	KAF6739542.1
ATP-binding cassette, sub-family B (MDR/TAP), member 4 [Sander lucioperca]	Pikeperch	99%	80.9	1285	XP_031155093.1
ATP-binding cassette, sub-family B (MDR/TAP), member 4 [Etheostoma cragini]	Arkansas darter	99%	80.8	1284	XP_034731183.1
P-glycoprotein [Poeciliopsis lucida]	Clearfin livebearer	99%	80.8	1286	ADQ20481.1
P-glycoprotein [Xiphophorus hellerii]	Green swordtail	96%	80.8	1286	AEV93606.1
multidrug resistance protein 1 [Oryzias latipes]	Japanese medaka	96%	80.7	1286	XP_023819737.1
multidrug resistance protein 1-like [Perca flavescens]	Yellow perch	100%	80.6	1285	XP_028437503.1
multidrug resistance protein 1 [Xiphophorus maculatus]	Southern platyfish	99%	80.5	1294	XP_014328020.1
ATP-binding cassette, sub-family B (MDR/TAP), member 4 isoform X2 [Micropterus salmoides]	Largemouth bass	99%	80.5	1287	XP_038567411.1
multidrug resistance protein 1-like isoform X1 [Salmo trutta]	River trout	96%	80.5	1287	XP_029592305.1
PREDICTED: multidrug resistance protein 1-like [Poecilia mexicana]	Shortfin molly	99%	80.4	1295	XP_014861382.1
ATP-binding cassette, sub-family B (MDR/TAP), member 4 [Notolabrus celidotus]	New Zealand spotty	99%	80.4	1287	XP_034553324.1
ATP-dependent translocase ABCB1-like [Mastacembelus armatus]	Zig-zag eel	99%	80.3	1289	XP_026172544.1
P-glycoprotein [Gambusia affinis]	Western mosquitofish	99%	80.3	1294	QKW91241.1
multidrug resistance protein [Platichthys flesus]	European flounder	100%	80.3	1292	CAC86600.1
ATP-dependent translocase ABCB1-like [Oncorhynchus kisutch]	Coho salmon	96%	80.2	1279	XP_031643356.1
ATP-binding cassette, sub-family B (MDR/TAP), member 4 [Hippoglossus hippoglossus]	Atlantic halibut	99%	80.2	1301	XP_034467969.1
ATP-binding cassette, sub-family B (MDR/TAP), member 4 [Neolamprologus brichardi]	Brichard's Lyretail Fairy Cichlid	95%	80.1	1215	XP_006801536.2
ATP-binding cassette, sub-family B (MDR/TAP), member 4 [Nematolebias whitei]	Rio pearlfish	98%	80.1	1337	XP_037532573.1
ATP-dependent translocase ABCB1-like [Oreochromis aureus]	Blue tilapia	90%	80.1	1124	XP_031601846.1
ATP-binding cassette, sub-family B (MDR/TAP), member 4 [Hippoglossus stenolepis]	Pacific halibut	99%	80.0	1301	XP_035019497.1
PREDICTED: multidrug resistance protein 1-like [Poecilia latipinna]	Sailfin molly	99%	80.0	1298	XP_014878632.1

Phylogenetic analysis of *Ta*-ABCB1

The phylogenetic analysis based on amino acid sequence alignments revealed that *Ta*-ABCB1 clusters with ABCB1/B4 orthologs of fish and other vertebrate species (Figure 1). This subcluster is distinct from the group of ABCB5 orthologues and the evolutionarily distant *S. purpuratus* ABCB1a protein. The topology of the tree suggests that the annotated *X. tropicalis* ABCB1 transporter (XP_017951387) has a closer evolutionary relationship to the ABCB5 orthologues. Amino acid sequence comparison between *Ta*-ABCB1 and vertebrate ABCB1 orthologs (Figure 1, inserts) shows 74% sequence identity to ABCB4 of model fish species *D. rerio* and only 53% sequence identity to *D. rerio* ABCB5, the two identified P-glycoproteins in zebrafish (Fischer et al. 2013; Gordon et al. 2019; Robey et al. 2021). Interestingly, the freshwater livebearer Southern Platyfish (*X. maculatus*) ABCB1/4 had 80.1% sequence identity to *Ta*-ABCB1.

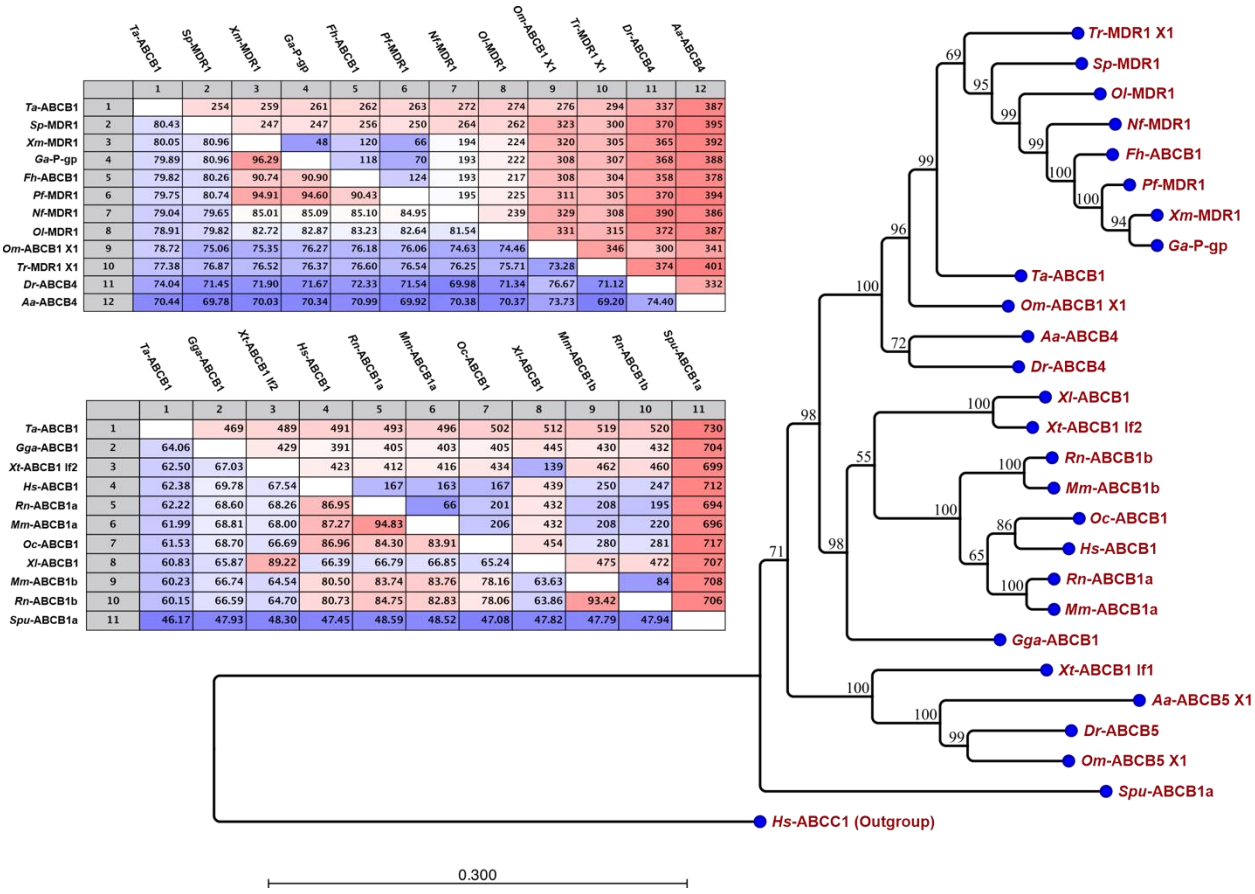


Figure 1: Phylogenetic analysis and sequence comparison of full length *T.a.*-ABCB1 with ABCB1 homologs of vertebrates. The percentage concordance based on 1,000 bootstrap iterations is shown at the nodes. Table inserts: Pairwise comparison of amino acid sequences and their percent identities (lower comparison) and differences (upper comparison) separated by fish (upper panel) and other vertebrate (lower panel) ABCB1 homologs. Ta, *Thunnus albacares*; Sp, *Stegastes partitus*; Xm, *Xiphophorus maculatus*; Ga, *Gambusia affinis*; Fh, *Fundulus heteroclitus*; Pf, *Poecilotheria formosa*; Nf, *Nothobranchius furzeri*; Ol, *Oryzias latipes*; Om, *Oncorhynchus mykiss*; Tr, *Takifugu rubripes*; Dr, *Danio rerio*; Aa, *Anguilla anguilla*; Gga, *Gallus gallus*; Xt, *Xenopus tropicalis*; Hs, *Homo sapiens*; Rn, *Rattus norvegicus*; Mm, *Mus musculus*; Oc, *Oryctolagus cuniculus*; Xl, *Xenopus laevis*; Spu, *Strongylocentrotus purpuratus*.

Tuna ABCB1 showed slightly higher sequence identity to human ABCB1 (62.4%) versus rat ABCB1a (62.2%) or mouse ABCB1a (62.0%). Indeed, in rat a total of 491 alignment positions differed from tuna ABCB1, while mouse ABCB1a differed by 496 positions (Figure 1, lower insert). The comparison of all non-fish vertebrate species revealed that chicken ABCB1 has the highest sequence identity to tuna ABCB1 with 64% and only 469 amino acid positions differing

(Figure 1, lower insert). Despite being an evolutionarily distant deuterostome, the sequence identity between *Ta*-ABCB1 and sea urchin ABCB1a is still 46.2%. The pairwise comparison of fish model organisms ABCB1 orthologs showed high amino acid sequence identities to tuna ABCB1, ranging from 70.4% in the catadromous European eel (*A. anguilla*) to 80.4% in bicolor Damselfish (*S. partitus*), a tropical reef fish (Figure 1, upper insert).

Although we refer to the identified tuna gene as *abcb1*, we note the fact that several teleost fish possess at least two ABCB/P-glycoprotein-like co-orthologues with xenobiotic efflux function, commonly referred to as *abcb4* and *abcb5* (Fischer et al. 2013; Liu, Li, and Liu 2013; Gordon et al. 2019; Luckenbach, Fischer, and Sturm 2014). While these proteins share many functions with mammalian ABCB1, the designation of *D. rerio* ABCB4 is based on synteny analysis, rather than shared function in export of bile acids. As such further nomenclature for the identified tuna ABCB transporter must await successful chromosomal analysis of *Ta*-ABCB1.

Ta-ABCB1 expression and purification

A challenge of cellular assays of transporter activity against pollutants is the confounding effect of background transporters (Nicklisch and Hamdoun 2020b). To address this issue and better characterize the activity of *Ta*-ABCB1 against pollutants we expressed the protein at mg scale in yeast (*Pichia pastoris*) and purified it using combined affinity tag and size exclusion chromatography. While a mixture of βDDM, CHS, and CHAPS were used during purification of *Ta*-ABCB1, use of βDDM alone was sufficient for subsequent functional assays. Native *Ta*-ABCB1 with a molecular mass of approximately 170-200 kDa was detected in liver, gill, brain, and gonads using the C219 anti-P-glycoprotein monoclonal antibody (Figure 2A). The predicted molecular mass of recombinant *Ta*-ABCB1 with the four mutated N-glycosylation sites, a protease

cleavage site, and affinity tag is 146 kDa which could be confirmed using MALDI TOF mass spectrometry (Figure 2B). The 7.5% SDS-gel and Anti-His_6-tag Western blot in Figure 2B (insert) show that no major contaminants or degradation products were detected during the purification process.

ATPase activity of detergent-solubilized *Ta*-ABCB1

To examine whether the *Ta*-ABCB1 gene we cloned encodes a functional transporter, we used an optimized ATPase assay based on the sensitive malachite green method requiring only 1 μg of total protein per well (Nicklisch et al. 2016b). Figure 2C shows the respective dose–response curves for the model substrates verapamil and the model inhibitor cyclosporine A with tuna ABCB1. Activating *Ta*-ABCB1 ATPase activity using verapamil resulted in a half-maximal stimulation concentration (EC_{50} value) of 8.8±0.6 μM, similar to that found for mouse ABCB1a (Bai et al. 2011; Swartz, Weber, and Urbatsch 2013). Verapamil-stimulated (100 μM) *Ta*-ABCB1 was inhibited in the presence of increasing concentrations of cyclosporine A with an EC_{50} value of 1.3±0.1 μM. Cyclosporine A restored *Ta*-ABCB1 back to the basal activity level (~0.1 μmol/min/mg protein).

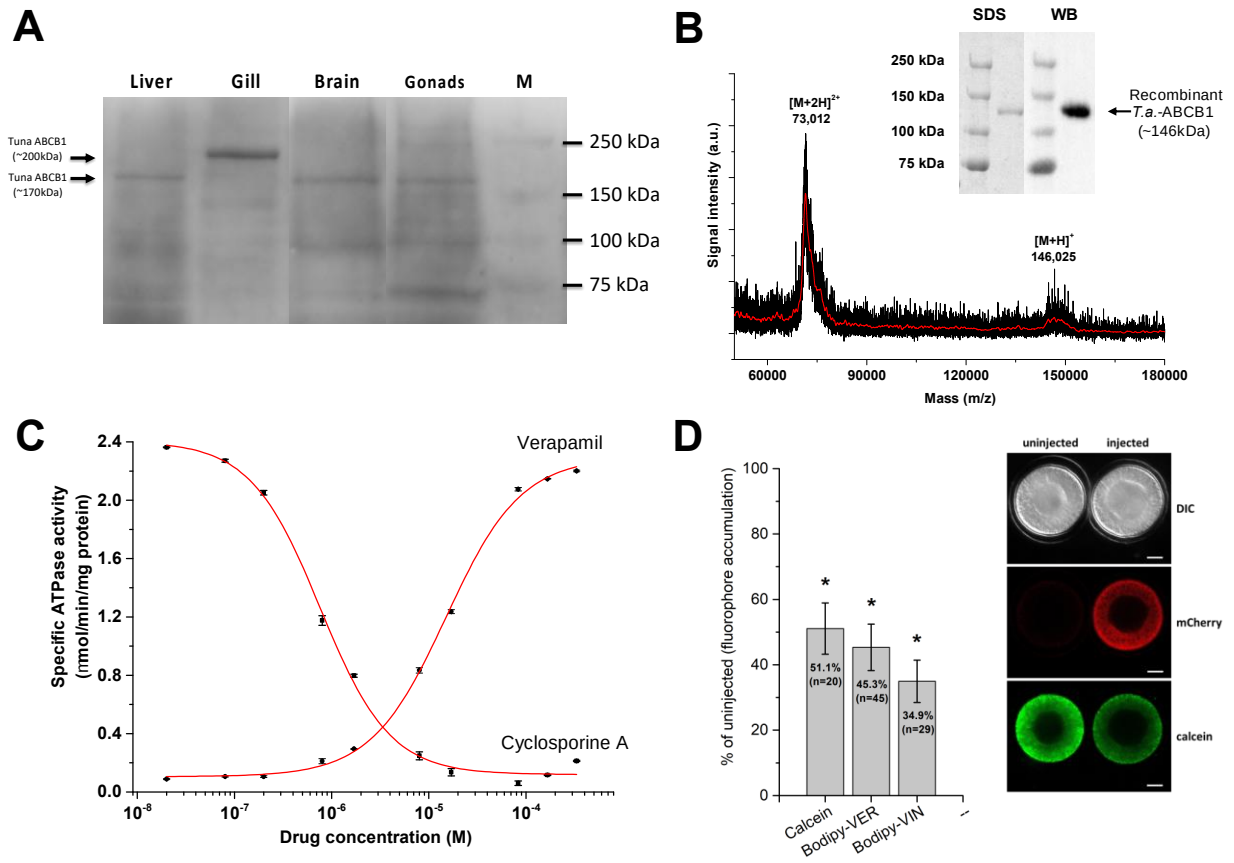


Figure 2: Detection, purification, and activity of recombinant tuna ABCB1 (*T.a.*-ABCB1). A: PVDF Immunoblot of yellowfin tuna tissue extracts using C219 mAB, Extracts were separated with a 7.5% SDS PAGE gel. Liver, gonad, and brain extracts show a sharp protein band at ~170 kDa with minor degradation bands, and gills have a band at ~200kDa indicating protein glycosylation. **B:** MALDI TOF mass spectrum of the purified *Ta*-ABCB1 with two differently charged species. The addition of a C-terminal 3C protease site (LEVLFQGP) and His10–tag (HHHHHHHHHH) leads to an observed mass of about 146 kDa. Noise reduction of the data was done by adjacent averaging (weighted average) with a window of n = 100 points (red trace). Matrix = Sinapinic acid, Accelerating voltage = 25,000 V, Grid voltage = 93%, Guide wire voltage = 0.3, Delay time = 700 ms. Insert: 7.5% SDS-PAGE with Coomassie blue staining (left) and an immunoblot of *Ta*-ABCB1 fused to a C-terminal His_{10}-tag by anti-His antibody (right) of purified *Ta*-ABCB1. **C:** ATPase activity of purified *Ta*-ABCB1 using the malachite green method. ATPase activation and inhibition were determined with increasing concentrations of verapamil and cyclosporine A. Data points indicate the average specific activity ±SEM from three to six independent experiments. Where not visible, error bars are smaller than the symbols. Lines represent non-linear regression analysis of the data points with a Hill equation ($y = v_1 + (v_2 - v_1) * x_n / (k_n + x_n)$). R^2 values for the data fits were between >0.99. **D:** Quantitative analysis of intracellular fluorophore accumulation in purple sea urchin (*S. purpuratus*) embryos expressing *Ta*-ABCB1. Asterisk indicates that the difference between the means of uninjected and injected embryos was significant at the level of $\alpha = 0.05$ (one-way ANOVA). Representative apical localization of N-terminal mCherry-tagged *Ta*-ABCB1 transporter in 16 hpf embryos (right panel). The DIC image shows blastulae with a single cell layer of polarized cells. Scale bars: 20µm. n = 37 embryos from 3 separate batches.

Dye efflux assays of *Ta*-ABCB1 expressed in sea urchin embryos

To validate transporter localization and efflux activity in a marine cell, we used a well-established transporter overexpression method in purple sea urchin embryos (Gökirmak et al. 2012; 2014; Gokirmak et al. 2016; Shipp et al. 2015). In this assay the mRNA encoding the transporter fused to a fluorescent protein reporter is injected into sea urchin embryos. At 16h after fertilization the embryo forms a polarized blastula, and the localization and efflux function of the overexpressed transporter can readily be assayed using fluorescent substrates and confocal microscopy. The results revealed that embryos overexpressing *Ta*-ABCB1 have reduced intracellular accumulation of the three fluorescent ABCB1 substrates calcein, BODIPY-verapamil, and BODIPY-vinblastine (Gökirmak et al. 2014; Litman et al. 2000). Figure 2D shows the quantitative and qualitative analysis of intracellular accumulation in sea urchin embryos expressing tuna ABCB1 fused to an N-terminal mCherry tag. *Ta*-ABCB1 fused to an N- or C-terminal mCherry tag localized apically at 16 HPF (hours post fertilization) and led to reduction of intracellular accumulation of calcein. *Ta*-ABCB1 overexpressing embryos accumulated 51.1%, 45.3%, and 34.9% of BODIPY-verapamil, and BODIPY-vinblastine as compared to the control embryos ($p<0.05$, one-way ANOVA). The N-terminal mCherry fusion showed a more distinct apical localization of active *Ta*-ABCB1 as has been observed with other fluorescently tagged ABCB1 proteins (Gökirmak et al. 2012). Embryos injected with the C-terminal mCherry fusion did not produce mature tuna ABC transporter as gauged by fluorescence.

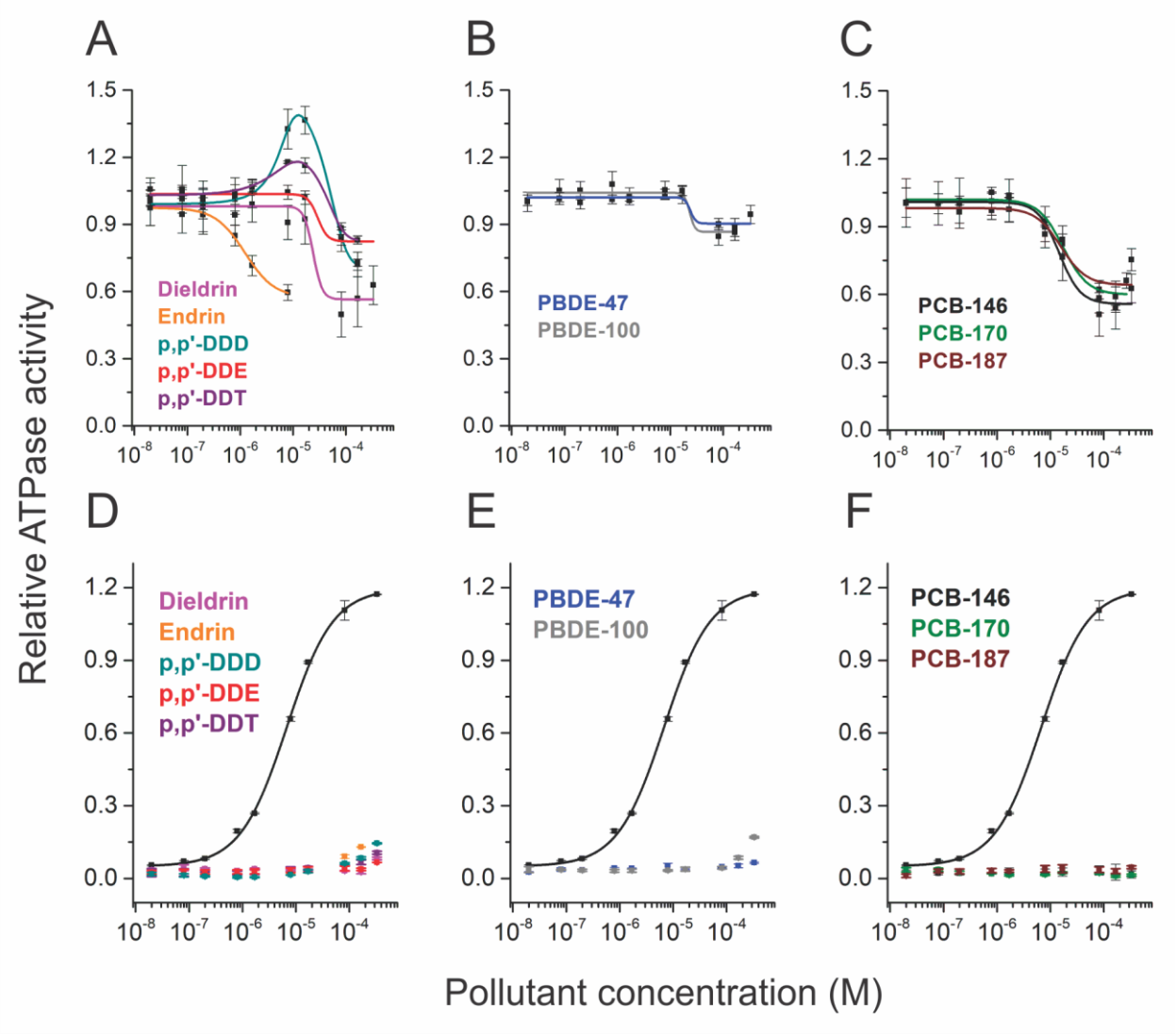


Figure 3: ATPase activity assays of *Ta*-ABCB1 with Transporter-Interfering Chemicals (TICs). Upper panels show the inhibition profiles of the five organochlorine pesticides (**A**), two flame retardants (**B**) and three polychlorinated biphenyl (PCB) congeners (**C**) that were previously identified to inhibit mouse ABCB1a. Lower panels show *Ta*-ABCB1 ATPase activity assays in stimulation mode. The five pesticides (**D**), two flame retardants (**E**), and three PCBs (**F**) were not able to stimulate ATPase activity. Black curves show verapamil stimulation. All data points were normalized to 100µM verapamil stimulation and indicate the average relative ATPase activity ±SEM from at least three to six independent experiments. Where not visible, error bars are smaller than the symbols. Lines represent non-linear regression analysis of the data points with a Hill equation ($y = v_1 + (v_2 - v_1) * x_n / (k_n + x_n)$). R^2 values for data fits were >0.99.

Molecular interactions of *Ta*-ABCB1 with environmental chemicals

Conserved inhibition kinetics with Transporter-interfering chemicals (TICs)

Next, we examined the interaction of known TICs with tuna ABCB1 (Figure 3). The ATPase assay can be conducted in activation mode to identify possible transporter substrates and in inhibition mode to identify both compounds that block transporter activity or do only weakly interact (Nicklisch and Hamdoun 2020b). Consistent to what was found with mouse ABCB1a (Nicklisch et al. 2016b), all TICs inhibited verapamil-stimulated *Ta*-ABCB1 ATPase activity (Figure 3A-C). In activation mode, TICs were unable to stimulate *Ta*-ABCB1 ATPase activity as compared to model stimulator verapamil (Figure 3D-F). The observed stereoselectivity for mouse ABCB1a inhibition by the two TICs Endrin and Dieldrin was also conserved in *Ta*-ABCB1, with Endrin having an EC_{50} value of 1.2±0.2 μM and Dieldrin of 26.4±6.5 μM (Table 1, Figure 3A). As observed for human ABCB1 (IC_{50} = 27.7 μM) and mouse ABCB1a (IC_{50} = 25.2 μM) (Nicklisch et al. 2016b), an environmental mixture of TICs showed additive inhibitory effects on *Ta*-ABCB1 with an EC_{50} of 24.7 μM (Table 1).

Differences in Ta-ABCB1 interaction kinetics with DDT and BDE compounds among TICs

The inhibition curves for p,p'-DDD and p,p'-DDT showed an unexpected pattern: At low concentrations (<0.17 μM), there was no change in verapamil-stimulated ATPase activity. However, at test concentrations of 0.33, 1.67 and 3.33 μM, a pronounced stimulation of ATPase activity was observed, that declined with further increase in pollutant concentrations at 16.67, 33.33 and 166.7 μM (Figure 3A). While both pollutants showed no stimulation in the absence of verapamil at these concentrations (Figure 3D), the results indicate a type of co-stimulation of verapamil and DDD or DDT at relatively low pollutant concentrations (Litman et al. 1997;

Orlowski et al. 1996; Adam B. Shapiro et al. 1999; a B. Shapiro and Ling 1997). The EC_{50} values of the brominated flame retardant BDE-100 (21.9±5.7 µM) was comparable to that observed for mouse ABCB1a (Figure 3B, Table 1). However, half-maximal inhibitory concentration of BDE-47 towards *Ta*-ABCB1 was about twice as high as with mouse ABCB1a (Figure 3B, Table 1).

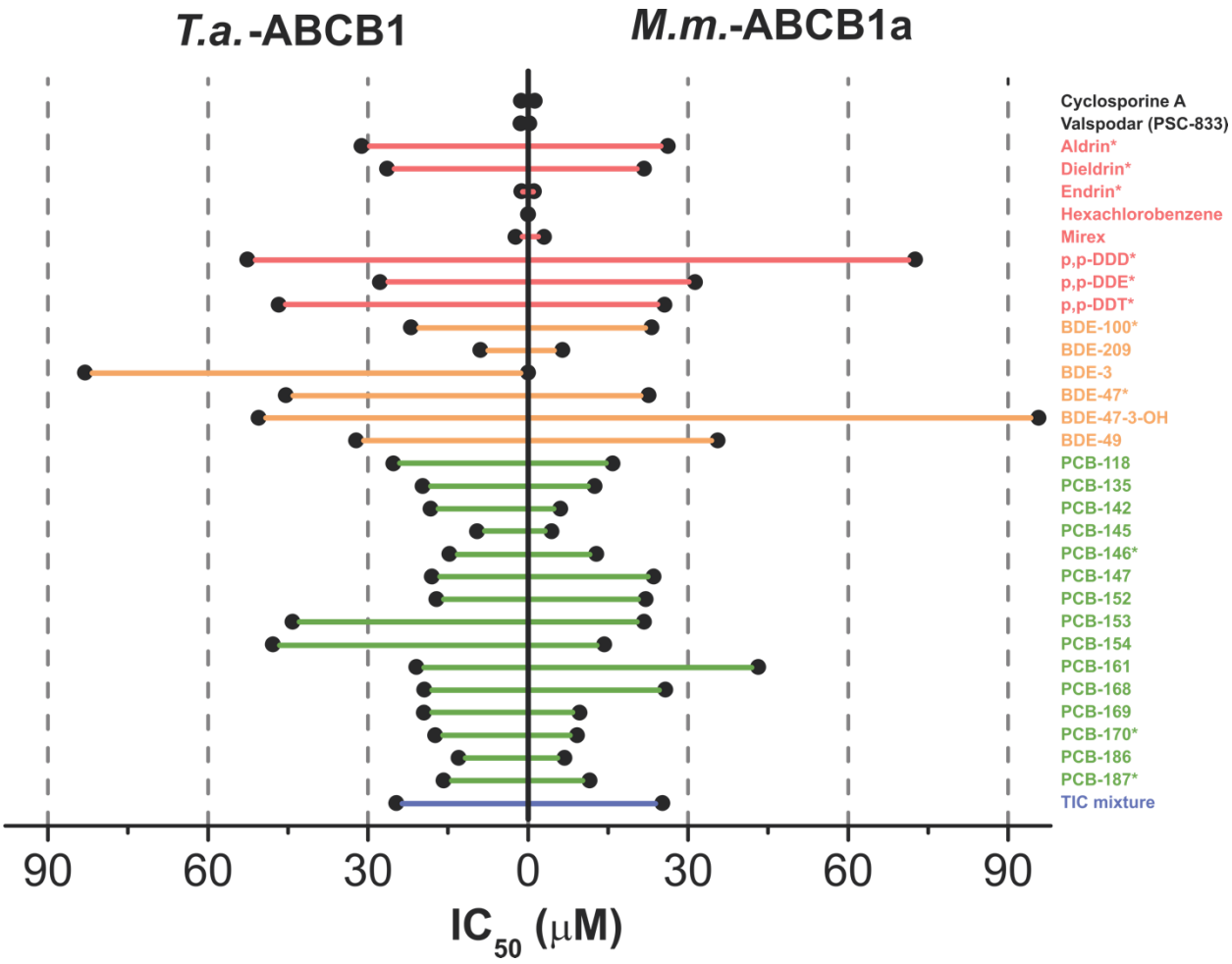


Figure 4: Comparison of ATPase inhibition coefficients of purified mouse ABCB1a and tuna ABCB1 for model inhibitor drugs and 30 POPs. The stiff diagram displays the determined IC_{50} vales for each tested pollutant compound relative to 100µM verapamil-stimulated ATPase activity. Marked with an asterisk are the previously identified ten TICs. The TIC mixture was prepared according to previous work (Nicklisch et al. 2016b). IC_{50} = inhibition coefficient or concentration of compound that inhibited 50% of maximal ATPase activity.

Non-TIC interactions show potent inhibition in mouse and tuna

We further examined the interaction profiles of 19 additional persistent organic pollutants (POPs) and an environmental TIC mixture with *Ta*-ABCB1 according to Nicklisch *et al.,* 2016 (Figure 4, Table 1). The results show that most transporter interaction kinetics were conserved (Figure 4), including potent inhibition by the OC pesticide Mirex in tuna (EC_{50} = 2.3±0.9 μM) and mouse (EC_{50} = 3.0±0.2 μM), and weak interaction of both transporters with OC pesticide Hexachlorobenzene (Table 1). The fully brominated flame retardant BDE-209 showed highly similar inhibition with mouse ABCB1a (EC_{50} = 6.5±0.4 μM) and tuna ABCB1 (EC_{50} = 8.9±2.4 μM). However, the singly brominated BDE-3 was able to inhibit verapamil-stimulated *Ta*-ABCB1 ATPase activity (EC_{50} = 83.0±2.0 μM) but showed weak interaction with the mouse transporter (Table 1).

Table 1: Observed kinetic parameters for the interaction of model drug stimulator, inhibitors and persistent organic pollutants (POPs) with purified *Ta*-ABCB1 protein. Marked in bold and italics are the 10 identified TICs from our previous study. MW = molecular weight in Da. Log K_{ow} = calculated octanol water partition coefficient according to PubChem (https://pubchem.ncbi.nlm.nih.gov). EC_{50} = effective concentration or concentration of a compound at which 50% of its maximum effect (i.e., inhibition or stimulation) is reached. Mouse ABCB1a EC_{50} values and TIC mixture composition according to (Nicklisch et al. 2016b). NI = no/weak interaction. NA = not available. S.D. = standard deviation.

Compound	MW	Log K_{ow}	Tuna EC_{50} ± S.D (µM)	Mouse EC_{50} ± S.D (µM)
Verapamil	454.6	3.8	8.8±0.6	9.4±0.4
Cyclosporine A	1202.6	7.5	1.3±0.1	1.3±0.1
Valspodar (PSC-833)	1214.6	7.7	1.4±0.2	0.3±0.1
Aldrin	364.9	6.5	31.2±7.2	26.2±1.9
Dieldrin	380.9	5.4	26.4±6.5	21.8±4.2
Endrin	380.9	5.1	1.2±0.2	1.1±0.7
Hexachlorobenzene	284.8	5.7	NI	NI
Mirex	545.6	5.3	2.3±0.9	3.0±0.2
p,p'-DDD	318.0	6.1	52.6±2.8	72.5±5.7
p,p'-DDE	318.0	6.8	27.7±15.7	31.3±3.7
p,p'-DDT	354.5	6.5	46.7±2.1	25.6±4.8
BDE-100	564.7	6.9	21.9±5.7	23.2±2.9
BDE-209	959.2	6.3	8.9±2.4	6.5±0.4
BDE-3	249.1	4.34	83.0±2.0	NI
BDE-47	485.8	6.2	45.4±52.7	22.6±6.2
BDE-47-3-OH	501.8	NA	50.5±8.1	95.7±3.6
BDE-49	485.8	6.2	32.2±5.2	35.6±5.4
PCB-118	326.4	6.6	25.2±2.5	15.9±1.0
PCB-134	360.9	6.6	19.7±3.9	12.5±0.8
PCB-142	360.9	6.6	18.2±2.2	6.1±0.7
PCB-145	360.9	6.2	9.5±0.9	4.4±0.4
PCB-146	360.9	6.9	14.7±2.3	12.8±1.9
PCB-147	360.9	6.5	18.0±1.7	23.6±3.1
PCB-152	360.9	6.1	17.1±3.8	22.1±4.2
PCB-153	360.9	6.8	44.1±43.6	21.8±3.1
PCB-154	360.9	6.7	47.8±29.0	14.3±1.1
PCB-161	360.9	6.8	20.9±5.6	43.2±8.3
PCB-168	360.9	6.8	19.4±0.3	25.8±3.7
PCB-169	360.9	7.4	19.5±3.3	9.7±0.5
PCB-170	395.3	7.1	17.4±4.2	9.2±0.8
PCB-186	395.3	6.7	13.0±1.4	6.9±0.5
PCB-187	395.3	7.0	15.8±6.9	11.6±0.6
TIC mixture	NA	NA	24.7±5.4	25.2±1.3

Discussion

Understanding the interactions of pharmaceutical compounds with xenobiotic transporters is part of how we predict how the human body will handle those pharmaceuticals. By the same token, understanding how xenobiotic transporters handle environmental compounds is likely to help increase our understanding of how these pollutants are handled and how they move through organisms and are ultimately transferred to humans. A fundamental difference between the scenario of pharmaceuticals and pollutants, is that environmental chemicals often move and amplify through multiple organisms to transfer from the environment to humans. As such the interactions of environmental chemicals with xenobiotic transporters from multiple species, at multiple levels of the food chain, are involved in the ultimate patterns of human exposure.

This study builds upon previous structural, functional, and environmental studies (Bruyere et al. 2017; Chedik, Bruyere, and Fardel 2019; Chedik et al. 2018; Epel et al. 2008; Fardel, Kolasa, and Le Vee 2012; Guéniche, Bruyere, Le Vée, et al. 2020; Guéniche, Bruyere, Ringeval, et al. 2020; Luckenbach and Epel 2005; Nicklisch et al. 2017a; 2016b; Smital et al. 2004; Stevenson et al. 2006) to probe potential similarities and differences in how XTs from humans and the species they consume may interact with common pollutants. It further sheds light on how chemicals that may interfere with these transporters in humans, i.e., TICs, might also act in the species that carry them. Our approach, using purified ABCB1 from wild yellowfin tuna (*Thunnus albacares*), enables direct comparison to this prior work with mammalian proteins.

Functional similarities and differences

The results revealed some important similarities and differences in the activity of *Ta*-ABCB1. Most of the tested pollutants were inhibitors of *Ta*-ABCB1, however, several interaction patterns were different for mouse and tuna (Figure 4, Table 1). For example, at low concentrations the organochlorine pesticide TICs p,p'-DDT and its metabolite p,p'-DDD co-stimulated verapamil-induced *Ta*-ABCB1 ATPase activity, while verapamil pre-stimulated mouse ABCB1a ATPase activity was inhibited across all tested DDT and DDD concentrations (Nicklisch et al. 2016b). Likewise, the flame retardant BDE-3 was able to inhibit verapamil-stimulated *Ta*-ABCB1 ATPase activity while pre-stimulated mouse ABCB1a showed only weak or no interaction with the compound. In contrast, the fungicide Hexachlorobenzene showed only weak interaction while the insecticide Mirex showed potent inhibition with both mouse and tuna ABCB1.

When tested against the ten previously identified TICs, *Ta*-ABCB1 showed highly similar inhibition profiles and IC_{50} values as compared to mouse, indicating a conserved mode of TIC interaction among vertebrate ABCB1. For example, the organochlorine pesticides Endrin was a strong inhibitor of ATPase activity in both tuna and mouse, with an IC_{50} value in the range of model drug inhibitor cyclosporin A. The flame retardant BDE-100, which was previously co-crystallized with mouse ABCB1a (Nicklisch et al. 2016b; Le, Harvey, and Aller 2020), showed similar interaction parameters in mouse and tuna. Furthermore, both the interaction patterns and EC_{50} values of the major DDT metabolite and TIC p,p'-DDE were similar in mouse and tuna ABCB1. Notably, the pollutant mixture representing environmental levels of nine TICs detected in yellowfin tuna caught in the Gulf of Mexico, inhibited *Ta*-ABCB1 to the same extent as mouse ABCB1a and human ABCB1 (Nicklisch et al. 2016b; 2017a).

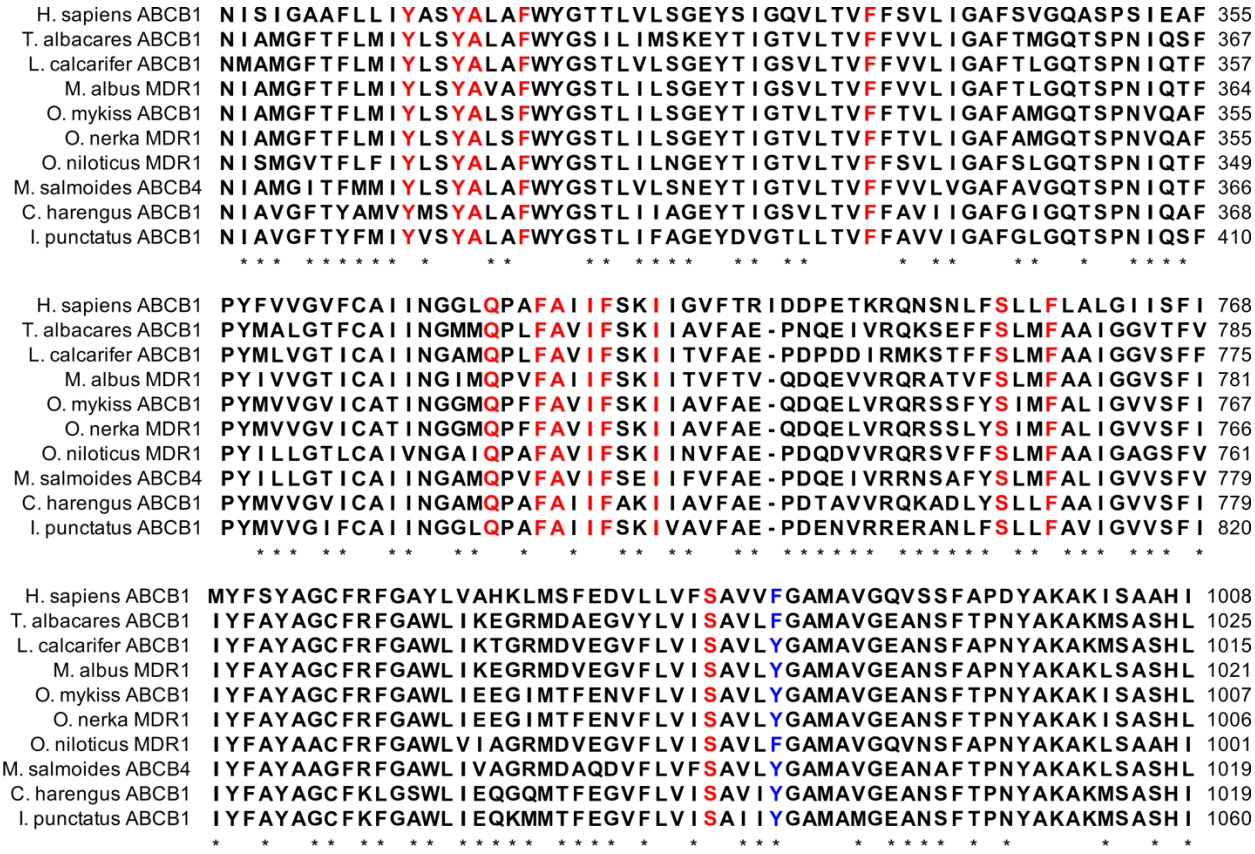


Figure 5: Amino acid sequence alignment of BDE-100 binding residues in ABCB1 orthologs of human and commercial fish species. Highlighted are the 15 residues that interact with the bound flame retardant in the mouse ABCB1a co-crystal structure (PDB: 4XWK) (Nicklisch et al. 2016b). Except for F983 (blue) in human ABCB1, all other fourteen BDE-interacting residues (red) are highly conserved across commercial fish ABCB1 proteins. Asterisks mark non-conserved residues.

The co-crystal structure of mouse ABCB1a with BDE-100 revealed an intricate network of transporter:environmental chemical interactions, mediated by eleven hydrophobic and four hydrophilic amino acids (Nicklisch et al. 2016b; Nicklisch and Hamdoun 2020b). Ten of these fifteen interacting residues are different from known ABCB1 inhibitor interaction sites and have not been described before (Table S3), indicating that environmental chemicals inhibit ABCB1 function by a novel mode of interaction that is distinct from pharmaceutical inhibitors. Interestingly, an amino acid alignment of ABCB1 orthologs from eight common commercial fish species with tuna and human ABCB1 shows that 14 out of the 15 BDE-interacting residues are

identical (Figure 5), suggesting that the TIC effects we describe here could be widespread. Notably, the only residue that is different is in all species either a phenylalanine (F979 in mouse, F983 in human) or a tyrosine (Y), two aromatic amino acids only differing in the hydroxyl group on tyrosine. Previous studies have shown that ABCB1 often uses both tyrosine and phenylalanine residues to bind to structurally diverse ligands via a combination of hydrophobic and hydrogen bonding interactions (Chufan, Kapoor, and Ambudkar 2016; Gutmann et al. 2010), suggesting that either of these aromatic amino acids could interact with BDE-100 and possibly other TICs. The conservation of TIC-interacting residues in human ABCB1 and its orthologs could be an opportune way to predict trophic transfer and pollutant bioaccumulation in humans and food organisms.

Table S3: Binding residues of BDE-100 and known drug inhibitors in mouse ABCB1a. Shown are residues near binding sites of two QZ59 compounds, BDE-100, and residues that are protected from MTS labeling by verapamil binding (Aller et al. 2009; Nicklisch et al. 2016b; Nicklisch and Hamdoun 2020b). Marked in blue are ten newly identified binding sites for BDE-100 in TM 5, 6, 7, 8, and 12. Residue F724 (red) interacts with all four inhibitory compounds. Residues marked in green represent residues that only interact with the "lower" binding site of QZ59-SSS. Residues marked with and asterisk are conserved among vertebrates, including human, mouse, rat, claw frog, chicken, and rabbit.

Transmembrane Domains (TMDs)	QZ59-RRR	QZ59-SSS	Verapamil	BDE-100
TMD 1			H60	
			A63	
			L64	
	M68	M68		
TMD 4			S218	
TMD 5		L300		
			I302	
	Y303	Y303		Y303*
				Y306*
				A307
				F310*
				F331*
TMD 6	F332	F332		
	L335		L335	
	I336	I336		
			A338	
	F339	F339		
TMD 7	Q721	Q721		Q721*
	F724	F724	F724	F724*
				S725
				I727
	F728			F728*
				V731
TMD 8				S752*
				F755*
		L758		
TMD 9		F833		
TMD 10			I864	
			G868	
TMD 11			F938	
			T941	
	Y949	Y949		
TMD 12			L971	
	F974	F974		
	S975			S975*
	V978	V978	V978	
				F979
			G980	
		A981	A981	
		M982		
		G985		
		Q986		
		S989		

Conclusions and Implications of conserved TIC effects for yellowfin tuna

While the mechanisms governing accumulation of POPs in tuna and human are poorly understood, our data suggest that bio-accumulative TICs can inhibit ABCB1 function in mammals and fish and each class of TICs can interact at different ligand binding sites within ABCB1. The consequences of the inhibitory action of TICs on fish xenobiotic transporters (XTs) can be manifold. Modulating XT efflux activity can substantially increase the intracellular concentration and toxic effects of other xenobiotic substrates of these transporters, including PAHs and OCPs (Popovic et al. 2014; Valton et al. 2013; Lu et al. 2014). This is particularly important during early development where efflux transporters are highly expressed in embryos and juvenile fish to prevent xenobiotic uptake and toxicity (Brette et al. 2014; Fischer et al. 2013; Gordon et al. 2019; Incardona et al. 2014). Once in the body, TICs and other XT-evading free rider chemicals could exert sublethal toxic actions at much lower levels, specifically by impairing crucial fish sensory systems (Besson et al. 2020; Lari et al. 2020; Maryoung et al. 2015; Schlenker, Welch, Meredith, et al. 2019; Schlenker, Welch, Mager, et al. 2019; Tierney et al. 2010). As structures from other xenobiotic transporters become available, the results of this study will serve as a framework to pave the way to identify additional TICs and to investigate their interactions with both ABC-type efflux transporters, including ABCB1, ABCG2, ABCC1 and ABCC2, and SLC-type uptake transporters, including Organic anion-transporting polypeptides (OATPs), Organic anion transporters (OATs), and Organic cation transporters (OCTs).

Chapter 2: Targeting insect xenobiotic defense transporters for precision pest control and pollinator protection

Abstract

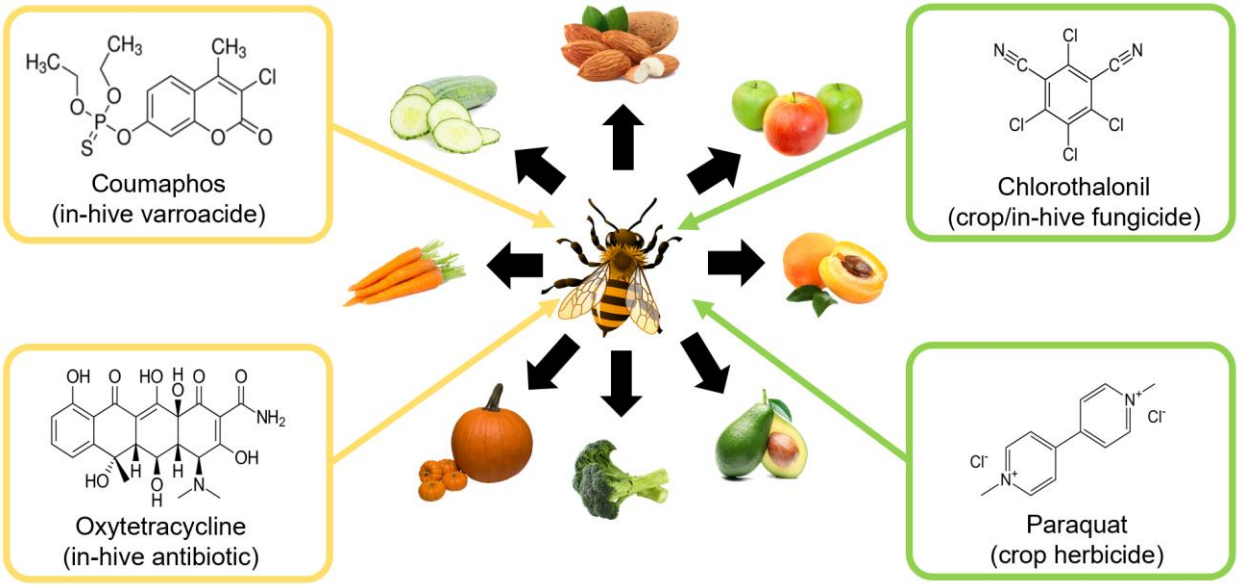


Figure 6: Unintentional honeybee exposures to mixtures of agricultural pesticides from crops and in-hive medicine. Honeybees are instrumental for pollinating important commodity crops across the globe. However, bees often get exposed to a mixture of agricultural pesticides applied to crops (green squares) and medications applied in the hives (yellow squares). Crop pictures and chemical structures are licensed under Attribution-NonCommercial 2.0 Generic (CC BY-NC 2.0).

All organisms have developed biochemical defenses to combat toxic insult from xenobiotics. ABC-type efflux transporters such as ABCB1 typically act as a first line of defense at biological barriers to expel toxic xenobiotics before they can enter and cause harm to the organism. The Multidrug resistance protein homolog 49 or MDR49 is the ABCB1 homolog in insects and has been shown to confer resistance to broad-spectrum insecticides in fruit flies and mosquitos. However, it is unknown to what extent the ABCB1 homologs in beneficial pollinator insects can recognize and eliminate toxic pesticides.

In this chapter, we will discuss current and emerging threats to honeybee health and discuss novel strategies for identifying and predicting chemical uptake and toxicities in the bee. Using the highly conserved drug efflux transporter, ABCB1, as an example, we propose novel approaches that use structure-function analysis across insect MDR transporters to inform sustainable pest management and precision pesticide application with the goal of preserving honeybee health.

Introduction

Economic Importance of Honeybee Pollination

Agricultural technologies are constantly improving and have changed rapidly over the last 50 years. Changes in seed biotechnology, analytical software, and tilling practices have all contributed to increased crop yield and quality (Dutia 2014). In the last 20 years, the emergence of cover crop practices has been shown to increase nutrient cycling and soil and water quality (Snapp et al. 2005). However, even with these technological advancements, farmers still rely on insect pollinators such as the European Honeybee, *Apis mellifera*, to help produce top commodities like almonds, apples, and cucumbers (Table 2)(Morse and Calderone 2000; Klein et al. 2007). It is estimated that almost $215 billion a year in global agricultural production can be attributed to honeybees (Smith et al. 2013). Additionally, honey, one of the products most associated with bees, amounted to $320 million in US sales alone (US Department of Agriculture 2019).

Emerging threats to bee survival

Unfortunately, bees have suffered massive population decline in recent years, causing justified public concern over the future of bees and the crops they pollinate. A 2011 study reported up to a 96% decrease in the number of North American bumble bees (Cameron et al. 2011). In late

2006, a phenomenon characterized by a sudden loss of 30-90% of adult worker bees was identified by US beekeepers, which was subsequently referred to as colony collapse disorder or CCD (vanEngelsdorp et al. 2009; Ellis, Evans, and Pettis 2010; Evans and Chen 2021). Massive worker loss leaves behind a weakened hive, queen, and brood with fewer forager bees to gather required food and water (US EPA 2013a). Several studies have shown that exposures to multiple pesticides may contribute to CCD (Farooqui 2013; Johnson et al. 2010; Magal, Webb, and Wu 2019; 2020). For instance, Thiamethoxam, a popular neonicotinoid insecticide used to treat cherries, cucumbers, and pears, along with several other neonicotinoids, were shown to alter honeybee and bumble bee feeding rates, motor skills and visual learning (Ludicke and Nieh 2020; Laycock et al. 2014). As a result, Thiamethoxam, was recently banned in Europe for application to flowering plants (Stokstad 2018).

However, pesticides are not the only chemical stressor acting on commercially kept honeybees. There is a growing body of evidence that medications applied to beehives to treat infection and infestation might also be detrimental to bee health. Analogous to drug-drug interactions, mixtures of pesticides applied to crops and bee medications could have unanticipated synergistic effects. The effects of unintended co-exposures to pesticide/pesticide and pesticide/hive medications on honeybee health are not well understood. For example, Zhu et al. (2017) demonstrated that binary pesticide mixtures dramatically increased mortality when compared to applications of single pesticides. Johnson and coworkers found that administering Fumagillin, an antibiotic used to treat European foulbrood disease, or the common agricultural fungicide Chloranthalonil together with Fluvalinate, an acaricide used to treat for varroa mite infestations, can actually increase mortality rates in the honeybees (Johnson et al. 2013). Pesticide exposure to hives already battling pathogens may also have detrimental effects. Doublet et al.

(2015) found that larvae exposed to sub-lethal doses of the neonicotinoid insecticide, thiacloprid, in conjunction with Black Queen Cell Virus have a lower survival rate (65%) than the control (90%), just thiacloprid (80%), or just virus (85%) (Doublet et al. 2015). Agricultural pesticides not only affected physical health, but they also altered behavior in these pollinators. Bees exposed to non-lethal doses of the neuroactive neonicotinoid imidacloprid had impaired short-term (10-minute) and long-term (24-hour) memory when their responses to a conditioned stimulus were tested (Williamson and Wright 2013). Exposure to another neonicotinoid, thiamethoxam, in non-lethal doses still caused an increase in mortality by affecting the ability of the bees to navigate home (Henry et al. 2012). Even in non-lethal doses, pesticide exposure is shown to be detrimental to bee health. Vigilance and communication could be key to minimizing honeybee exposure to toxic pesticide mixtures. To add another layer of complexity, commercial beekeeping operations are often migratory (Simone-Finstrom et al. 2016; Traynor et al. 2016; Alger et al. 2018). Colonies are often moved regionally and nationally to pollinate different types of monocultures in the US. This constant migration likely exposes the same bee colonies to different types of crop pesticides and/or hive medications within only a few months each year.

Chemicals of concern

A plethora of pesticides have been approved for US agricultural crops, often applied in concert to combat several pests at once (Table 2). Insecticides are classified according to their chemistry and mode of action. Neonicotinoids and organophosphates, for example, are common examples of neuroactive classes of pesticides. Neonicotinoids act on the Nicotinic Acetylcholine Receptor in the insect central nervous system and have been shown to cause respiratory depression in honeybees (Hatjina et al. 2013). Neonicotinoids also cause reduced colony weight gain during

the growth phase and reduced production of new queen bees in bumble bee hives (Whitehorn et al. 2012). Organophosphate exposure also leads to reproductive harm via lowered sperm viability, smaller ovaries, and decreased weight in queen bees (Chaimanee et al. 2016). Organophosphates work by inhibiting the enzyme acetylcholinesterase (AchE) that breaks down the neurotransmitter acetylcholine (Minton and Murray 1988). Sub-lethal doses of organophosphates administered to honeybees lead to impaired learning and memory (Williamson and Wright 2013).

Another major compound frequently studied is the phenylpyrazole insecticide Fipronil. Although it has been banned in the European Union since 2013 (European Food Safety Authority 2013), it is still used in other parts of the world, including the US. Fipronil is an insecticide targeting GABA- and glycine-gated chloride channels, causing hyperstimulation of the central nervous system, leading to seizures, paralysis, and death (Islam and Lynch 2012). Fipronil has been cited as the cause for a mass bee mortality event in France in the 1990s (Holder et al. 2018), leading to the eventual ban of the compound in France in 2003 and the rest of the European Union in 2017 (Comoretto, Arfib, and Chiron 2007; Erickson 2018). Insecticide toxicity to bees is often increased when combined with adjuvants, a common agricultural practice of adding additional compounds like surfactants and oils to pesticides to boost their absorption or distribution. A 2015 study found that field-applied Fipronil with added surfactant adjuvants increased bee mortality by up to 80% (D. F. Mayer and Lunden 1999). Similar adjuvants, that are typically considered biologically inert, have also been shown to promote viral pathogenicity in chronically exposed honeybee larvae (Fine, Cox-Foster, and Mullin 2017).

Table 2: Pesticides used on fifteen major bee-pollinated commodity crops. The list was generated from the UC Agriculture & Natural Resources Integrated Pest Management (ANR IPM) program database (https://www2.ipm.ucanr.edu/agriculture/). Known vertebrate and insect MDR transporter interactions for each pesticide are shown (Zuo et al. 2018; Wu et al. 2019; Luo, Sun, and Wu 2013; Lespine et al. 2007; Guéniche, Bruyere, Le Vée, et al. 2020; Pan et al. 2020; Schrickx 2014; Pivčević and Žaja 2006; Jin et al. 2019; Meng et al. 2020; Chedik et al. 2018; Hawthorne and Dively 2011). Upregulation under Transporter Interactions refers to increased expression. MDR under Transporter Interactions refers to undefined transporters of the MDR family (e.g. P-gp, MRP, and BCRP). Bee mortality is based on LD_{50} data from https://ecotox.ipmcenters.org/index.cfm?menuid=3 ("OPP Pesticide Ecotoxicity Database" 2017). Blue highlight denotes compounds of the 2018 top 100 pesticides by pound used in California according to the California Department of Pesticide Regulation (https://www.cdpr.ca.gov/docs/pur/pur18rep/top_100_sites_lbs_2018.htm). Bold and italicized compounds were previously shown *in vivo* to cause increased mortality in combinations (Johnson et al. 2013; Guseman et al. 2016; Zhu et al. 2017; Wade et al. 2019). N/A = data not available.

	Pesticide	Class	Almonds	Apples	Apricots	Avocados	Broccoli	Carrots	Cherries	Cucumber	Peaches	Pears	Plums	Pumpkins	Rapeseed	Raspberries	Sunflowers	Oral LD_{50} (µg/bee)	Contact LD_{50} (µg/bee)	Transporter Interaction Vertebrate	Transporter Interaction Insect
Insecticides	Abamectin	Avermectin	–	x	x	x	–	–	–	x	x	x	–	x	–	–	–	0.004	0.002	P-gp Inhibitor	P-gp Upregulator
	Acetamiprid	Neonicotinoid	x	x	–	–	x	–	x	x	–	x	–	x	–	–	–	15.1	8.1	N/A	MDR Substrate
	Bacillus thuringiensis	Biological	x	x	x	x	x	–	x	x	x	x	x	x	–	x	x	N/A	N/A	N/A	Possible MRP2 Inhibitor
	Bifenazate	Carbazate	x	–	x	–	–	–	–	x	x	x	–	x	–	–	–	N/A	7.9	N/A	N/A
	bifenthrin	Synthetic pyrethroid	–	–	–	–	x	–	–	x	x	–	–	x	x	x	–	N/A	0.015	MRP2 Inhibitor	N/A
	Carbaryl	Carbamate	x	–	x	–	x	–	x	x	x	–	–	x	–	x	x	0.15	1.1	P-gp Non-Interactor	N/A
	Chlorantraniliprole	Carboxamide	x	x	x	–	x	–	–	x	x	x	–	x	–	x	x	N/A	N/A	N/A	N/A
	Chlorpyrifos	Organophosphate	x	–	–	–	x	x	–	–	–	–	–	–	–	–	x	0.25	0.059	MDR Non-Interactor	N/A
	Cryolite	Inorganic	–	–	–	–	x	–	–	x	–	–	–	x	–	–	–	N/A	217.55	N/A	N/A
	Cyantraniliprole	Synthetic Ryanoid	x	–	–	–	–	–	–	x	–	–	–	x	–	–	–	>0.1	0.093	N/A	N/A
	Diazinon	Organophosphate	–	x	x	–	x	–	x	x	x	x	x	x	–	–	–	0.2	0.2	P-gp Inhibitor	N/A
	Esfenvalerate	Synthetic pyrethroid	–	–	x	–	x	–	x	x	x	x	–	x	–	–	x	N/A	0.017	MDR Non-Interactor	N/A
	Imidacloprid	Neonicotinoid	–	x	x	–	–	x	x	x	–	–	x	x	–	–	–	0.004	0.078	N/A	MDR Substrate
	Indoxacarb	Organophosphate	–	–	–	–	x	–	–	x	x	–	–	x	–	–	–	0.204	0.068	N/A	P-gp Substrate
	Lambda-Cyhalothrin	Synthetic pyrethroid	–	–	–	–	–	–	x	x	–	x	–	x	x	–	x	0.909	0.038	MDR Non-Interactor	P-gp Non-Interactor
	Methomyl	Carbamate	–	–	–	–	x	–	–	x	x	–	–	x	–	–	–	0.29	0.162	P-gp Non-Interactor	N/A
	Methoxyfenazide	Insect growth regulator	x	x	x	–	x	–	x	x	x	x	x	x	–	–	–	>100	>100	P-gp Inhibitor	N/A
	Spinetoram	Actinomycete	x	x	x	x	x	–	x	x	x	x	–	x	–	x	–	0.11	0.024	N/A	N/A
	Spinosad	Actinomycete	x	x	x	x	x	–	x	x	x	x	x	x	–	x	–	N/A	0.003	P-gp Inhibitor	P-gp Non-Interactor
	Thiamethoxam	Neonicotinoid	–	–	–	–	x	x	x	x	–	x	x	x	–	–	–	0.005	0.024	N/A	P-gp Upregulator
	Zeta-Cypermethrin	Synthetic pyrethroid	–	–	–	–	–	x	–	–	–	–	–	–	x	–	x	0.172	0.023	N/A	N/A
Fungicides	Azoxystrobin	Methoxyacrylates	x	–	x	x	–	x	x	–	x	–	x	–	x	–	x	>25	>200	BCRP Inhibitor	N/A
	Captan	Phthalimides	x	x	x	–	–	–	x	–	x	–	x	–	–	–	–	N/A	>10	N/A	N/A
	Chlorothalonil	Chloronitriles	x	–	x	–	–	x	x	x	x	–	x	x	–	–	–	N/A	181	N/A	N/A
	Cyprodinil	Anilino-Pyrimadines	x	–	x	–	–	–	–	–	–	x	x	–	–	–	–	N/A	>787	N/A	N/A
	Fenbuconazole	Triazole	x	–	x	–	–	–	–	–	x	–	–	–	–	–	–	N/A	>292	N/A	N/A
	Iprodione	Dicarboximides	x	–	x	–	x	–	x	–	x	–	x	–	–	–	–	N/A	N/A	N/A	N/A
	Metconazole	Triazole	x	–	x	–	–	–	x	–	x	–	–	–	x	–	x	88	>95.3	N/A	N/A
	Myclobutanil	Triazole	x	x	x	–	–	–	x	x	x	–	x	x	–	x	–	>500	>500	P-gp Upregulator	N/A
	Potassium Bicarbonate	Inorganic	–	x	–	–	–	–	x	x	–	–	–	x	–	–	–	N/A	N/A	N/A	N/A
	Propiconazole	Triazole	x	–	x	–	–	–	x	–	x	–	x	–	x	–	–	N/A	>25	P-gp Inhibitor	N/A
	Pyraclostrobin	Methoxy Carbamates	x	x	x	–	–	x	x	x	x	x	x	x	x	x	x	>100	>100	N/A	N/A
	Boscalid	Carboxamides	x	x	x	–	–	–	x	x	x	x	x	x	x	x	x	>166	>200	N/A	N/A
	Pyrimethanil	Anilino-Pyrimadines	x	–	x	–	–	–	–	–	x	–	x	–	–	–	–	>100	>100	N/A	N/A
	Tebuconazole	Triazole	x	x	x	–	–	–	x	–	x	x	–	–	–	–	x	>83	>200	N/A	N/A
	Trifloxystrobin	Oximino Acetates	x	x	x	–	–	x	x	x	x	x	–	x	–	–	–	>200	>200	N/A	N/A
	Triflumizole	Triazole	–	x	–	–	–	x	x	–	x	–	x	–	–	–	–	N/A	>160	N/A	N/A
	Ziram	Dithiocarbamates	x	x	x	–	–	–	–	–	x	–	–	–	–	–	–	>100	>100	N/A	N/A
Herbicides	Carfentrazone	Triazole	x	–	x	x	x	–	x	x	x	–	–	x	–	x	x	N/A	>27.2	N/A	N/A
	Clethodim	Cyclohexanedione	–	–	–	–	x	x	–	x	–	–	–	x	x	–	x	N/A	>100	N/A	N/A
	Glyphosate	Glycine	x	–	x	x	–	–	x	–	x	–	x	–	x	x	x	>100	>100	MDR Non-Interactor	N/A
	Oryzalin	Dinitroaniline	x	–	x	x	–	–	x	–	x	–	x	–	–	x	–	N/A	>11	N/A	N/A
	Oxyfluorfen	Diphenylether	x	–	x	x	x	–	x	–	x	–	x	–	–	–	–	>100	>100	N/A	N/A
	Paraquat	Bipyridylium	x	–	x	x	–	–	x	–	x	–	x	–	–	x	x	51	>144	P-gp Substrate	N/A
	Sethoxydim	Cyclohexanedione	x	–	–	x	x	x	x	x	x	–	–	x	x	x	x	>200	>200	N/A	N/A
	Trifluralin	Dinitroaniline	x	–	x	–	–	–	x	x	x	–	x	x	x	–	x	>50	>24.17	N/A	N/A

Pesticides in the US are regulated by the Environmental Protection Agency (EPA) to ensure safety in humans, bees, and other non-target organisms (US EPA, OPP 2016). However, they are usually only tested one at a time (Levine and Borgert 2018). Although the EPA has set guidelines on the mixing of commercial pesticides, there is no standardized protocol to determine the toxicity of chemicals in combination even though combinations are more environmentally relevant

(Levine and Borgert 2018). Pesticides are often applied to crops in mixtures (known as Tank-Mixing) to treat for several different problems at once, (e.g. an aphid infestation alongside a fungal infection) (Holloway and Western 2003). There is also the risk of unintentional honeybee exposures to combinations of chemicals, including in-hive medications and crop pesticides (Hawthorne and Dively 2011).

Unintentional toxic effects of chemical co-exposures

Although intentional tank-mixing of agrochemicals can be monitored by the applicator, bees can also be exposed to unintentional pesticide mixtures due wind drift or bee foraging on neighboring crop fields with differential pesticide application (Davis and Williams, n.d.; Botías et al. 2015; Macri et al. 2021; Ucar and Hall 2001; Krupke et al. 2012). In-hive medications (Table 3), used to mitigate bee parasites and disease, can also be detrimental to honeybee health when, for example, foraging bees are pre-exposed to other agrochemicals in the fields (Hawthorne and Dively 2011).

Table 3: List of in-hive medications used to treat seven prominent honeybee pests or diseases. List of diseases and treatments generated from information obtained from the Wisconsin Department of Agriculture (https://datcp.wi.gov/Documents/TreatmentOptions.pdf). Bee mortality is based on LD_{50} data from various studies ("Pesticides & Bee Toxicity | Minnesota Department of Agriculture" n.d.; Calatayud-Vernich et al. 2018; Gregorc et al. 2018; Jack et al. n.d.). Known vertebrate and insect MDR transporter interactions for each pesticide are shown (Chedik et al. 2018; Hawthorne and Dively 2011). Bold and italicized compounds were previously shown *in vivo* to cause increased mortality in combinations (Johnson et al. 2013; Guseman et al. 2016; Zhu et al. 2017; Wade et al. 2019).

Compound	Class	Varroa Mite (*Varroa destructor*)	Tracheal Mite (*Acarapis woodi*)	Waxmoth (*Galleria mellonella*)	Small Hive Beetle (*Aethina tumida*)	American Foulbrood (*Paenibacillus larvae*)	European Foulbrood (*Melissococcus pluton*)	Nosema (*Nosema apis*)	Oral LD_{50} (µg/bee)	Contact LD_{50} (µg/bee)	Transporter Interaction Vertebrate	Insect
Amitraz	Formamidine	X	–	–	–	–	–	–	50	4.9	N/A	N/A
Coumaphos	Organophosphate	X	–	–	X	–	–	–	0.004	N/A	P-gp Inhibitor	P-gp Substrate
Fluvalinate	Synthetic pyrethroid	X	–	–	–	–	–	–	45	N/A	MDR Non-Interactor	P-gp Substrate
Formic Acid	Carbolic Acid	X	X	–	–	–	–	–	N/A	N/A	N/A	N/A
Fumagillin	Antibiotic	–	–	–	–	–	–	X	N/A	N/A	N/A	N/A
Lincomycin	lincosamide antibiotic	–	–	–	–	X	–	–	N/A	N/A	BCRP Substrate	N/A
Oxalic Acid	Carbolic Acid	X	–	–	–	–	–	–	N/A	N/A	N/A	N/A
Oxytetracycline	Tetracycline Antibiotic	–	–	–	–	X	X	–	>1600	N/A	N/A	N/A
Paradicholorbenzene	Chorinated Aromatic Hydrocarbo	X	X	X	–	–	–	–	N/A	N/A	N/A	N/A
Permethrin	Synthetic pyrethroid	–	–	–	X	–	–	–	0.024	0.131	MDR Non-Interactor	N/A
Thymol	Phenol	X	X	–	–	–	–	–	N/A	210.3	N/A	N/A
Tylosin Tartrate	Macrolide antibiotic	–	–	–	–	X	–	–	>2400	N/A	N/A	N/A

Such unintended co-exposures of agricultural pesticides has been suggested to affect bee colony health either directly by causing acute additive or synergistic toxicity to the bees or by indirectly sensitizing bees to other xenobiotics (Calatayud-Vernich et al. 2016; Hawthorne and Dively 2011). Increased toxicity due to co-exposure is a common theme in these studies: although testing shows limited toxicity to non-target pollinators for a single compound, these compounds can synergize and increase mortality rates dramatically. Pesticides are rarely applied alone; they are usually applied in conjunction with adjuvants or other pesticides to treat multiple pests and disease at the same time. For example, crop farmers might mix an insecticide together with an herbicide to stave off both parasitic insects that feed on the plant and weeds that compete for water and nutrients from the soil. Zhu and coworkers showed that when honeybees were exposed to eight different insecticides (imidacloprid, acephate, λ-cyhalothrin, oxamyl, tetraconazole, glyphosate, sulfoxaflor, and clothianidin) alone or in binary combinations, the mixtures showed synergistic toxicity that increased mortality between 15-26% (Zhu et al. 2017). Notably, when bees were exposed to a mixture of all eight pesticides, the mortality increased to 100%. Johnson and coworkers performed a similar study using pairwise combinations of acaricides, fungicides, and detoxification enzyme inhibitors (Johnson et al. 2013). The group reported that synergistic toxicity was observed with the acaricide tau-fluvalinate in combination with either the fungicide prochloraz (1980-fold), the model CYP450 inhibitor piperonyl butoxide (1980-fold), and the acaricide coumaphos (25-fold). Tau-fluvalinate also increased toxicity towards honeybees when combined with five different sterol biosynthesis inhibiting (SBI) fungicides, including prochloraz (41-fold) and myclobutanil (74-fold). Synergistic drug interactions have also been observed for binary combinations of hive medications. Hawthorne and Dively showed that bees pre-treated with oxytetracycline (OTC), an antibiotic treatment for foulbrood diseases, and sequentially exposed to

the acaricide coumaphos, a treatment for mite infestation, experienced a 44% increase in mean mortality (Hawthorne and Dively 2011). Likewise, bees fed with OTC and the acaricide tau-fluvalinate exhibited a 33% increase in mortality. The results of these studies strongly indicate that co-exposure of multiple environmental chemicals can sensitize bees towards toxicant accumulation. Common targets for drug-drug or chemical-chemical interactions are the xenobiotic defense systems present in all organisms, including metabolizing enzymes (CYPs) and the multi-xenobiotic/multidrug resistance (MXR/MDR) transporters of the ABC-type family.

Xenobiotic defense transporters systems in insects

Multidrug resistance (MDR) proteins from the ATP Binding Cassette (ABC) family are key determinants of drug and xenobiotic disposition in all organisms (The International Transporter Consortium 2010; Nicklisch and Hamdoun 2020a; Nigam 2015). Three of these ABC-type transporters, ABCB1 (aka P-glycoprotein), ABCC1, and ABCG2, are ubiquitously expressed at biological barriers and function to regulate chemical uptake, disposition, and elimination. Orthologs of these xenobiotic efflux transporters have been identified across the kingdoms of life, including animals, plants, fungi, and bacteria (Hwang et al. 2016). Yet, pharmacological, and toxicological studies of these important efflux systems in insects are scarce and have not been conducted in honeybees. The honeybee genome was fully sequenced and its annotation showed that bees possess orthologous genes for all three ABC transporters, including ABCB1, ABCC1, and ABCG2 (Kaplan and Linial 2006).

Among those, ABCB1 is arguably one of the best characterized MDR transporters. Insect homologs of ABCB1 have been identified and characterized in fruit flies (*Drosophila melanogaster)* (F. Mayer et al. 2009; A. Callaghan and Denny 2002; Vache et al. 2007; Groen et

al. 2017; Seong et al. 2016; I. Bosch et al. 1996), chironomid flies (Podsiadlowski, Matha, and Vilcinskas 1998), mosquitos (Porretta et al. 2008), Tobacco budworm (*Heliothis virescens*) (Lanning, Ayad, and Abou-Donia 1996), Tomato Hornworm (*Manduca sexta*) (Gaertner, Murray, and Morris 1998; Murray et al. 1994), Leaf Beetle (*Chrysomela tremula*) (Pauchet et al. 2016), Melon Fly (*Zeugodacus cucurbitae*) (H.-Q. Xu et al. 2021), red flour beetles (*Tribolium castaneum*), and diamondback moths (*Plutella xylostella*), and have been shown to have a major role in pesticide and environmental chemical resistance (Merzendorfer 2014; Dermauw and Van Leeuwen 2014; Heckel 2012; Wu et al. 2019; Gott et al. 2017; Buss and Callaghan 2007).

However, the effects of pesticides and their combinations on ABCB1 (and possibly other) transporter activity in honeybees have only recently been demonstrated *in vivo* (Hawthorne and Dively 2011, Guseman et al. 2016). Guseman and coworkers showed that the anti-microbial compound fumagillin, the crop fungicide Pristine, and the natural plant compound quercetin could sensitize honeybees towards toxic accumulation of the antiparasitic drug ivermectin, a known ABCB1 substrate (Guseman et al. 2016). An analogous increase in mortality was shown when bees were exposed to pesticides in combination with verapamil, a potent ABCB1 inhibitor (Hawthorne and Dively 2011, Guseman et al. 2016).

Functional diversification in honeybee ABCB1 splicing variants

Most insects, including bees, express one ABCB1 gene as their ABCB1 homolog. The annotation of the European honeybee's genome (The Honeybee Genome Sequencing Consortium 2006; Wallberg et al. 2014) identified a total of seven isoforms of the ABCB1 gene that cluster into two groups, only differing in the N-terminal stretch of approximately 60 amino acids (180 nucleotides), suggesting an alternative splicing mechanism (Figures 7 and 8). Specifically, the

gene variants X1-X4 contain identical sequences that code for ABCB1 protein isoform X1. Variants X5-X7 contain identical coding sequences for ABCB1 protein isoform X2. The alternative splicing pattern coupled with an alternative transcription start site (C. Xu, Park, and Zhang 2019) seen in the *in-silico* alignment leads to a frameshift at the N terminus causing the first 60 amino acids to misalign. However, the two isoforms fall back into the same frame after the splice junction then code for identical amino acids through the C terminus. Similar alternative splicing variants have been observed for the multidrug resistance-associated protein 1 (MRP1) and can increase functional diversity in an organism (Gökirmak et al. 2016). The question of whether only one or both honeybee isoforms are expressed and functional in the organism awaits successful isoform-specific immunolocalization and biochemical characterization.

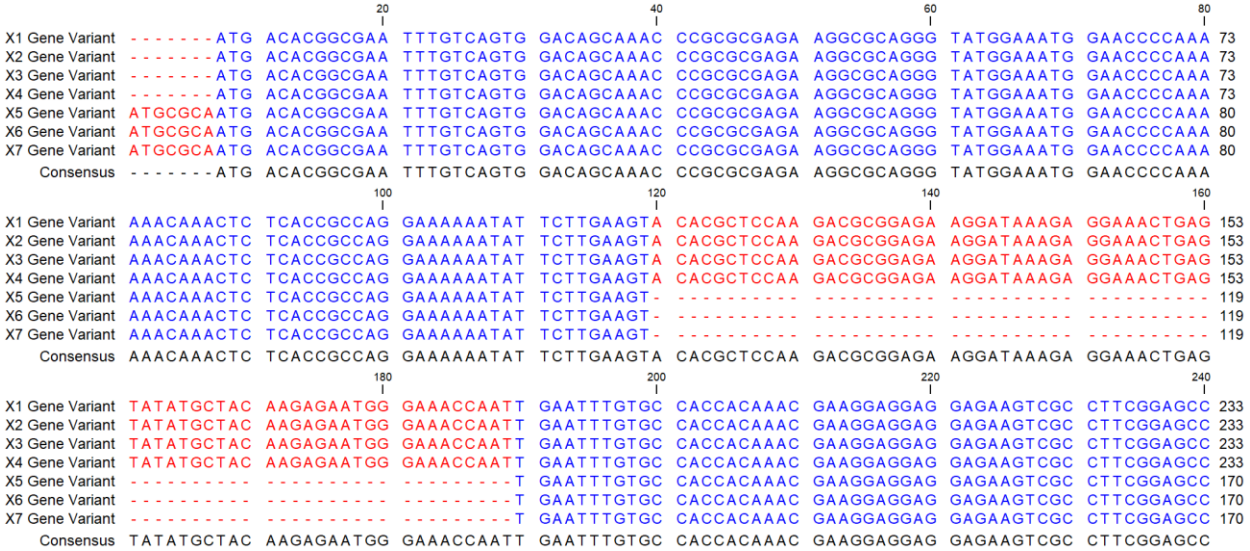


Figure 7: Partial nucleotide alignment showing all seven predicted *Apis mellifera* ABCB1 gene variants listed on NCBI. Conserved nucleotides are marked in blue, non-conserved nucleotides are marked in red. Full-length gene sequences are approximately 4kb each but have been truncated to showcase the alternative transcription start sites and the alternative splicing pattern. For NCBI accession number, see supplemental Table 4 (Table S4).

Table S4: Known ABCB1 gene isoforms of the European honeybee (*Apis mellifera*). The NCBI database (https://www.ncbi.nlm.nih.gov/) was used to determine all protein names, lengths, accession numbers, and gene IDs.

Protein	Isoform	Length (bp)	Accession	Gene ID
MDR49	X1	4032	XM_623561	551167
MDR49	X2	4032	XM_006568981	551167
MDR49	X3	4032	XM_026446478	551167
MDR49	X4	4032	XM_016918087	551167
MDR49	X5	3969	XM_006568983	551167
MDR49	X6	3969	XM_006568982	551167
MDR49	X7	3969	XM_016918088	551167

Further i*n silico* analysis of the amino acid sequence also revealed epitopes for the mouse monoclonal ABCB1 antibody C219, including VQRALD and VQAALD (Figure 8). Because both isoforms only differ in the N-terminus of the amino acid sequence, both isoforms have the same C219 epitopes. The conservation between isoforms allows western blotting and *in situ* hybridization to determine the localization and relative expression of *Am*-ABCB1 throughout the bee body (Figure 11).

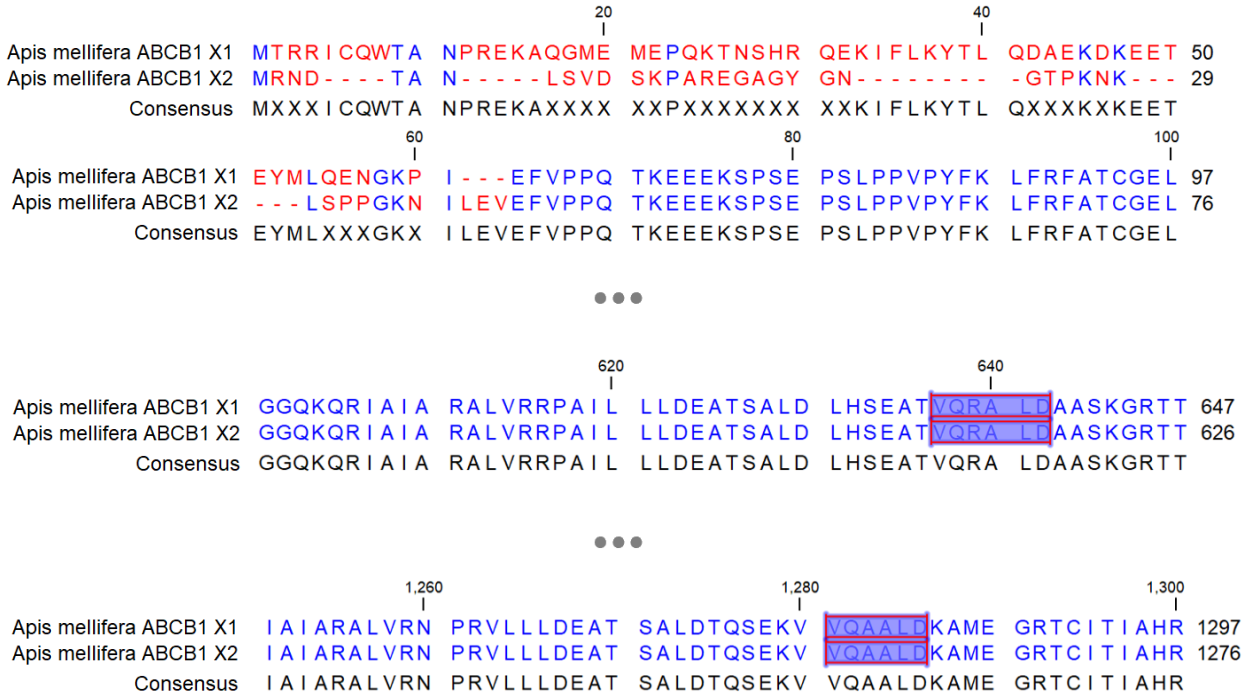


Figure 8: **Partial amino acid sequence alignment of the two groups (isoforms) of A*m*-ABCB1.** The European honeybee (*Apis mellifera*) genome annotation shows seven different gene isoforms of ABCB1 that can be clustered into two groups of isoforms (Table S1 and Figure 7). These two isoforms only differ in the N-terminal 60 amino acids. Conserved amino acids are marked in blue, non-conserved amino acids are marked in red. The sequence has been truncated to showcase the alternative splicing frameshift and C219 antibody epitope (highlighted).

The lack of pollinator model systems to study MDRs

Drosophila melanogaster is a well characterized model insect for evolutionary, genetic, and biochemical studies whose genome was fully sequenced in 2000 (Adams 2000; Roberts 2006; Wang et al. 2018; Tickoo 2002). Genome annotations and extensive functional characterization of the ABCB1 homologs showed that *Drosophila* expresses three different MDR proteins (MDR49, MDR50, and MDR65) (Vache et al. 2007; Irene Bosch and Croop 1998b). It is unclear as to why flies have evolved to express three ABCB1 homologs that convey multidrug resistance. Whether honeybees and other Hymenoptera possess additional ABCB1 homologs is not known and must await successful synteny analysis. While there is a wealth of resources and information available

about *Drosophila*, the model system could turn out to be inadequate to study how pesticides and other environmental chemicals interact with MDR transporters across insects, specifically pollinators.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Apis mellifera MDR49 X1	1		53	83	143	80	83	133	84	134	70	119	95	145	695	757	726
Apis mellifera MDR49 X6	2	96.07		133	131	130	132	128	133	129	120	107	144	138	681	761	714
Bombus impatiens MDR49 X1	3	93.83	90.13		65	8	112	160	110	158	97	145	123	172	700	760	740
Bombus impatiens MDR49 X2	4	89.47	90.25	95.21		73	172	164	170	162	157	148	183	174	683	766	731
Bombus terrestris MDR49	5	94.06	90.36	99.40	94.62		106	154	104	152	94	142	120	169	699	760	738
Osmia lignaria MDR49 X1	6	93.84	90.22	91.68	87.34	92.12		54	3	57	53	102	105	155	709	763	728
Osmia lignaria MDR49 X2	7	90.13	90.46	88.11	87.82	88.56	95.99		57	3	104	93	155	130	693	755	707
Osmia bicornis bicornis MDR49 X1	8	93.77	90.15	91.83	87.49	92.27	99.78	95.77		54	52	101	104	154	710	764	730
Osmia bicornis bicornis MDR49 X2	9	90.06	90.39	88.26	87.96	88.71	95.77	99.77	95.99		103	92	154	129	694	756	709
Megachile rotundata MDR49 X1	10	94.81	91.11	92.79	88.45	93.02	96.06	92.27	96.14	92.35		52	91	142	702	756	726
Megachile rotundata MDR49 X2	11	91.17	92.00	89.23	89.00	89.45	92.42	93.03	92.50	93.11	96.14		140	110	684	751	707
Ceratina calcarata MDR49 X1	12	92.95	89.33	90.86	86.53	91.08	92.20	88.49	92.28	88.57	93.24	89.61		54	698	771	735
Ceratina calcarata MDR49 X2	13	89.24	89.71	87.22	87.06	87.44	88.49	90.27	88.57	90.34	89.46	91.73	95.99		680	764	713
Drosophila melanogaster MDR49	14	49.20	49.63	48.83	49.37	48.90	48.21	48.70	48.14	48.63	48.72	49.30	48.98	49.55		815	757
Drosophila melanogaster MDR50	15	45.22	44.86	44.97	44.61	44.97	44.79	44.81	44.72	44.74	45.30	45.30	44.21	44.19	40.25		837
Drosophila melanogaster MDR65	16	47.24	47.58	46.22	46.41	46.37	47.09	47.82	46.95	47.68	47.24	47.90	46.58	47.38	43.34	38.50	

Figure 9: Pairwise comparisons of amino acid sequences of AB homologs from seven insect pollinators and fruit fly. European Honeybee (*Apis mellifera*), Buff-Tailed Bumblebee (*Bombus terrestris*), Common Eastern Bumblebee (*Bombus impatiens*), Spurred Carpenter Bee (*Ceratina calcarata*), and fruit fly (*Drosophila melanogaster*). Upper comparison values note the number of amino acid differences between each pair while lower comparison values note the percent identity between each pair. See supplemental Table 5 (Table S5) for NCBI accession numbers for each sequence.

Figure 9 shows a pairwise comparison of the amino acid sequence of the ABCB1 homologs of *Apis mellifera* and three other key pollinator insects with *Drosophila* MDR49, MDR50 and MDR65. The analysis shows that ABCB1 from *D. melanogaster* only shares 48-49% sequence identity with the ABCB1 homologs of bee pollinators. On the other hand, the ABCB1 homologs across the seven bee species share 89-96% sequence identify. Given the large differences in the amino acid sequences of fruit fly and bee ABCB1, the results from functional assays performed with *Drosophila* ABCB1 are likely not transferable to honeybees. Therefore, the differences seen between *Drosophila* and honeybee indicate that *Drosophila* would be a poor model to evaluate the molecular interactions of chemicals with bee ABCB1 transporters. Given the large differences in the amino acid sequences of fruit fly and bee ABCB1, the results from functional assays performed

with *Drosophila* ABCB1 are likely not transferable to honeybees. Compounds found to be non-toxic due to non-interaction with *Drosophila* ABCB1 could be strong inhibitors of *A. mellifera* ABCB1 and promote toxic accumulation in bees.

Challenges and Opportunities for creating targeted insect pesticides

The low amino acid conservation in ABCB1 transporters of beneficial insect pollinators and other insects provides an opportunity to design more targeted pesticides and hive medicines. For instance, *Am*-ABCB1 only shares 40-45% sequence identity with ABCB1 homologs in Wax moths and the Small Hive Beetle, and only 21% identity with Varroa mite ABCB1 (Figure 10A). Similarly, the ABCB1 homologs of common disease vectors, including Anopheles, Aedes and Culex only share 56-57% sequence identity with *Am*-ABCB1 (Figure 10B). Using structure-function analysis with native or purified honeybee and pest ABCB1 proteins could provide a new, high-throughput avenue to predict toxic chemical accumulation potential in each of these species. Similar to modern "precision medicine", one could select for highly effective pesticides to control hive pests and disease vectors but with low accumulation potential in beneficial pollinator insects.

A

		1	2	3	4	5	6	7	8	9	10	11	12
Apis mellifera MDR49 X1	1		53	800	806	784	786	735	768	1307	1303	1297	1308
Apis mellifera MDR49 X6	2	96.07		779	800	778	778	714	747	1303	1299	1293	1294
Galleria mellonella ABCB1-Like	3	40.65	41.30		425	423	423	714	713	1310	1306	1301	1288
Galleria mellonella ABCB1-Like X1a	4	40.34	40.48	66.69		470	471	713	728	1337	1333	1328	1327
Galleria mellonella ABCB1-Like X1b	5	41.84	41.98	66.35	63.19		13	681	719	1320	1316	1311	1308
Galleria mellonella ABCB1-Like X2b	6	41.69	41.98	66.35	63.12	98.96		681	718	1320	1316	1311	1308
Aethina tumida MDR1-Like IF1*	7	45.56	46.23	43.82	45.11	47.37	47.37		621	1307	1303	1297	1293
Aethina tumida MDR1-Like IF2*	8	43.24	43.88	44.03	43.96	44.56	44.64	51.22		1298	1294	1290	1285
Varroa destructor MDR1-Like X1	9	21.60	21.60	19.83	19.70	19.81	19.81	20.30	21.29		4	10	22
Varroa destructor MDR1-Like X2	10	21.65	21.65	19.88	19.75	19.85	19.85	20.35	21.34	99.75		14	26
Varroa destructor MDR1-Like X3	11	21.77	21.78	19.94	19.81	19.91	19.91	20.48	21.34	99.38	99.13		32
Varroa destructor MDR1-Like X4	12	21.54	21.72	20.10	19.72	19.95	19.95	20.48	21.41	98.63	98.38	98.01	

B

		1	2	3	4	5	6	7	8	9	10	11	12	13
Apis mellifera MDR49 X1	1		53	581	583	581	575	573	582	578	574	589	589	591
Apis mellifera MDR49 X6	2	96.07		571	573	573	565	564	572	572	568	580	580	582
Anopheles coluzzii MDR49	3	56.87	57.29		6	58	118	117	120	230	227	300	300	304
Anopheles gambiae ABCB1	4	56.72	57.14	99.54		62	121	120	125	232	229	302	302	306
Anopheles stephensi MDR49	5	56.87	57.14	95.55	95.25		127	122	119	230	226	304	304	308
Anopheles albimanus MDR49-Like	6	57.31	57.77	90.96	90.74	90.28		8	127	222	217	300	301	301
Anopheles darlingi ABC	7	57.46	57.85	91.04	90.81	90.66	99.39		125	220	215	298	299	298
Anopheles stephensi ABCB1-Like	8	56.79	57.22	90.80	90.42	90.88	90.27	90.42		226	224	294	296	298
Aedes aegypti MDR49	9	57.09	57.25	82.43	82.28	82.43	83.05	83.21	82.75		27	255	256	259
Aedes albopictus MDR49-like	10	57.39	57.55	82.66	82.51	82.73	83.44	83.59	82.90	97.93		252	253	256
Culex pipiens pallens MDR49-Like IF2	11	56.27	56.65	77.19	77.03	76.88	77.19	77.34	77.63	80.59	80.82		2	8
Culex quinquefasciatus MDR2	12	56.27	56.65	77.19	77.03	76.88	77.11	77.26	77.47	80.52	80.75	99.85		10
Culex pipiens pallens MDR49-Like IF1	13	56.12	56.50	76.88	76.73	76.58	77.11	77.34	77.32	80.29	80.52	99.39	99.24	

Figure 10: Pairwise comparisons of ABCB1 amino acid sequences from honeybees and in hive pests (A) versus disease vectors (B). Upper comparison values note the number of amino acid differences between each pair while lower comparison values note the percent identity between each pair. See Supplemental Table 5 (Table S5) for NCBI accession numbers for each species shown.

Differential expression of *Am*-ABCB1 variants to protect CNS and gut

Eusocial organisms like bees are organized into castes, each with specific jobs. Nurse bees are newly pupated adults that remain in the hive and care for the queen and larvae. Forager bees are older bees that have aged out of nursing and venture out of the hive in search of water, nectar and pollen (Wright, Nicolson, and Shafir 2018). Although forager bees are just older nurse bees, they go through significant physiological, morphological, biochemical, and gene expression change when they transition between castes (Robinson 2002). For example, higher expression of immune and detoxification genes have been seen in forager bees *versus* nurse bees, likely reflecting gene induction due to higher exposures to xenobiotics during foraging (Vannette, Mohamed, and Johnson 2015).

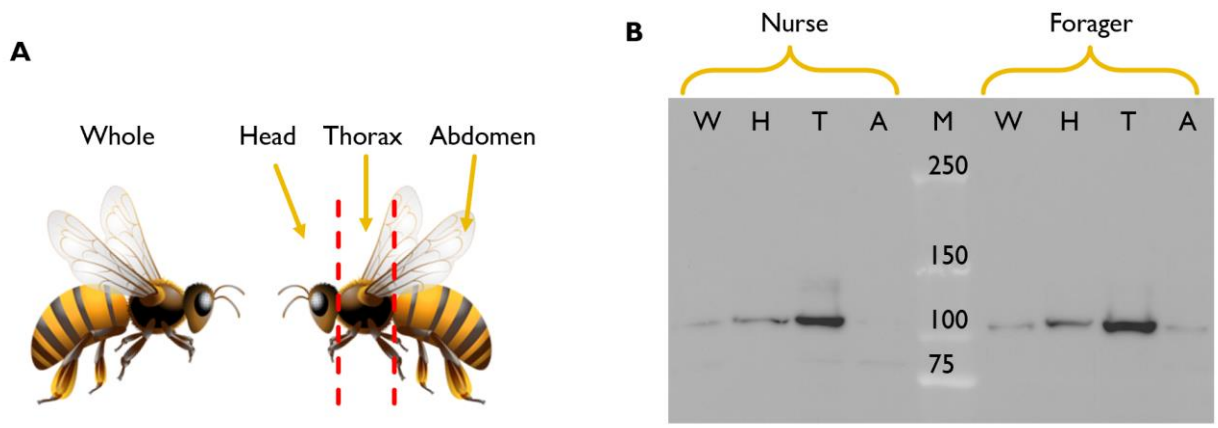


Figure 11: Immunoblotting of whole and partial honeybee extracts show highest abundance of *Am*-ABCB1 in the thorax and head. **[A]** Diagram showing a whole bee and the dissection pattern for immunoblotting individual extracts of the head, thorax, and abdomen of nurse and forager bees. **[B]** Western blot of Honeybee nurse and foragers as whole bee samples (W), heads (H), thorax (T), and abdomen (A). Proteins were blotted onto 0.22 µm PVDF membrane and probed with the C219 anti-ABCB1 primary antibody. The blot shows protein bands at ~100 kDa. However, the calculated molecular weight of *Am*-ABCB1 is ~145 kDa.

A western blot was conducted on both nurse and forager bees to determine the localization of *Am*-ABCB1. Samples included preparations of whole bees, heads, thoraxes, and abdomens (Figure 11A). Each dissected portion of the bee body contains important organs and biological barriers. Bee heads contain the brain-hemolymph barrier, eyes-hemolymph barrier, and the secretory hypopharyngeal gland. The thoraxes contain muscle, the esophageal tract, the aorta, and a portion of the nerve ganglia-hemolymph barrier. The abdomen of the bee houses the heart, stomach, gut-hemolymph barrier, and the rest of the nerve ganglia-hemolymph barrier (Carreck et al. 2013; Faux 2021). Our results showed high expression of *Am*-ABCB1 in the head and thorax, suggesting that *Am*-ABCB1 might be localized within the hemolymph-brain or hemolymph-ganglia barriers of the central nervous system in the bees (Figure 11B). However, the SDS-PAGE migration pattern on the immunoblot shows *Am*-ABCB1 at 100 kDa and does not align with the calculated molecular weight of 147 kDa for the X1 isoform and 144 kDa for X2. Since we did not detect any significant degradation bands, further analysis is needed to determine the cause of the

peculiar migration pattern. These analyses will include purification of the *Am*-ABCB1 from whole bee extracts and determining the intact protein molecular weight using MALDI-TOF mass spectrometry. Since both isoforms of *Am*-ABCB1 in the honeybee have identical C219 epitopes, the immunoblotting technique was not able to further differentiate which isoform is more abundant in the honeybee. As such, we designed isoform-specific primers and conducted qPCR analysis to determine relative *Am*-ABCB1 isoform expression in head, thorax, and abdomen of the honeybees.

The qPCR analysis showed that bees expressed both isoforms of *Am*-ABCB1 in different ratios throughout the body (Figure 12-13). The X2 isoform is more abundant in all samples, but there are elevated levels of the X1 isoform in the abdomen when compared to X1 levels in the head and thorax.

Relative qPCR				
	Nurse		**Forager**	
	X1	**X2**	**X1**	**X2**
Whole	4%	96%	63%	37%
Head	8%	92%	8%	92%
Thorax	10%	90%	8%	92%
Abdomen	27%	73%	85%	15%

Figure 12: Ratios of *Am*-ABCB1 isoforms in nurse and forager bee sections using relative qPCR. The results of the relative qPCR show the X2 isoform is dominant in all parts of the nurse bee anatomy whereas in the forager bee, X2 is only dominant in the Head and Thorax. Similar abundance is seen the head and thorax of both nurse and forager, but inverse proportions are seen in abdomen of nurse and foragers. These numbers are reflected in the differences between nurse and forager whole. Relative qPCR was normalized to a primer pair that bound to both isoforms and GAPDH was used as a reference gene. The values represent the mean of duplicate measurements using cDNA prepared from 1 bee per whole sample and 2-3 bees per dissected sample.

A

Absolute qPCR - cDNA Standard				
	Nurse		Forager	
	X1	X2	X1	X2
Whole	14%	86%	25%	75%
Head	7%	93%	10%	90%
Thorax	8%	92%	6%	94%
Abdomen	33%	67%	27%	73%

C

Absolute qPCR - Plasmid Standard				
	Nurse		Forager	
	X1	X2	X1	X2
Whole	2.81%	97.19%	1.48%	98.52%
Head	0.44%	99.56%	0.04%	99.96%
Thorax	0.44%	99.56%	0.07%	99.93%
Abdomen	6.35%	93.65%	0.69%	99.31%

B

Absolute qPCR - cDNA Standard				
	Nurse		Forager	
	X1	X2	X1	X2
Whole	2901 ng/µL	18241 ng/µL	9050 ng/µL	27772 ng/µL
Head	182 ng/µL	2343 ng/µL	287 ng/µL	2460 ng/µL
Thorax	611 ng/µL	7043 ng/µL	1162 ng/µL	17923 ng/µL
Abdomen	8412 ng/µL	16985 ng/µL	4824 ng/µL	12842 ng/µL

D

Absolute qPCR - Plasmid Standard				
	Nurse		Forager	
	X1	X2	X1	X2
Whole	34.5 C/µL	1194.1 C/µL	223.1 C/µL	14818.7 C/µL
Head	0.5 C/µL	101.9 C/µL	0.8 C/µL	2113.5 C/µL
Thorax	2.9 C/µL	653.5 C/µL	7.0 C/µL	10592.1 C/µL
Abdomen	184.2 C/µL	2717.2 C/µL	83.8 C/µL	11987.1 C/µL

Figure 13: Ratios of *Am*-ABCB1 isoforms in nurse and forager bee sections using absolute qPCR. [A] The results of the absolute qPCR using a cDNA standard show higher levels of X2 isoform in all samples and similar isoform abundance in both nurse and forager bees. **[B]** Results of the Absolute qPCR with cDNA standard shown as DNA concentration. **[C]** The results of the Absolute qPCR with the plasmid standard show much lower levels of the X1 variant overall. **[D]** Results of the Absolute qPCR with Plasmid standards shown as copy number per uL of DNA. The values represent the mean of duplicate measurements using cDNA prepared from 1 bee per whole sample and 2-3 bees per dissected sample as unknown samples. cDNA from a single whole nurse bee was used as a standard for A&B. A plasmid containing a codon-optimized version of the cloned *Am*-ABCB1 X1 gene variant was used as a standard for C&D.

Conclusions

Ecological surveys by the USDA's National Agricultural Statistics Service (NASS) have detected a 12% decline in bees within a 5-year period. The sharp decline has appropriately caused alarm and scientific inquiries that have identified common agricultural pesticides as a possible contributing factor for the decline in bee numbers. Pesticide/pesticide and pesticide/hive medication mixtures are of great concern due to their additive and synergistic toxic effects of honeybee health. Although there are plenty of studies showing increased mortality due to pesticide mixtures, there is a significant data gap explaining the molecular mechanisms of this phenomenon. Multidrug resistance transporters, meant to keep drugs and toxins out, are a logical protein of interest for this line of research.

Although *Drosophila melanogaster* has verified and well-researched MDR proteins, these proteins are not suitable to study honeybee interactions. Sequence analysis of *Drosophila* ABCB1 and *Am*-ABCB1 showed less than 50% amino acid similarity. Such low similarity likely will reflect differences in ligand binding residues and ligand specificity. Honeybee also shows a 40% difference from disease-carrying mosquitos and a 60-80% difference from hive pests, including the greater wax moth and varroa mite. These differences could offer a chance to create specific pest management programs detrimental to pests and safe for honeybees.

The presence of ABCB1 in honeybees was validated through western blot then cloned and sequenced. It was discovered that both the X1 and X2 isoforms can be found in a single bee. Proportions of each isoform were determined through qPCR. Results from the qPCR showed similar proportions of X1 and X2 in nurse and forager bees. There was a 1:10 ratio of X1 to X2 in the head and thorax of the bee but 3:10 ratio of X1 to X2 in the abdomens, showing that X1 might be more associated with gastro-intestinal protection. The cloned *Am*-ABCB1 sequences will be expressed as protein and purified for use in determining *in vitro* pesticide interactions and their implications for honeybee health.

The data acquired from *in vitro* pesticide mixture screening could be used to create a centralized, public database of safe and unsafe chemical mixtures for bees. This database could help both professional farmers and amateur gardeners practice safer pest management while preserving the bees. Bringing pesticide mixture concerns to the public eye would also help start a conversation between beekeepers and local farmers to ensure deadly unintentional mixtures do not happen.

Table S5: Table listing species, proteins, and protein accession numbers for bees, hive pests, and disease vectors. The NCBI database was used to determine all protein names, lengths, accession numbers, and gene IDs. Known proteins that have been validated by publications are denoted with NP in the accession number, experimental or model proteins determined by annotation software are denoted with XP in the accession number and "Direct Submit" proteins are not validated by NCBI or their annotation software but are submitted directly to NCBI by researchers.

Scientific name	Common Name	Protein	Length	Accession	Gene ID
Anopheles albimanus	Mosquito	MDR49-Like	1304	XP_035789861	118465598
Anopheles coluzzii	Mosquito	MDR49	1304	XP_040221247	120948693
Anopheles darlingi	Mosquito	ABC	1304	ETN61204	Direct Submit
Anopheles gambiae	Mosquito	AGAP005639-PA	1301	XP_315658	1276325
Anopheles sinensis	Mosquito	AGAP005639-PA-Like	1297	KFB50603	Direct Submit
Anopheles stephensi	Asian malaria mosquito	MDR49	1304	XP_035913596	118512787
Aedes aegypti	yellow fever mosquito	MDR49	1307	XP_001654492	5573277
Aedes albopictus	Asian tiger mosquito	MDR49-Like	1307	XP_029735703	109408676
Culex quinquefasciatus	southern house mosquito	MDR49	1311	XP_038117776	6050364
Culex pipiens pallens	northern house mosquito	MDR49-Like IF1	1311	XP_039451126	120430107
Culex pipiens pallens	northern house mosquito	MDR49-Like IF2	1311	XP_039451145	120430126
Drosophila melanogaster	fruit fly	MDR65	1302	NP_476831	38726
Drosophila melanogaster	fruit fly	MDR49a	1302	NP_523724	36428
Drosophila melanogaster	fruit fly	MDR50	1313	NP_523740	36582
Musca domestica	house fly	MDR65	1303	XP_005186344	101899244
Galleria mellonella	Wax Moth	ABCB1-Like X1	1274	XP_026762002	113520794
Galleria mellonella	Wax Moth	ABCB1-Like	1183	XP_026762069	113520845
Galleria mellonella	Wax Moth	ABCB1-Like X1	1254	XP_026765038	113523317
Galleria mellonella	Wax Moth	ABCB1-Like X2	1254	XP_026765039	113523317
Varroa destructor	Varroa Mite	MDR1-Like X1	1607	XP_022661976	111250671
Varroa destructor	Varroa Mite	MDR1-Like X2	1603	XP_022661977	111250671
Varroa destructor	Varroa Mite	MDR1-Like X3	1598	XP_022661978	111250671
Varroa destructor	Varroa Mite	MDR1-Like X4	1585	XP_022661980	111250671
Aethina tumida	Small Hive Beetle	MDR1-Like IF1	1252	XP_019879179	109607095
Aethina tumida	Small Hive Beetle	MDR1-Like IF2	1260	XP_019874216	109602313
Apis mellifera	European honeybee	MDR49 X1	1343	XP_006569044	551167
Apis mellifera	European honeybee	MDR49 X2	1322	XP_006569046	551167
Bombus impatiens	eastern bumblebee	MDR49 X1	1344	XP_012242648	100745824
Bombus impatiens	eastern bumblebee	MDR49 X2	1323	XP_012242651	100745824
Bombus terrestris	buff-tailed bumblebee	MDR49	1344	XP_020723751	100650108
Ceratina calcarata	carpenter bee	MDR49 X1	1346	XP_017884014	108627333
Ceratina calcarata	carpenter bee	MDR49 X2	1325	XP_026671324	108627333
Megachile rotundata	alfalfa leafcutting bee	MDR49 X1	1346	XP_003701514	100877577
Megachile rotundata	alfalfa leafcutting bee	MDR49 X2	1325	XP_012136740	100877577
Osmia bicornis bicornis	Red Mason Bee	MDR49 X1	1346	XP_029036184	114872770
Osmia bicornis bicornis	Red Mason Bee	MDR49 X2	1325	XP_029036190	114872770
Osmia lignaria	orchard mason bee	MDR49 X1	1346	XP_034170893	117600059
Osmia lignaria	orchard mason bee	MDR49 X2	1325	XP_034170899	117600059

Appendix 1: Cloning

1.1. Honeybee ABCB1 Sequence Verification

One of the first issues I ran into with this project was the lack of consistency in the naming of insect transporter proteins. The *Am*-ABCB1 sequence was listed as ABCB4 in an article, as ABCB1 in the KEGG database, and as ABCB1 in NCBI (Table A1). Due to these inconsistencies, I had to individually verify every sequence I pulled by performing a BLAST search and gene or amino acid sequence alignments. Each sequence from Dermauw *et al.* 2014 was aligned with a corresponding sequence from KEGG. The amino acid sequences for each comparison between the Dermauw *et al.* paper and KEGG were aligned using the CLC Main Workbench Software.

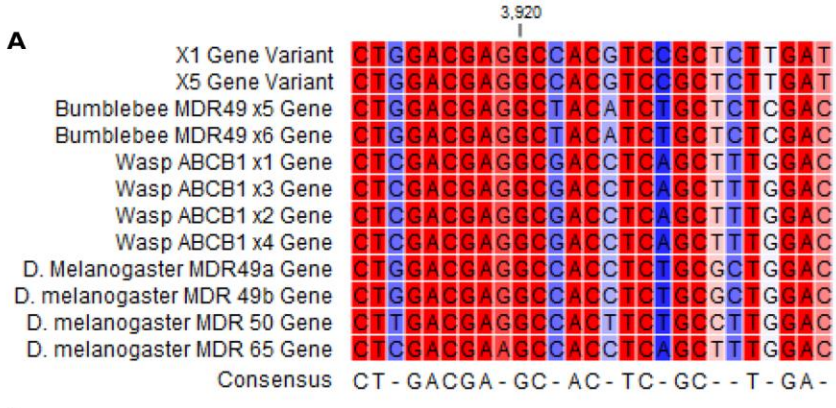
Table A1: Comparison of protein sequences from literature, KEGG, and NCBI. Inconsistencies in the transporter nomenclature are highlighted. Accession numbers refer to NCBI. C219 epitope lists which C219 epitopes are present in the protein and N/A denotes no C219 epitope. The percent identity or %ID is the percentage of identical amino acids between the sequences in the alignment. Known proteins that have been validated by publications are denoted with NP in the accession number, experimental or model proteins determined by annotation software are denoted with XP in the accession number and "Direct Submit" proteins are not validated by NCBI or their annotation software but are submitted directly to NCBI by researchers. Direct submit proteins do not follow the "XP/NP" nomenclature. N/A denotes the lack of a comparison protein for ABCB8 in the Dermauw *et al.* paper.

		Dermauw et al (2014)			KEGG (accessed March 2021)				
Species Name	**Common Name**	**Dermauw et al**	**Accession Name**	**Accession Number**	**KEGG**	**Accession Name**	**Accession Number**	**C219 Epitope**	**Alignment % ID**
Apis meillifera	Eastern Honeybee	**AmABCB4**	**MDR49 (P-gp 49)**	**XP_623564.2**	ABCB1	MDR49 X1	XP_006569044	VQRALD, VQAALD	93%
		AmABCB1	ABCB7 X1	XP_396202.3	ABCB7	ABCB7 X1	XP_396202	N/A	100%
		AmABCB2	CG5225-PA Like	XP_001122583.1	ABCB6	ABCB6	XP_001122583	N/A	89%
		AmABCB3	ABCB10	XP_625122.2	ABCB10	ABCB10	XP_006566708	N/A	93%
		AmABCB7	ABCB8	XP_624810	ABCB8	ABCB8	XP_006561654	VQKALD	97%
Anopheles gambiae	Mosquito	**AGAP005639-PA**	**AGAP005639-PA**	**XP_315658.3**	ABCB1	AGAP005639-PA	XP_315658	VQNALD	100%
		AGAP002717-PA	AGAP002717-PA	XP_312209.3	ABCB10	AGAP002717-PA	XP_312209	N/A	100%
		AGAP006364-PA	AGAP006364-PA	XP_001688853.1	ABCB7	AGAP006364-PA	XP_001688853	N/A	100%
		AGAP002278-PA	AGAP002278-PA	XP_307900.4	ABCB6	AGAP006273-PA	XP_307900	N/A	100%
		N/A	N/A	N/A	ABCB8	AGAP002278-PA	XP_316337	VQRALD	N/A
Drosophila melanogaster	Fruit Fly	**CG10226**	**CG10226 IFa**	**AAF50670**	ABCB1	Dme_CG10226 IFb	NP_001261473	VQAALD, VQQALD	91%
		Mdr49	**Mdr49 IFa**	**NP_523724**	ABCB1	Mdr49 IFa	NP_523724	VQQALD	100%
		Mdr50	**Mdr50**	**NP_523740**	ABCB1	Mdr50	NP_523740	VQQALD, VQQALD	100%
		Mdr65	**Mdr65**	**NP_476831**	ABCB1	Mdr65	NP_476831	VQQALD, VQQALD	100%
		CG1824	CG1824	AAF48177	ABCB8	Dmel_CG1824	NP_572810	VQKALD	100%
		CG3156	CG3156	NP_569844	ABCB10	Dmel_CG3156	NP_569844	VQNALD	100%
		CG4225	HMT-1	AAF55241	ABCB6	HMT-1	NP_650503	N/A	100%
		CG7955	ABCB7 IFa	AAF47525	ABCB7	ABCB7 IFa	NP_728642	N/A	100%
Homo sapiens	Human	**hABCB1**	**ABCB1**	**P08183**	ABCB1	ABCB1 IF2	NP_000918	VQVALD, VQEALD	100%
		hABCB2	ABCB2	Q03518	ABCB2	TAP1 IF1	NP_000584	N/A	93%
		hABCB3	ABCB3	Q03519	ABCB3	TAP2 IF3	NP_001276972	N/A	100%
		hABCB4	**ABCB4**	**P21439**	ABCB4	ABCB4	NP_061337	VQAALD, VQEALD	100%
		hABCB5	**ABCB5**	**Q2M3G0**	ABCB5	ABCB5 IF1	NP_001157413	VQHALD	100%
		hABCB6	ABCB6	Q9NP58	ABCB6	ABCB6 IF1	NP_005680	N/A	100%
		hABCB7	ABCB7	O75027	ABCB7	ABCB7 IF2	NP_001258625	N/A	100%
		hABCB8	ABCB8	Q9NUT2	ABCB8	ABCB8 IFa	NP_001269220	VQEALD	100%
		hABCB9	ABCB9	Q9NP78	ABCB9	ABCB9 IF1	NP_062571	N/A	100%
		hABCB10	ABCB10	Q9NRK6	ABCB10	ABCB10	NP_036221	VQEALD	100%
		hABCB11	**ABCB11**	**O95342**	ABCB11	ABCB11	NP_003733	N/A	100%

1.2. Bee ABCB1 Degenerate Primer Design

When cloning ABCB1 out of live honeybees, I started with end-to-end primers that were designed to anneal to the 5' and 3' ends of the full coding sequence (5'-ATGACACGGCGAATTTGTCA-3' and 5'-TTATGTAGCAAGGCCTGCGTC-3'). After several tries, the end-to-end primers proved to be unsuccessful. From here, I thought the problem was that the genes I was referencing from NCBI (XM_623561 and XM_006568983) was labeled as a "predicted" gene. This could have meant that the gene that I used to design the end-to-end primers did not reflect the real gene sequence found in honeybees. To remedy this, I decided to design degenerate primers.

Degenerate primers (Table A2, Figure A2) were designed by first aligning closely related Hymenoptera species to find consensus sequences (see Figure A1). Species included were *Apis mellifera* X1 and X5 (XM_623561 and XM_006568983), *Bombus impatiens* X5 and X6 (XM_012387228 and XM_012387229), *Nasonia vitripennis* X1-4 (XM_031925341, XM_031925342, XM_016986704, and XM_031925343), and *Drosophila melanogaster* MDR49a, MDR49b, MDR50, and MDR65 (NM_079000, NM_001169661, NM_079016, and NM_057483). Highly conserved portions of the alignment between 17 and 23 base pairs were selected and converted into degenerate primers using degenerate nucleotide codes (see figure A1). The degeneracy value for each sequence was calculated by multiplying the number of possible nucleotides for each base of a primer. Primers with values under 100 were ordered from Eurofins. In addition to those generated from the sequence alignments, I also made degenerate primers against the C219 epitope that I know are in the bees due to previous western blot testing. Once again, I tried to clone the ABCB1 gene out of the honeybees using new end-to-end degenerate primers. This also did not work (Figure A3).



A	C	G	T	A/C	A/g	A/T	C/g	C/T	g/T	A/C/g	A/C/T	A/g/T	C/g/T	A/C/g/T
A	C	G	T	M	R	W	S	Y	K	V	H	D	B	N

Figure A1: Degenerate sequence alignment and degeneracy codes. [A] Segment of the alignment used to make the degenerate primers. Species used are *Apis mellifera* X1 and X5 (XM_623561 and XM_006568983), *Bombus impatiens* X5 and X6 (XM_012387228 and XM_012387229), *Nasonia vitripennis* X1-4 (XM_031925341, XM_031925342, XM_016986704, and XM_031925343), and *Drosophila melanogaster* MDR49a, MDR49b, MDR50, and MDR65 (NM_079000, NM_001169661, NM_079016, and NM_057483). Red shows higher identity between sequences and blue shows lower identity. **[B]** Table of degeneracy codes.

Table A2: Degenerate Primers and calculated degeneracy values. Table shows original sequence from the consensus sequence of *Apis mellifera* ABCB1 X1 and X6, the name of the ordered primers, the sequences of the ordered primers, and the degeneracy values. Two C219 epitopes were designed and ordered because there are two different C219 Epitopes in *Am*-ABCB1 (see Figure 8, Table A1).

Sequence template	Name	Degenerate Seq	Name	Reverse Complement	Degeneracy
C219 Epitope 1	C219_Epi1_BeePgp_FW	GTNCARGARGCNYTNGAY	C219_Epi1_BeePgp_RV	RTCNARNGCYTCYTGNAC	
C219 Epitope 2	C219_Epi2_BeePgp_FW	GTNCARGCNGCNYTNGAY	C219_Epi2_BeePgp_RV	RTCNARNGCNGCYTGNAC	
TGGATGTTGGGACAAGC	HymPgp_Deg_FW_1	TGGATGTTGGGMCARGC	HymPgp_Deg_RV_1	GCYTGKCCCAACATCCA	4
CCGGTGCTCTTTGACAC	HymPgp_Deg_FW_2	CCGGTKCTCTTYGMCAC	HymPgp_Deg_RV_2	GTGKCRAAGAGMACCGG	8
CAGGATATGACGTGGTACGA	HymPgp_Deg_FW_3	CAGGAYATSRCSTGGTACGA	HymPgp_Deg_RV_3	TCGTACCASGYSATRTCCTG	16
GATTTGGACAAGATGAAGG	HymPgp_Deg_FW_4	GAYYTGGACAARMTGAAGG	HymPgp_Deg_RV_4	CCTTCAKYTTGTCCARRTC	16
TCGCACATAGGCGTGGTCGG	HymPgp_Deg_FW_5	TCGCASATMGGMGTGGTSGG	HymPgp_Deg_RV_5	CCSACCACKCCKATSTGCGA	16
ACGATACGGGAGAATATCCG	HymPgp_Deg_FW_6	ACSATWSGSGAGAATATCCG	HymPgp_Deg_RV_6	CGGATATTCTCSCSWATSGT	16
TGCGGCAAATCCACCTG	HymPgp_Deg_FW_7	TSYGGCAARTCSACCTG	HymPgp_Deg_RV_7	CAGGTSGAYTTGCCRSA	16
AAGGTGGTGCAAGCTGC	HymPgp_Deg_FW_8	AAGGTSGTGCAASMKGC	HymPgp_Deg_RV_8	GCMKSTTGCACSACCTT	16
CATTCGTTTACGGGTGGAA	HymPgp_Deg_FW_9	CDTTCGTHTACGGVTGGAA	HymPgp_Deg_RV_9	TTCCABCCGTADACGAAHG	27
GGCGCCTCGTTCCCAGC	HymPgp_Deg_FW_10	GGCGCCWCRTTCCCRSY	HymPgp_Deg_RV_10	RSYGGGAAYGWGGCGCC	32
ATCACCATAGCGCATCG	HymPgp_Deg_FW_11	MTSACSATWGCKCATCG	HymPgp_Deg_RV_11	CGATGMGCWATSGTSAK	32
GAGATGGGTACTCACGACGA	HymPgp_Deg_FW_12	GAGMWSGGYACYCACGATGA	HymPgp_Deg_RV_12	TCATCGTGRGTRCCSWKCTC	32
CTGGTGGTGCTAAGCTGCGC	HymPgp_Deg_FW_13	CTGGTSGTDCTRAGYTGY	HymPgp_Deg_RV_13	RCARCTYAGHACSACCAG	48
GTCGCCGAGGAGGTGTTAGG	HymPgp_Deg_FW_14	GTSGYCGAGGARGTRTTRRG	HymPgp_Deg_RV_14	CYYAAYACYTCCTCGRCSAC	64
TCATCATATACATCAGC	HymPgp_Deg_FW_15	TSATCATMTACMTSWGY	HymPgp_Deg_RV_15	RCWSAKGTAKATGATSA	64
CGTGATCGTGTTCTTCG	HymPgp_Deg_FW_16	SGTSATYGTSYTSTTCG	HymPgp_Deg_RV_16	CGAASARSACRATSACS	64
GCCCAGAACATGGGTCT	HymPgp_Deg_FW_17	GCYCAGAAYMTSGGYYT	HymPgp_Deg_RV_17	ARRCCSAKRTTCTGRGC	64
GAGACCGTTGCCCTCGTCGG	HymPgp_Deg_FW_18	SAGACSGTKGCMYTCGTSGG	HymPgp_Deg_RV_18	CCSACGARKGCMACSGTCTS	64
AAGAAGAAGATGGAGGC	HymPgp_Deg_FW_19	AAGRMGWMGATSGAGGM	HymPgp_Deg_RV_19	KCCTCSATCKWCKYCTT	64
GAGGCGTTCGCGGTCGC	HymPgp_Deg_FW_20	GARGCSWTSGCBGTSGC	HymPgp_Deg_RV_20	GCSACVGCSAWSGCYTC	96
TCGGAGGCGTTGATCTTTGG	HymPgp_Deg_FW_21	**KCSGARGCHYTGATCTTYGG**	HymPgp_Deg_RV_21	CCRAAGATCARDGCYTCSGM	96

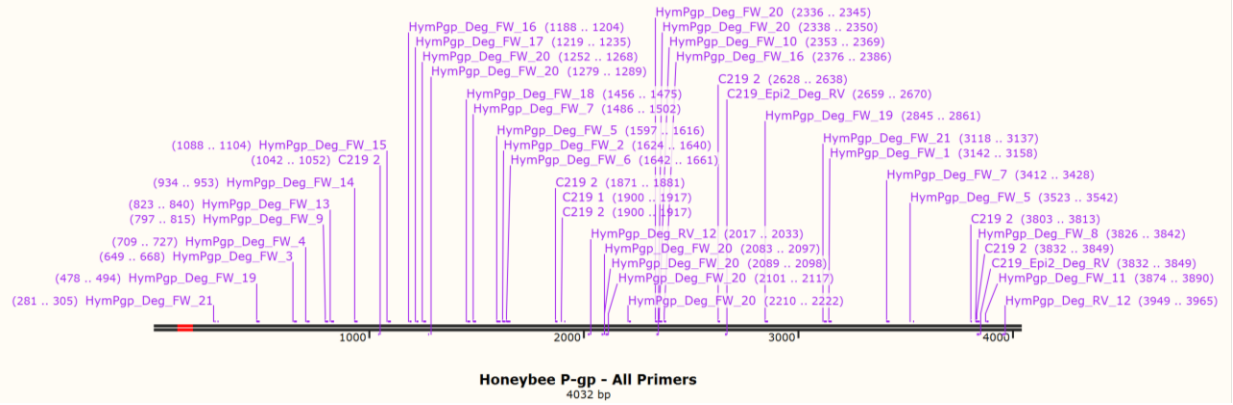
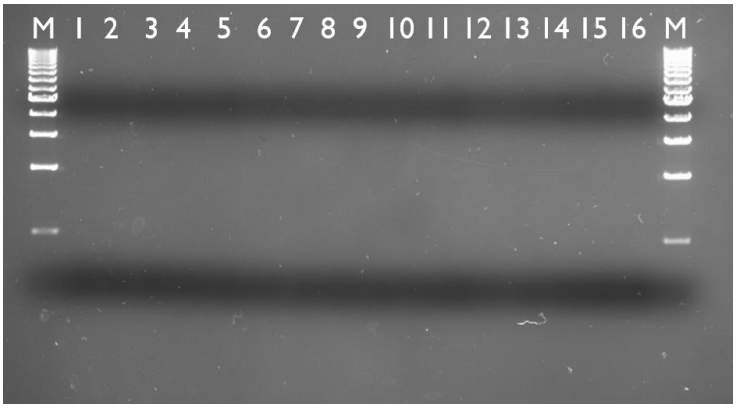


Figure A2: Degenerate Primer alignment to Honeybee ABCB1 X1 coding sequence. Shown are primers from Table A2 aligned to the *Am*-ABCB1 coding sequence. Only one forward or reverse primer is shown for each degenerate primer sequence but both forward and reverse primers were ordered. The red portion near the 3' end of the gene is the alternative splicing region only seen in the X1-X4 gene variants. Because the degenerate primers are not specific, there are multiple binding sites for several of the degenerate primers. This figure also shows the binding sites for the C219 epitope.

A



B

	Forward Primer	Reverse Primer
1	HymPgp_Deg_Fw_20	HymPgp_Deg_Rv_11
2	HymPgp_Deg_Fw_20	C219_Epi2_BeePgp_RV
3	HymPgp_Deg_Fw_20	HymPgp_Deg_Rv_8
4	HymPgp_Deg_Fw_20	HymPgp_Deg_Rv_5
5	HymPgp_Deg_Fw_3	HymPgp_Deg_Rv_11
6	HymPgp_Deg_Fw_3	C219_Epi2_BeePgp_RV
7	HymPgp_Deg_Fw_3	HymPgp_Deg_Rv_8
8	HymPgp_Deg_Fw_3	HymPgp_Deg_Rv_5
9	HymPgp_Deg_Fw_4	HymPgp_Deg_Rv_11
10	HymPgp_Deg_Fw_4	C219_Epi2_BeePgp_RV
11	HymPgp_Deg_Fw_4	HymPgp_Deg_Rv_8
12	HymPgp_Deg_Fw_4	HymPgp_Deg_Rv_5
13	HymPgp_Deg_Fw_9	HymPgp_Deg_Rv_11
14	HymPgp_Deg_Fw_9	C219_Epi2_BeePgp_RV
15	HymPgp_Deg_Fw_9	HymPgp_Deg_Rv_8
16	HymPgp_Deg_Fw_9	HymPgp_Deg_Rv_5

Figure A3: Degenerate primer Test PCR gel. [A] 1% Agarose gel with 1x TAE Buffer and SYBR safe dye. Markers are 1kb standards from BioRad (Cat# 1708355EDU). Gel was run at 100V for 1 hour. **[B]** Table Showing primer pairs used in test PCR run with proofreading Phusion polymerase (cat# F530S) using the "Sascha Touch-Down" PCR program (see section 1.6).

1.3. Primer Pair optimization

Further investigation led me to believe that our 4kb gene was too big of an amplicon to clone all at once. From here I designed some gene specific internal primers (Table A3, figure A4) to clone the gene out in fragments. A few test PCRs showed that the largest fragment that I could

clone out as one time hovered around 1kb. After sending out these first few fragments for sequencing, I found that the fragments that I cloned out were very similar, if not identical, to the "predicted" gene sequence taken off NCBI. After the success of the first set of internal primers, more were designed to cover the rest of the gene and offer more flexibility with fragment size and location.

Table A3: Honeybee ABCB1 Primer inventory. Listed are all the gene-specific honeybee primers ordered. Primers include 5' and 3' primers for X1-X4 and X5-X7 variants and internal gene-specific primers.

Seq Name	Seq 5'-3'		Seq Name	Seq 5'-3'
GSP-bB1X1-4-FW17	ATGACACGGCGAATTTG		GSP-bB1X5-7-FW18	ATGCGCAATGAACACGGCG
GSP-bB1X1-4-FW19	ATGACACGGCGAATTTGTC		GSP-bB1X5-7-FW24	ATGCGCAATGAACACGGCGAATTTG
GSP-bB1X1-4-FW20	ATGACACGGCGAATTTGTCA		GSP-bB1X5-7-FW26	ATGCGCAATGAACACGGCGAATTTGTC
GSP-bB1X1-4-REV18	CTACTCCATCGCGGCCTC		GSP-bB1X5-7-REV18	CTACTCCATCGCGGCCTC
GSP-bB1X1-4-REV21	CTACTCCATCGCGGCCTCTTG		GSP-bB1X5-7-REV22	CTACTCCATCGCGGCCTCTTGG
GSP-bB1X1-4-REV22	CTACTCCATCGCGGCCTCTTGG		GSP-bB1X5-7-REV24	CTACTCCATCGCGGCCTCTTGGAG
BeePgp_3329_F	ACA TCT CCT CGG TCT CGT		BeePgp_1167_R	CTCCTTGCTCAAACTGTCG
BeePgp_35_F	CACAAACGAAGGAGGAGGAG		BeePgp_1408_F	GAAGCTGAACGTGCAGTG
BeePgp_2870_F	CGTTCTTCGGTTACGCTTTG		BeePgp_1408_R	CACTGCACGTTCAGCTTC
BeePgp_2922_F	CGAGGGGTTGAATTATCAGG		BeePgp_1562_F	CGACTTCATCAGCAAACTGC
BeePgp_1893_F	CAAGCATTATTACGGATTGGTGT		BeePgp_1562_R	GCAGTTTGCTGATGAAGTCG
BeePgp_2376_F	GACCACGAGGATCAGGAAGA		BeePgp_2007_F	AATTCTCCACTCTGTCGATG
BeePgp_27_F	GTGCCACCACAAACGAAGG		BeePgp_2007_R	CATCGACAGAGTGGAGAATT
BeePgp_3699_R	CCTTCCATCGCTTTGTCC		BeePgp_2670_F	GCAGGAGAAGAAGAAGATGG
BeePgp_186_R	GAGATGGGGATGCACAGG		BeePgp_2670_R	CCATCTTCTTCTTCTCCTGC
BeePgp_1243_R	TCTGGCCGGATACTGGAA		BeePgp_3011_F	CCAATTTCAACACCGCCAAG
BeePgp_3725_R	TGAGCTATGGTGATGCAGGT		BeePgp_3011_R	CTTGGCGGTGTTGAAATTGG
BeePgp_235_R	ATGTTTCGATCCACCAGCA		BeePgp_3433_F	CCGATGGACGAGATTATAGA
BeePgp_2252_R	AAGACCCAGCACGTAGTAAACC		BeePgp_3433_R	TCTATAATCTCGTCCATCGG
BeePgp_2246_R	CCCAGCACGTAGTAAACCTC		BeePgp_35_R	CTCCTCCTCCTTCGTTTGTG
BeePgp_3842_R	GTGCAGATGGGCGTAGAGG		BeePgp_186_F	CCTGTGCATCCCCATCTC
BeePgp_-88_F	CAAACTCTCACCGCCAGG		BeePgp_235_F	TGCTGGTGGATCGAAACAT
BeePgp_-88_R	CCTGGCGGTGAGAGTTTG		BeePgp_1243_F	TTCCAGTATCCGGCCAGA
BeePgp_439_F	AGACAAATCGTTCGGGTACG		BeePgp_1893_R	ACACCAATCCGTAATAATGCTTG
BeePgp_439_R	CGTACCCGAACGATTTGTCT		BeePgp_2252_F	GGTTTACTACGTGCTGGGTCTT
BeePgp_637_F	GTCTACGGATGGAAGCTG		BeePgp_2376_R	TCTTCCTGATCCTCGTGGTC
BeePgp_637_R	CAGCTTCCATCCGTAGAC		BeePgp_2870_R	CAAAGCGTAACCGAAGAACG
BeePgp_828_F	GAAGGAGGTGAACAGATACG		BeePgp_2922_R	CCTGATAATTCAACCCCTCG

BeePgp_828_R	CGTATCTGTTCACCTCCTTC		BeePgp_3329_R	ACGAGACCGAGGAGATGT
BeePgp_1022_F	GCTGGTGATCGTGTTCTTC		BeePgp_3699_F	GGACAAAGCGATGGAAGG
BeePgp_1022_R	GAAGAACACGATCACCAGC		BeePgp_3725_F	ACCTGCATCACCATAGCTCA
BeePgp_1167_F	CGACAGTTTGAGCAAGGAG		BeePgp_3842_F	CCTCTACGCCCATCTGCAC

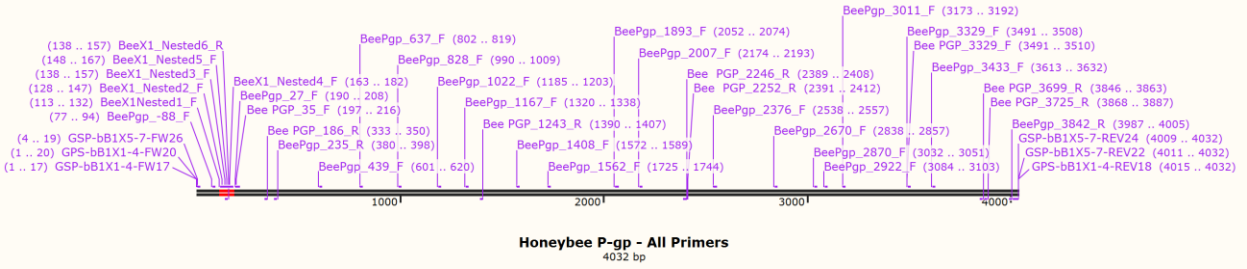


Figure A4: Gene-specific primers aligned to *Am*-ABCB1. Shown are primers from Table A3 aligned to the *Am*-ABCB1 coding sequence. Only one forward or reverse primer is shown for each internal GSP sequence but both forward and reverse primers were ordered. The red portion near the 3' end of the gene is the alternative splicing region only seen in the X1-X4 gene variants.

1.4. Honeybee Housekeeping Gene Primers

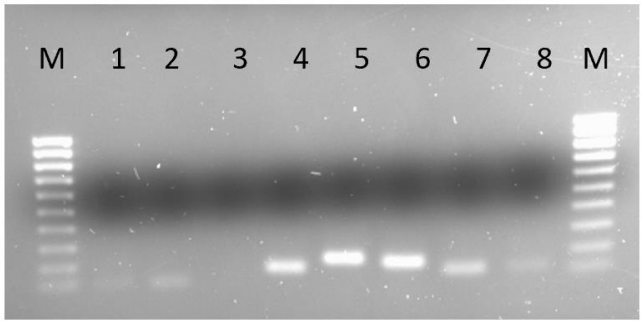
I also ordered primers for honeybee housekeeping genes (Table A4) to use as controls for PCR reactions. These primers were pulled from literature (see legend in Table A4), so they all worked well (Figure A5), except for the two I designed myself for Melittin, MeltA. The ones I designed for Melittin might not have worked because they spanned the signaling sequence and this was probably cleaved so the primers could not bind (Figure A6).

Table A4: Honeybee Housekeeping Gene Primers. Shown are housekeeping genes for honeybees and the primers designed for each gene. These primers, aside from MeltA, were pulled directly from published articles (Lourenço et al. 2008; Moon, Lee, and Kim 2018; Park et al. 2014; Scharlaken et al. 2008).

Gene	Name	Sequence
TATA-Box Binding Protein	Tbp_F	TTGGCAGCAAGAAAGTATGC
TATA-Box Binding Protein	Tbp_R	TCACATCACAGCTGCCTACC
Melittin	Melt_F	GGAATTGGAGCAGTTCTGAAGG
Melittin	Melt_R	TAACCCTGTTGCCTCTTACG
Melittin	MeltA_F	GGTCGTGTACATTTCTTAC
Melittin	MeltA_R	GCCTCTTACGTTTAATCC
Actin	Act_F	TGCCAACACTGTCCTTTCTG
Actin	Act_R	AGAATTGACCCACCAATCCA
GAPDH	Gapdh_F1	GATGCACCCATGTTTGTTTG
GAPDH	Gapdh_R1	TTTGCAGAAGGTGCATCAAC
GAPDH	Gapdh_F2	CACCTTCTGCAAAATTATGGCG
GAPDH	Gapdh_R2	ACCTTTGCCAAGTCTAACTGTTAA
Ribosomal Protein S18	Rps18_F	GATTCCCGATTGGTTTTTGAATAG
Ribosomal Protein S18	Rps18_R	AACCCCAATAATGACGCAAACC
TATA-Box Binding Protein Associated Factor 10	Taf10_F	TTGGTTTCATTAGCTGCACAA

TATA-Box Binding Protein Associated Factor 10	Taf10_R	ACTGCGGGAGTCAAATCTTC

A



B

	Forward	Reverse
1	Tbp_F	Tbp_R
2	Melt_F	Melt_R
3	MeltA_F	MeltA_R
4	Act_F	Act_R
5	Gapdh_F1	Gapdh_R1
6	Gapdh_F2	Gapdh_R2
7	Rps18_F	Rps18_R
8	Taf10_F	Taf10_R

Figure A5: Housekeeping gene test PCR gel. [A] 1% Agarose gel with 1x TAE Buffer and SYBR safe dye. Markers are 100bp BioRad Marker (Cat# 1708352EDU). Gel was run at 100V for 30 minutes. **[B]** Table showing the primers used for the PCR Reaction run with proofreading Phusion polymerase (cat# F530S) using the "Sascha Touch-Down" PCR program (see section 1.6).

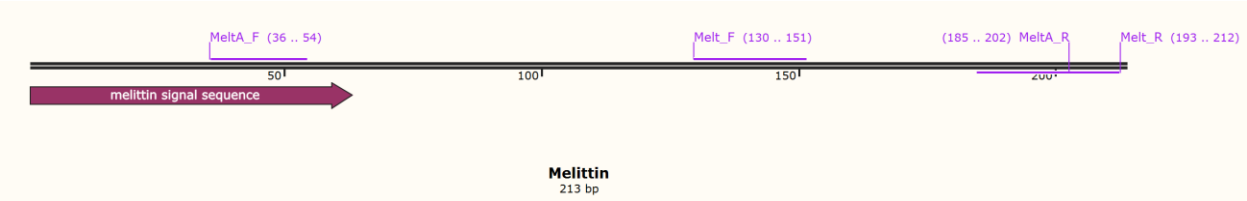
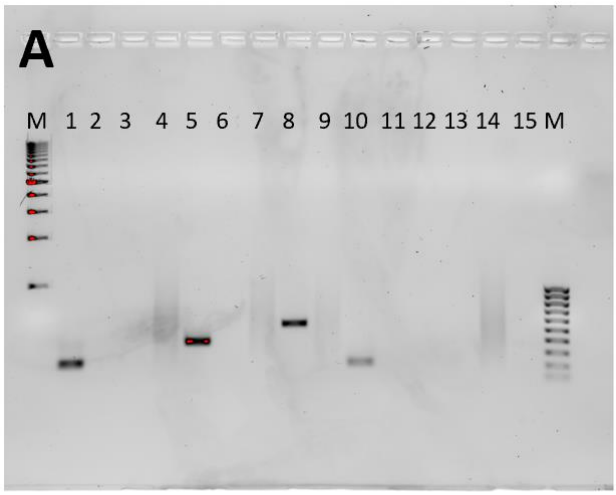


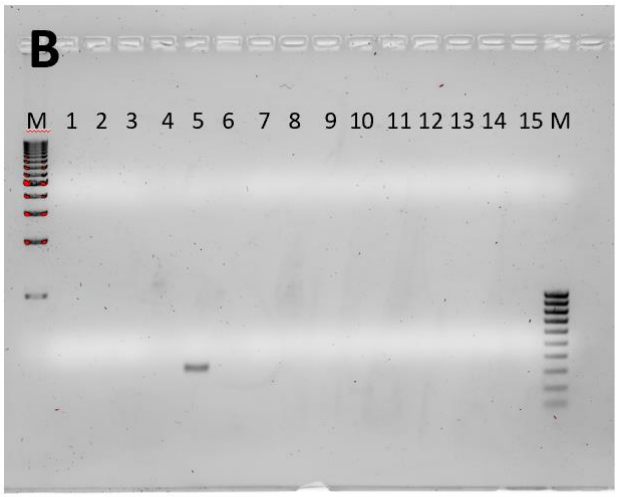
Figure A6: Melittin gene sequence aligned with the melittin primers from Table A4. MeltA_F anneals to the melittin signaling sequence that might be cleaved off from the mRNA/cDNA. This might explain why the MeltA primer pair might not have produced any fragments (see Figure A5).

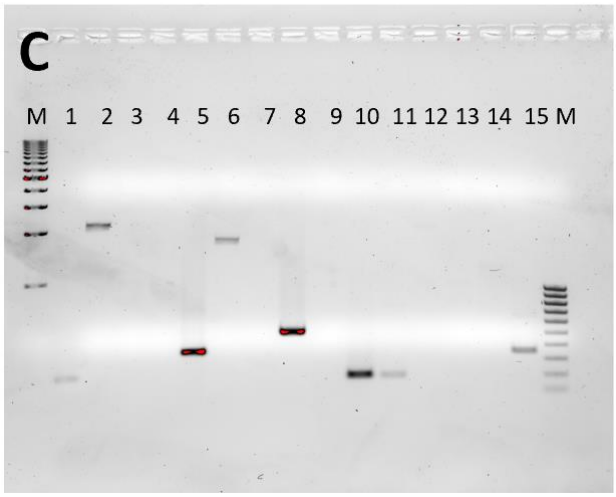
1.5. Full-length Honeybee ABCB1 Cloning: PCR Optimization

Partway through the cloning process, the PCR started to lose efficiency. I tested several different variables to determine the optimal PCR protocol. I changed the polymerase, the PCR program, and I optimized primer pairs *in silico* using Primer3Plus to pick primers and Beacon Designer to predict dimers (See section 3.4).

The polymerase I was working with at the beginning, Phusion polymerase from Thermo, started to fail. At first, I thought it was just a bad batch, so I opened a new box and tried again. This also did not yield any results, so I decided to order other polymerases to test (see results in figure A7, A8).







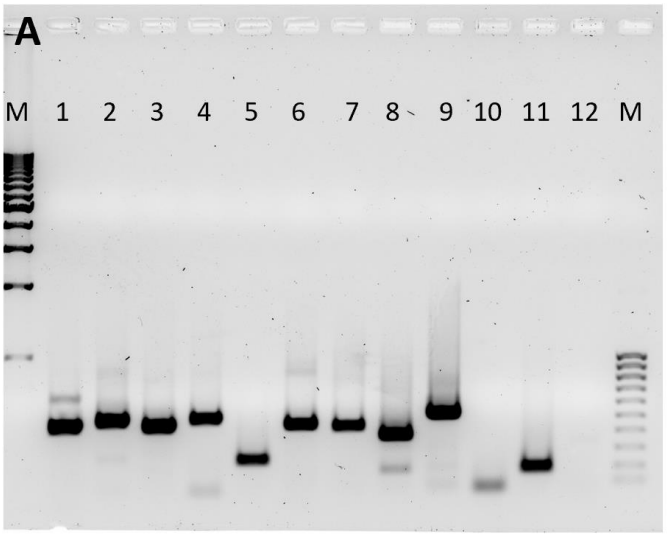
D

	Forward	Reverse
1	BeePgp_35_F	BeePgp_235_R
2	BeePgp_35_F	BeePgp_2252_R
3	BeePgp_35_F	BeePgp_3699_R
4	BeePgp_35_F	Poly dT
5	BeePgp_1893_F	BeePgp_2252_R
6	BeePgp_1893_F	BeePgp_3699_R
7	BeePgp_1893_F	Poly dT
8	BeePgp_3329_F	BeePgp_3842_R
9	BeePgp_3329_F	Poly dT
10	GAPDH_F1	GAPDH_R1
11	BeePgp_35_F	BeePgp_235_R
12	BeePgp_35_F	BeePgp_2252_R
13	BeePgp_35_F	BeePgp_3699_R
14	BeePgp_35_F	Poly dT
15	BeePgp_1893_F	BeePgp_2252_R

Figure A7: Test PCR using three different DNA polymerases. [A] GoTaq Green from Promega (Cat# EM7122) **[B]** Phusion polymerase from Thermo (cat# F530S). **[C]** Phusion Hot Start from Thermo (Cat# F549S). All polymerases were tested with the same cDNA and primers. Each polymerase had a different thermocycler program that followed the protocol included with each polymerase. See section 1.6 for thermocycler programs. GoTaq polymerase used the "GoTaq" program and both Phusion polymerases used the "Sascha Touch-Down" program. **[D]** Table showing primer pairs for each reaction. All gels are 1% Agarose gel with 1x TAE Buffer and SYBR safe dye. Markers are 1kb and 100bp BioRad Marker (Cat# 1708355EDU Cat# 1708352EDU). Gel was run at 100V for 1 hour.

The Phusion polymerase I had been using was not performing well. The Taq polymerase performed much better; however, Taq polymerase is not proofreading so I could not use it to clone fragments for sequencing. The Taq could unknowingly insert mutations at a much higher rate than proofreading enzymes. The Phusion Hot Start performed very well with the test PCR. However, it is one of the more expensive enzymes and would not be a sustainable choice since I would be

doing a lot of PCRs to fully clone out ABCB1. From here, I ordered some free samples of Q5 polymerase from NEB. Q5 is proofreading and is cheaper than the Phusion Hot Start. The test PCR using Q5 showed very nice bands and solidified my choice of polymerase.



B	Forward	Reverse
1	BeePgp_1562_F	BeePgp_235_R
2	BeePgp_1893_F	BeePgp_2252_R
3	BeePgp_2252_F	BeePgp_3699_R
4	BeePgp_2376_F	Poly dT
5	BeePgp_2670_F	BeePgp_2252_R
6	BeePgp_2870_F	BeePgp_3699_R
7	BeePgp_3011_F	Poly dT
8	BeePgp_3329_F	BeePgp_3842_R
9	BeePgp_3329_F	Poly dT
10	Melt_F	Melt_R
11	GAPDH_F1	GAPDH_R1
12	GSP-bC1X1-4-FW20	GSP-bC1X1-4-REV21

Figure A8: PCR using Q5 polymerase from NEB. [A] 1% Agarose gel with 1x TAE Buffer and SYBR safe dye. Markers are 1kb and 100bp BioRad Marker (Cat# 1708355EDU Cat# 1708352EDU). Gel was run at 100V for 1 hour. **[B]** Table showing primer pairs for each PCR reaction run with proofreading Q5 polymerase (M0491S) and the same cDNA but different thermocycler program and primer pairs than the previous test from Figure A7. Thermocycler program was pulled from the Q5 product insert (See Section 1.6 for Q5 program).

1.6. Thermocycler Programs and Annealing Temperatures

When I was having trouble with the PCR, Dr. Rice suggested I play with the annealing temperature to see if that helps. Lowering the annealing temperature increasing primer binding but it can also lead to higher instances of non-specific primer binding. Having a higher annealing temperature leads to more specific binding but lower binding all together. Touch-Down PCR lowers the annealing temperature every cycle, so you get both high temperature specificity and low temperature binding potential. Touch-Down is especially good if you are working with a bunch of primer pairs that have many different melting temperatures.

Sometimes I like to use the NEB Tm calculator (https://tmcalculator.neb.com/#!/main) to determine a good single annealing temperature in maximize fragment amplification. If you use a Touch-Down, the primers will not start to anneal until the temperature lowers to the correct level. This leads to fewer cycles that could amplify that fragment.

All the polymerases I used came in kits with protocol inserts. For the most part, I use the set programs that come with each polymerase, but I change the annealing temperature to optimize primer binding or change it to Auto-Delta to make it Touch-Down. See Figure A9 for thermocycler programs that I used.

A

Sascha Touch-Down		
Temp	**Time**	**Cycle**
98°C	00:45	1x
94°C	00:15	30x
70°C (Auto-delta, -0.5/cycle)	00:30	
72°C	00:30	
72°C	05:00	1x
4°C	∞	1x

B

Q5 Polymerase		
Temp	**Time**	**Cycles**
98°C	00:30	1x
98°C	00:05	30x
50°C (Variable)	00:10	
72°C	00:30	
72°C	02:00	1x
4°C	∞	1x

C

GoTaq Green		
Temp	**Time**	**Cycle**
95°C	02:00	1x
95°C	00:30	30x
50°C (Variable)	00:30	
72°C	01:00	
72°C	05:00	1x
4°C	∞	1x

D

RACE Touch-Down		
Temp	**Time**	**Cycle**
98°C	00:30	1x
94°C	00:30	30x
70°C (Auto-delta, -0.5/cycle)	00:30	
72°C	04:00	
72°C	05:00	1x
4°C	∞	1x

Figure A9: PCR thermocycler programs for general Touch-Down, Q5 polymerase, GoTaq Green polymerase, and RACE Touch-Down. Each program lists the temperature and time for each step and the number of cycles for each step. For the Thermo Scientific ProFlex PCR system, you can set the final step to hold the samples at 4ºC until you are ready to retrieve it.

1.7. RACE: Common 5' UTR between all the Bees

I was having quite a bit of trouble cloning the 5' end of the bee genes so we decided to use a RACE kit for Takara. RACE-ready cDNA was made from both nurse and forager bee RNA extracted earlier. Although Sascha had success cloning the Tuna ABCB1 end-to-end using RACE-ready cDNA, the honeybees were more stubborn. I could not get the bees to clone end-to-end using just the RACE-ready cDNA. I was, however, able to clone out the 5' ends of both ABCB1 and ABCC1. This allowed us to finish our cloned *Am*-ABCB1 and C1 sequences.

A few things we learned from the RACE PCR is that a single honeybee expresses both isoforms of ABCB1. Further analysis of the sequences show that the specific gene variants of our bees are X1 and X6. Sequencing analysis also showed that an 18-base sequence of ABCB1 5' UTR is common between many if not all bee species, 5'-AGTTGTTAATTAAGAATG-3'.

1.8. Non-Target Genes Cloned

When cloning the bee genes, I sometimes got multiple bands from the same reaction (see Figure A8). I sent the multiple bands out for sequencing to see what the extra fragements were. In some cases, the extra bands were different isoforms of the same gene, like X1 and X6 variants of ABCB1. In other cases, the extra bands cloned out non-target gene sequences like honeybee myosin (XM_026442162), an uncharacterized honeybee protein like Synaptotagmin and Neuromodulin (XM_006558545), and several bacterial fragments. A lot of the non-target fragments that were sequenced were fragments from the TOPO plasmids or general microbial genes that could have come from bee microflora. There was even a fragment that showed *Hordeum vulgare* (barley), most likely from pollen stuck to the bees. The most interesting non-target microbe that I sequences was *Bombilactobaccilus bombi* (CP031513), a common bee gut microbe.

Some other interesting non-targets that I sequenced were fragments of ABCB1 from other bee species like *Apis cerana* (XM_017064674), *Colletes gigas* (XM_043397307), and *Nomia melanderi* (XM_031983745). Because I did not have samples for these bees, I assume that the fragments I cloned out are highly conserved between bees. There is also the possibility that my bee samples were contaminated by other bees, but that seems unlikely. With such high conservation, there is a possibility that some of the honeybee primers I designed could also be used to clone other bee species.

Table A5: BLAST results for non-target fragments sequenced from honeybee PCRs. This is a list of results from the CLC BLAST function. Query is the fragment I cloned and sequenced and Greatest Identity % describes the percent identity of the query to the subject that BLAST thinks is most similar. The results also list the accession number and name of the gene BLAST matched to the samples. All these sequencing results are in the CLC sync Box.com folder under Bees>Analyzed Data>Eurofins>Raw Data.

Query	Greatest identity %	Accession (identity %)	Description (identity %)
bPgpF35xR186x2R_M13R_BCGP35_27	89.47	CP053618	Achromobacter xylosoxidans strain GN008 chromosome, complete genome
bPgpF35xR186x4_M13F_BCGP37_29	97.65	OU342944	Andrena haemorrhoa genome assembly, chromosome: 5
bC1N10xRACEx604R_PREMIX_BQQR62_3	95.35	CP031513	Bombilactobacillus bombi strain BI-2.5 chromosome, complete genome
bPgpOF6x8R_PREMIX_BQQS82_30	88.37	CP027080	Bos mutus isolate yakQH1 chromosome 12
bPgpRACEPxF3x186R2_M13F_H07	78.29	LC519320	Botrytis cinerea 18-053 HSP60 gene for heat shock protein 60, partial sequence
bPgpF3329xR3725x3_M13F_BCGP12_4	98.73	LN864495	Campylobacter jejuni partial 16S rRNA gene, strain MTG14
bPgpF35xR186x5_M13F_BCGP38_30	100.00	LN864495	Campylobacter jejuni partial 16S rRNA gene, strain MTG14
bPgpF35xR235x2_M13F_BCGP47_39	100.00	KX036765	Cloning vector pXF20pemIK-GW, complete sequence
bPgpxN10x2922Fx3433RxF_PREMIX_G11	86.05	LR778285	Coregonus sp. 'balchen' genome assembly, chromosome: 33
bC1N10xRACEx387R_PREMIX_BQQR60_5	82.35	NM_176363	Drosophila melanogaster wallenda (wnd), transcript variant C, mRNA
BPGPF35xR1243Rv_PREMIX_BCGQ00_1	75.55	CP034522	Eukaryotic synthetic construct chromosome 19
bPgpF3329xR3842x3_M13F_BCGP27_19	100.00	EU919404	Himar1-delivery and mutagenesis vector pHBurk5, complete sequence
bPgpF35xR1243xH2_M13F_BCGP40_32	100.00	EU919404	Himar1-delivery and mutagenesis vector pHBurk5, complete sequence
bPgpF35xR1243xH3_M13F_BCGP41_33	100.00	EU919404	Himar1-delivery and mutagenesis vector pHBurk5, complete sequence
bPgpF35xR186x3_M13F_BCGP36_28	100.00	EU919404	Himar1-delivery and mutagenesis vector pHBurk5, complete sequence
bPgpF35xR235x3_M13F_BCGP48_40	100.00	EU919404	Himar1-delivery and mutagenesis vector pHBurk5, complete sequence
bPgpF3329xR2252x2R_M13R_BCGP14_6	100.00	AB219366	Hordeum vulgare HvPIP2;1 mRNA for PIP aquaporin, complete cds
bC1F3xRACEx604R_PREMIX_BQQT47_10	96.97	AB167744	Numida meleagris hspa8 mRNA for heat shock protein, complete cds
bC1RACExF3x387RA_PREMIX_A08	72.92	XM_017064676	PREDICTED: Apis cerana multidrug resistance protein homolog 49 (LOC108002801), transcript variant X7, mRNA
bPgp1167Fx1408RxFA_PREMIX_C01	100.00	XM_026442162	PREDICTED: Apis mellifera myosin heavy chain, muscle (LOC409843), transcript variant X34, mRNA
bPgp1167Fx1408RxRA_PREMIX_D01	98.66	XM_026442162	PREDICTED: Apis mellifera myosin heavy chain, muscle (LOC409843), transcript variant X34, mRNA
bPgpRACExF3x88D_PREMIX_B09	99.70	XM_016915947	PREDICTED: Apis mellifera uncharacterized LOC100577515 (LOC100577515), mRNA
bPgp2870Fx3329RxF_PREMIX_E03	84.31	XM_043397307	PREDICTED: Colletes gigas multidrug resistance protein homolog 49 (LOC122397857), mRNA
bC1x105Fx742RxR_PREMIX_D08	85.11	XM_023175508	PREDICTED: Drosophila willistoni neurobeachin (LOC6638028), mRNA
bC1N10xRACEx387R_PREMIX_BQQR63_2	82.69	XM_015577048	PREDICTED: Dufourea novaeangliae multidrug resistance protein homolog 49 (LOC107188703), mRNA
bPgpOF5x7R_PREMIX_BQQS64_12	77.53	XM_015577048	PREDICTED: Dufourea novaeangliae multidrug resistance protein homolog 49 (LOC107188703), mRNA
bPgpRACExF3x35D_PREMIX_D09	100.00	XM_031983745	PREDICTED: Nomia melanderi multidrug resistance protein homolog 49 (LOC116430079), transcript variant X4, mRNA
bPgpRACExN10x35C_PREMIX_D08	100.00	XM_031983745	PREDICTED: Nomia melanderi multidrug resistance protein homolog 49 (LOC116430079), transcript variant X4, mRNA
bC1F3xRACEx387R_PREMIX_BQQT48_9	89.37	XM_024505106	PREDICTED: Physcomitrella patens uncharacterized LOC112275090 (LOC112275090), transcript variant X2, mRNA
bC1N10xRACEx105R_PREMIX_BQQR64_1	89.97	XM_024505106	PREDICTED: Physcomitrella patens uncharacterized LOC112275090 (LOC112275090), transcript variant X2, mRNA
bPgpF1893xR3399x66H1xF2376_PREMIX_BCGP89_41	95.92	XM_039449844	PREDICTED: Solenopsis invicta multidrug resistance protein homolog 49 (LOC105195263), transcript variant X7, mRNA
bPgpF3329xGSPR21xD_M13F_BCGP77_29	94.74	MN212799	Spodoptera frugiperda clone 10 transposon piggyBac, complete sequence
bPgpF1893xR3399xC1_M13F_BBHX77_52	100.00	MN212801	Spodoptera frugiperda clone 12 transposon piggyBac, complete sequence
bPgpF35xR1243xL1_M13F_BCGP45_37	97.27	KY217990	Tetrahymena borealis isolate 20771-1 cytochrome oxidase subunit 1 (cox1) gene, partial cds; mitochondrial
bPgpF35xR1243xL3_M13F_BCGP46_38	89.20	KY217992	Tetrahymena borealis isolate 20956-1 cytochrome oxidase subunit 1 (cox1) gene, partial cds; mitochondrial
bPgpF3329xR3842x6R_M13R_BCGP31_23	98.18	HG917720	Uncultured bacterium partial 16S rRNA gene, isolate MBR, T1, clone E10
bPgpF3329xR2252x5F_M13F_BCGP19_11	100.00	FR670376	Uncultured gamma proteobacterium partial 16S rRNA gene, clone LSmat.B29
bPgpF35xR186x1F_M13F_BCGP32_24	98.68	FR670376	Uncultured gamma proteobacterium partial 16S rRNA gene, clone LSmat.B29
bPgpF3329xR3842x6F_M13F_BCGP30_22	97.37	FR670386	Uncultured gamma proteobacterium partial 16S rRNA gene, clone LSmat.B39
bPgpF3329xR3842x1R_M13R_BCGP24_16	100.00	AM905372	Xanthomonas arboricola pv. juglandis partial integron InXaj76583, partial ilvD gene and intI pseudogene, strain DAR76583
bPgpF3329xR3842x5R_M13R_BCGP29_21	100.00	AM905372	Xanthomonas arboricola pv. juglandis partial integron InXaj76583, partial ilvD gene and intI pseudogene, strain DAR76583

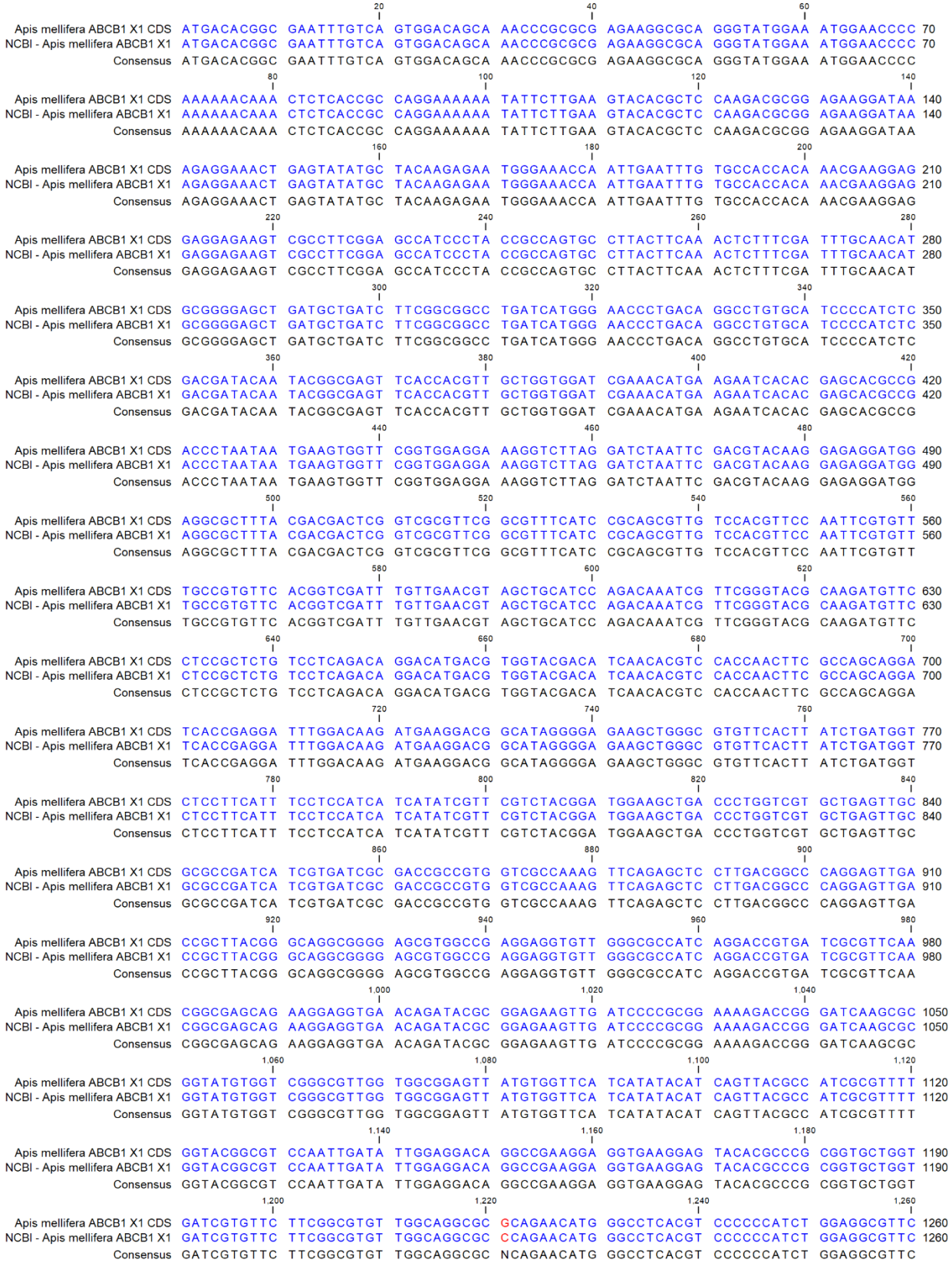
1.9. Consensus Sequences: Honeybee ABCB1 and ABCC1

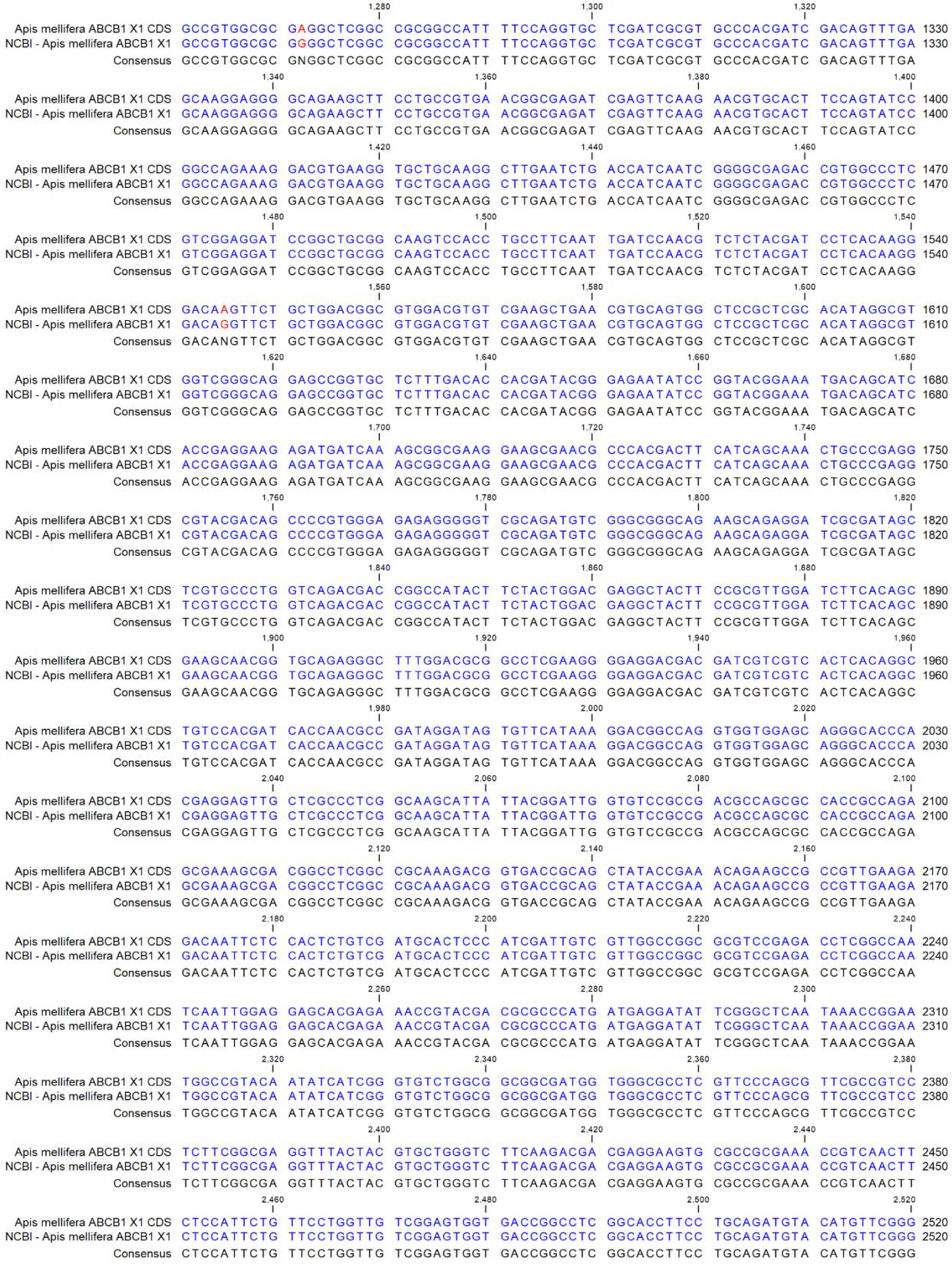
After countless PCRs returned several hundred sequencing fragments, I had to find a good software to concatenate all the fragments into one whole sequences. I tried U-Gene, which was too complicated and confusing. I tried DNA Dragon, which was helpful but was too expensive and only did one task. Eventually we settled on the CLC Main workbench from Qiagen. CLC had much more functionality for the price we paid.

From the CLC software, I was able to load in all my sequenced fragments and create a fully cloned sequence gene. There are a few single nucleotide polymorphisms (SNPs) in both ABCB1 isoforms (see Figures A10, A11), however, these seem to be silent mutations because the amino acid sequences are identical to the sequences pulled from NCBI. With the ABCC1 gene, there was just one silent SNP but there were also two fragments of the gene that were missing (see Figure A13).

Note: NCBI nomenclature list 7 different Am-ABCB1 gene sequences that code for two different protein isoforms. X1-4 code for protein isoform X1. Gene variants X5-X7 code for protein isoform X2. Following this nomenclature and the results of the RACE sequencing, Figures A11-A12 show the cloned gene variants X1 and X6 that were translated into the amino acid sequences for the protein isoforms X1 and X2 (Figure A12).

Apis mellifera ABCB1 X1

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NCBI - Apis mellifera ABCB1 X1 GCCGTGGCGC GGGGCTCGGC CGCGGCCATT TTCCAGGTGC TCGATCGCGT GCCCACGATC GACAGTTTGA 1330
Consensus GCCGTGGCGC GNGGCTCGGC CGCGGCCATT TTCCAGGTGC TCGATCGCGT GCCCACGATC GACAGTTTGA
1,340 1,360 1,380 1,400
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NCBI - Apis mellifera ABCB1 X1 GCAAGGAGGG GCAGAAGCTT CCTGCCGTGA ACGGCGAGAT CGAGTTCAAG AACGTGCACT TCCAGTATCC 1400
Consensus GCAAGGAGGG GCAGAAGCTT CCTGCCGTGA ACGGCGAGAT CGAGTTCAAG AACGTGCACT TCCAGTATCC
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1,480 1,500 1,520 1,540
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NCBI - Apis mellifera ABCB1 X1 GTCGGAGGAT CCGGCTGCGG CAAGTCCACC TGCCTTCAAT TGATCCAACG TCTCTACGAT CCTCACAAGG 1540
Consensus GTCGGAGGAT CCGGCTGCGG CAAGTCCACC TGCCTTCAAT TGATCCAACG TCTCTACGAT CCTCACAAGG
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NCBI - Apis mellifera ABCB1 X1 GACAGGTTCT GCTGGACGGC GTGGACGTGT CGAAGCTGAA CGTGCAGTGG CTCCGCTCGC ACATAGGCGT 1610
Consensus GACANGTTCT GCTGGACGGC GTGGACGTGT CGAAGCTGAA CGTGCAGTGG CTCCGCTCGC ACATAGGCGT
1,620 1,640 1,660 1,680
Apis mellifera ABCB1 X1 CDS GGTCGGGCAG GAGCCGGTGC TCTTTGACAC CACGATACGG GAGAATATCC GGTACGGAAA TGACAGCATC 1680
NCBI - Apis mellifera ABCB1 X1 GGTCGGGCAG GAGCCGGTGC TCTTTGACAC CACGATACGG GAGAATATCC GGTACGGAAA TGACAGCATC 1680
Consensus GGTCGGGCAG GAGCCGGTGC TCTTTGACAC CACGATACGG GAGAATATCC GGTACGGAAA TGACAGCATC
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Consensus ACCGAGGAAG AGATGATCAA AGCGGCGAAG GAAGCGAACG CCCACGACTT CATCAGCAAA CTGCCCGAGG
1,760 1,780 1,800 1,820
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NCBI - Apis mellifera ABCB1 X1 CGTACGACAG CCCCGTGGGA GAGAGGGGGT CGCAGATGTC GGGCGGGCAG AAGCAGAGGA TCGCGATAGC 1820
Consensus CGTACGACAG CCCCGTGGGA GAGAGGGGGT CGCAGATGTC GGGCGGGCAG AAGCAGAGGA TCGCGATAGC
1,840 1,860 1,880
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NCBI - Apis mellifera ABCB1 X1 TCGTGCCCTG GTCAGACGAC CGGCCATACT TCTACTGGAC GAGGCTACTT CCGCGTTGGA TCTTCACAGC 1890
Consensus TCGTGCCCTG GTCAGACGAC CGGCCATACT TCTACTGGAC GAGGCTACTT CCGCGTTGGA TCTTCACAGC
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NCBI - Apis mellifera ABCB1 X1 TGTCCACGAT CACCAACGCC GATAGGATAG TGTTCATAAA GGACGGCCAG GTGGTGGAGC AGGGCACCCA 2030
Consensus TGTCCACGAT CACCAACGCC GATAGGATAG TGTTCATAAA GGACGGCCAG GTGGTGGAGC AGGGCACCCA
2,040 2,060 2,080 2,100
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Consensus CGAGGAGTTG CTCGCCCTCG GCAAGCATTA TTACGGATTG GTGTCCGCCG ACGCCAGCGC CACCGCCAGA
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2,180 2,200 2,220 2,240
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NCBI - Apis mellifera ABCB1 X1 TCAATTGGAG GAGCACGAGA AACCGTACGA CGCGCCCATG ATGAGGATAT TCGGGCTCAA TAAACCGGAA 2310
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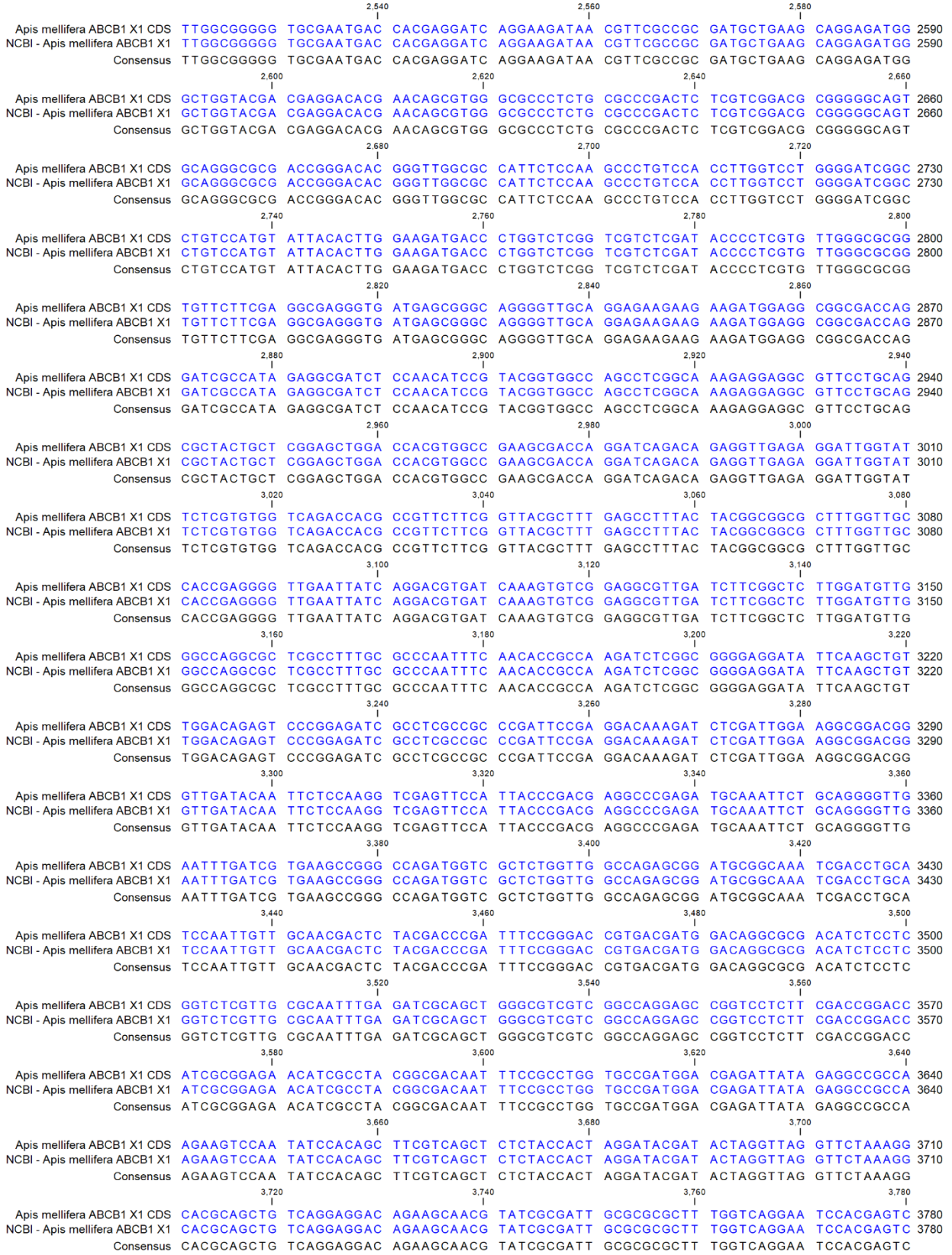
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Consensus TTGGCGGGGG TGCGAATGAC CACGAGGATC AGGAAGATAA CGTTCGCCGC GATGCTGAAG CAGGAGATGG
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NCBI - Apis mellifera ABCB1 X1 TGGACAGAGT CCCGGAGATC GCCTCGCCGC CCGATTCCGA GGACAAAGAT CTCGATTGGA AGGCGGACGG 3290
Consensus TGGACAGAGT CCCGGAGATC GCCTCGCCGC CCGATTCCGA GGACAAAGAT CTCGATTGGA AGGCGGACGG
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Consensus GTTGATACAA TTCTCCAAGG TCGAGTTCCA TTACCCGACG AGGCCCGAGA TGCAAATTCT GCAGGGGTTG
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NCBI - Apis mellifera ABCB1 X1 AATTTGATCG TGAAGCCGGG CCAGATGGTC GCTCTGGTTG GCCAGAGCGG ATGCGGCAAA TCGACCTGCA 3430
Consensus AATTTGATCG TGAAGCCGGG CCAGATGGTC GCTCTGGTTG GCCAGAGCGG ATGCGGCAAA TCGACCTGCA
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Consensus TCCAATTGTT GCAACGACTC TACGACCCGA TTTCCGGGAC CGTGACGATG GACAGGCGCG ACATCTCCTC
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NCBI - Apis mellifera ABCB1 X1 GGTCTCGTTG CGCAATTTGA GATCGCAGCT GGGCGTCGTC GGCCAGGAGC CGGTCCTCTT CGACCGGACC 3570
Consensus GGTCTCGTTG CGCAATTTGA GATCGCAGCT GGGCGTCGTC GGCCAGGAGC CGGTCCTCTT CGACCGGACC
3,580 3,600 3,620 3,640
Apis mellifera ABCB1 X1 CDS ATCGCGGAGA ACATCGCCTA CGGCGACAAT TTCCGCCTGG TGCCGATGGA CGAGATTATA GAGGCCGCCA 3640
NCBI - Apis mellifera ABCB1 X1 ATCGCGGAGA ACATCGCCTA CGGCGACAAT TTCCGCCTGG TGCCGATGGA CGAGATTATA GAGGCCGCCA 3640
Consensus ATCGCGGAGA ACATCGCCTA CGGCGACAAT TTCCGCCTGG TGCCGATGGA CGAGATTATA GAGGCCGCCA
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Consensus AGAAGTCCAA TATCCACAGC TTCGTCAGCT CTCTACCACT AGGATACGAT ACTAGGTTAG GTTCTAAAGG
3,720 3,740 3,760 3,780
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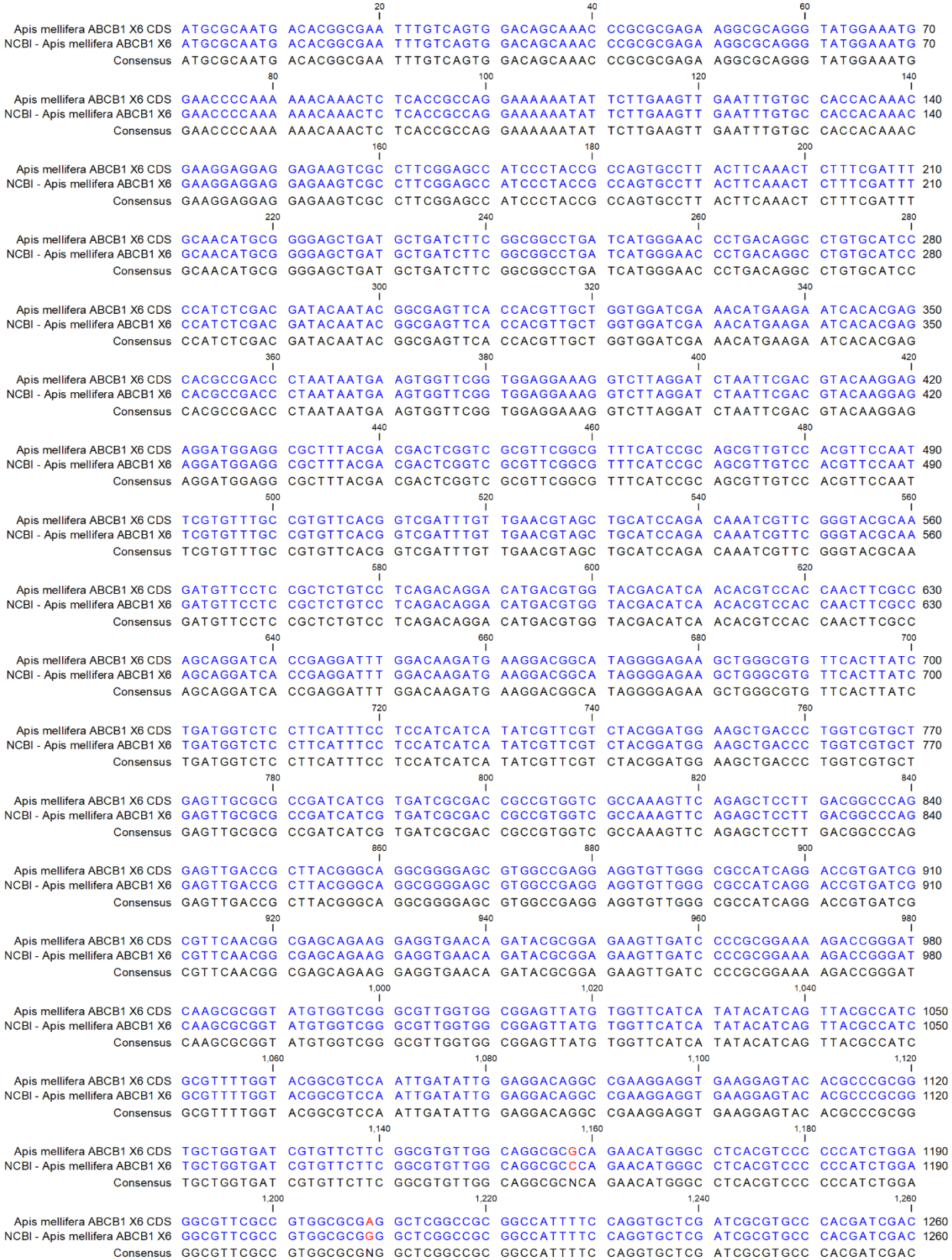
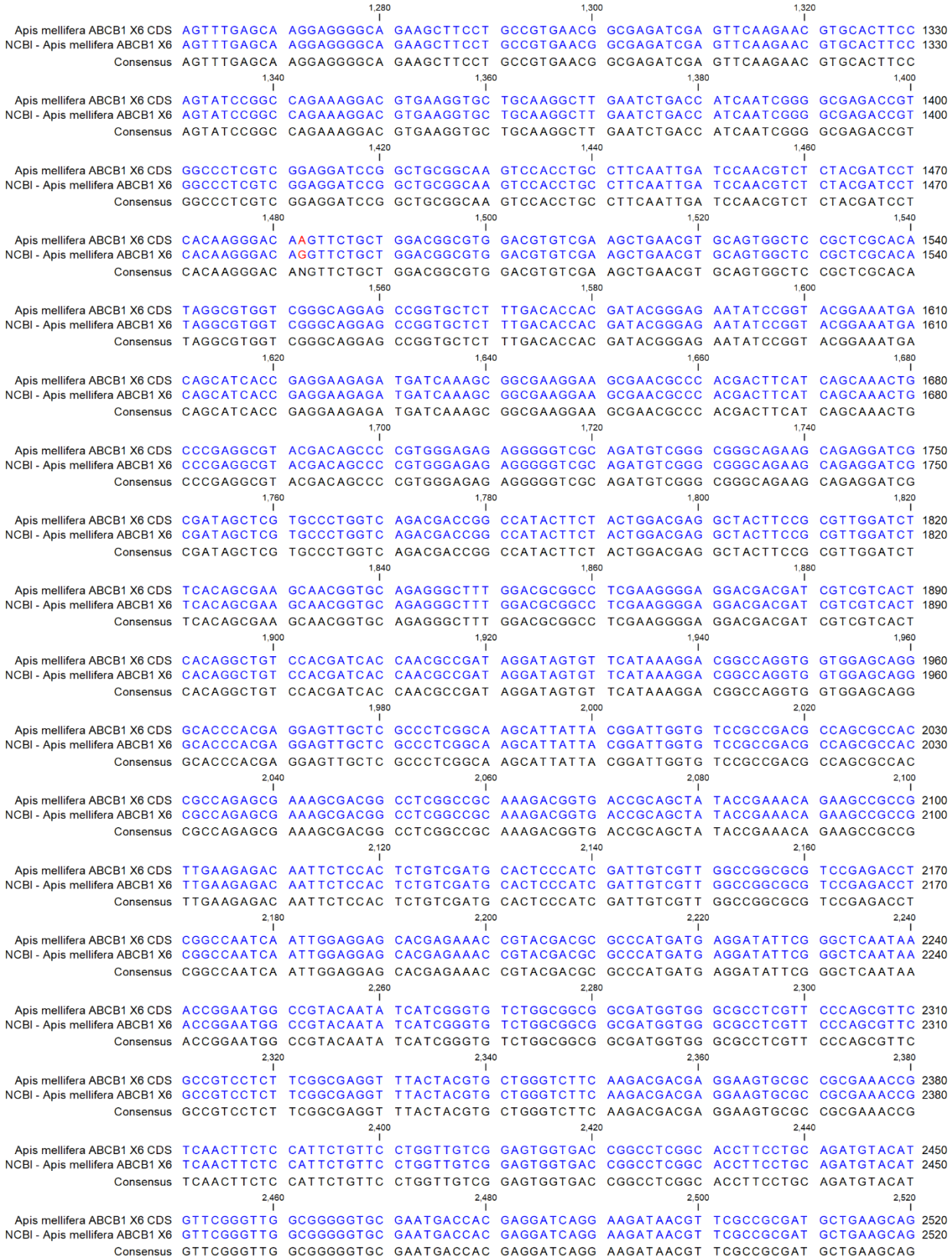
Figure A10: Alignment of fully cloned *Apis mellifera* ABCB1 X1 gene with *Apis mellifera* ABCB1 X1 gene pulled from NCBI. Both sequences are identical aside from three SNPs that do not result in any amino acid changes.

Apis mellifera ABCB1 X6

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NCBI - Apis mellifera ABCB1 X6 AGTTTGAGCA AGGAGGGGCA GAAGCTTCCT GCCGTGAACG GCGAGATCGA GTTCAAGAAC GTGCACTTCC 1330
Consensus AGTTTGAGCA AGGAGGGGCA GAAGCTTCCT GCCGTGAACG GCGAGATCGA GTTCAAGAAC GTGCACTTCC
1,340 1,360 1,380 1,400
Apis mellifera ABCB1 X6 CDS AGTATCCGGC CAGAAAGGAC GTGAAGGTGC TGCAAGGCTT GAATCTGACC ATCAATCGGG GCGAGACCGT 1400
NCBI - Apis mellifera ABCB1 X6 AGTATCCGGC CAGAAAGGAC GTGAAGGTGC TGCAAGGCTT GAATCTGACC ATCAATCGGG GCGAGACCGT 1400
Consensus AGTATCCGGC CAGAAAGGAC GTGAAGGTGC TGCAAGGCTT GAATCTGACC ATCAATCGGG GCGAGACCGT
1,420 1,440 1,460
Apis mellifera ABCB1 X6 CDS GGCCCTCGTC GGAGGATCCG GCTGCGGCAA GTCCACCTGC CTTCAATTGA TCCAACGTCT CTACGATCCT 1470
NCBI - Apis mellifera ABCB1 X6 GGCCCTCGTC GGAGGATCCG GCTGCGGCAA GTCCACCTGC CTTCAATTGA TCCAACGTCT CTACGATCCT 1470
Consensus GGCCCTCGTC GGAGGATCCG GCTGCGGCAA GTCCACCTGC CTTCAATTGA TCCAACGTCT CTACGATCCT
1,480 1,500 1,520 1,540
Apis mellifera ABCB1 X6 CDS CACAAGGGAC AAGTTCTGCT GGACGGCGTG GACGTGTCGA AGCTGAACGT GCAGTGGCTC CGCTCGCACA 1540
NCBI - Apis mellifera ABCB1 X6 CACAAGGGAC AGGTTCTGCT GGACGGCGTG GACGTGTCGA AGCTGAACGT GCAGTGGCTC CGCTCGCACA 1540
Consensus CACAAGGGAC ANGTTCTGCT GGACGGCGTG GACGTGTCGA AGCTGAACGT GCAGTGGCTC CGCTCGCACA
1,560 1,580 1,600
Apis mellifera ABCB1 X6 CDS TAGGCGTGGT CGGGCAGGAG CCGGTGCTCT TTGACACCAC GATACGGGAG AATATCCGGT ACGGAAATGA 1610
NCBI - Apis mellifera ABCB1 X6 TAGGCGTGGT CGGGCAGGAG CCGGTGCTCT TTGACACCAC GATACGGGAG AATATCCGGT ACGGAAATGA 1610
Consensus TAGGCGTGGT CGGGCAGGAG CCGGTGCTCT TTGACACCAC GATACGGGAG AATATCCGGT ACGGAAATGA
1,620 1,640 1,660 1,680
Apis mellifera ABCB1 X6 CDS CAGCATCACC GAGGAAGAGA TGATCAAAGC GGCGAAGGAA GCGAACGCCC ACGACTTCAT CAGCAAACTG 1680
NCBI - Apis mellifera ABCB1 X6 CAGCATCACC GAGGAAGAGA TGATCAAAGC GGCGAAGGAA GCGAACGCCC ACGACTTCAT CAGCAAACTG 1680
Consensus CAGCATCACC GAGGAAGAGA TGATCAAAGC GGCGAAGGAA GCGAACGCCC ACGACTTCAT CAGCAAACTG
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Consensus CCCGAGGCGT ACGACAGCCC CGTGGGAGAG AGGGGGTCGC AGATGTCGGG CGGGCAGAAG CAGAGGATCG
1,760 1,780 1,800 1,820
Apis mellifera ABCB1 X6 CDS CGATAGCTCG TGCCCTGGTC AGACGACCGG CCATACTTCT ACTGGACGAG GCTACTTCCG CGTTGGATCT 1820
NCBI - Apis mellifera ABCB1 X6 CGATAGCTCG TGCCCTGGTC AGACGACCGG CCATACTTCT ACTGGACGAG GCTACTTCCG CGTTGGATCT 1820
Consensus CGATAGCTCG TGCCCTGGTC AGACGACCGG CCATACTTCT ACTGGACGAG GCTACTTCCG CGTTGGATCT
1,840 1,860 1,880
Apis mellifera ABCB1 X6 CDS TCACAGCGAA GCAACGGTGC AGAGGGCTTT GGACGCGGCC TCGAAGGGGA GGACGACGAT CGTCGTCACT 1890
NCBI - Apis mellifera ABCB1 X6 TCACAGCGAA GCAACGGTGC AGAGGGCTTT GGACGCGGCC TCGAAGGGGA GGACGACGAT CGTCGTCACT 1890
Consensus TCACAGCGAA GCAACGGTGC AGAGGGCTTT GGACGCGGCC TCGAAGGGGA GGACGACGAT CGTCGTCACT
1,900 1,920 1,940 1,960
Apis mellifera ABCB1 X6 CDS CACAGGCTGT CCACGATCAC CAACGCCGAT AGGATAGTGT TCATAAAGGA CGGCCAGGTG GTGGAGCAGG 1960
NCBI - Apis mellifera ABCB1 X6 CACAGGCTGT CCACGATCAC CAACGCCGAT AGGATAGTGT TCATAAAGGA CGGCCAGGTG GTGGAGCAGG 1960
Consensus CACAGGCTGT CCACGATCAC CAACGCCGAT AGGATAGTGT TCATAAAGGA CGGCCAGGTG GTGGAGCAGG
1,980 2,000 2,020
Apis mellifera ABCB1 X6 CDS GCACCCACGA GGAGTTGCTC GCCCTCGGCA AGCATTATTA CGGATTGGTG TCCGCCGACG CCAGCGCCAC 2030
NCBI - Apis mellifera ABCB1 X6 GCACCCACGA GGAGTTGCTC GCCCTCGGCA AGCATTATTA CGGATTGGTG TCCGCCGACG CCAGCGCCAC 2030
Consensus GCACCCACGA GGAGTTGCTC GCCCTCGGCA AGCATTATTA CGGATTGGTG TCCGCCGACG CCAGCGCCAC
2,040 2,060 2,080 2,100
Apis mellifera ABCB1 X6 CDS CGCCAGAGCG AAAGCGACGG CCTCGGCCGC AAAGACGGTG ACCGCAGCTA TACCGAAACA GAAGCCGCCG 2100
NCBI - Apis mellifera ABCB1 X6 CGCCAGAGCG AAAGCGACGG CCTCGGCCGC AAAGACGGTG ACCGCAGCTA TACCGAAACA GAAGCCGCCG 2100
Consensus CGCCAGAGCG AAAGCGACGG CCTCGGCCGC AAAGACGGTG ACCGCAGCTA TACCGAAACA GAAGCCGCCG
2,120 2,140 2,160
Apis mellifera ABCB1 X6 CDS TTGAAGAGAC AATTCTCCAC TCTGTCGATG CACTCCCATC GATTGTCGTT GGCCGGCGCG TCCGAGACCT 2170
NCBI - Apis mellifera ABCB1 X6 TTGAAGAGAC AATTCTCCAC TCTGTCGATG CACTCCCATC GATTGTCGTT GGCCGGCGCG TCCGAGACCT 2170
Consensus TTGAAGAGAC AATTCTCCAC TCTGTCGATG CACTCCCATC GATTGTCGTT GGCCGGCGCG TCCGAGACCT
2,180 2,200 2,220 2,240
Apis mellifera ABCB1 X6 CDS CGGCCAATCA ATTGGAGGAG CACGAGAAAC CGTACGACGC GCCCATGATG AGGATATTCG GGCTCAATAA 2240
NCBI - Apis mellifera ABCB1 X6 CGGCCAATCA ATTGGAGGAG CACGAGAAAC CGTACGACGC GCCCATGATG AGGATATTCG GGCTCAATAA 2240
Consensus CGGCCAATCA ATTGGAGGAG CACGAGAAAC CGTACGACGC GCCCATGATG AGGATATTCG GGCTCAATAA
2,260 2,280 2,300
Apis mellifera ABCB1 X6 CDS ACCGGAATGG CCGTACAATA TCATCGGGTG TCTGGCGGCG GCGATGGTGG GCGCCTCGTT CCCAGCGTTC 2310
NCBI - Apis mellifera ABCB1 X6 ACCGGAATGG CCGTACAATA TCATCGGGTG TCTGGCGGCG GCGATGGTGG GCGCCTCGTT CCCAGCGTTC 2310
Consensus ACCGGAATGG CCGTACAATA TCATCGGGTG TCTGGCGGCG GCGATGGTGG GCGCCTCGTT CCCAGCGTTC
2,320 2,340 2,360 2,380
Apis mellifera ABCB1 X6 CDS GCCGTCCTCT TCGGCGAGGT TTACTACGTG CTGGGTCTTC AAGACGACGA GGAAGTGCGC CGCGAAACCG 2380
NCBI - Apis mellifera ABCB1 X6 GCCGTCCTCT TCGGCGAGGT TTACTACGTG CTGGGTCTTC AAGACGACGA GGAAGTGCGC CGCGAAACCG 2380
Consensus GCCGTCCTCT TCGGCGAGGT TTACTACGTG CTGGGTCTTC AAGACGACGA GGAAGTGCGC CGCGAAACCG
2,400 2,420 2,440
Apis mellifera ABCB1 X6 CDS TCAACTTCTC CATTCTGTTC CTGGTTGTCG GAGTGGTGAC CGGCCTCGGC ACCTTCCTGC AGATGTACAT 2450
NCBI - Apis mellifera ABCB1 X6 TCAACTTCTC CATTCTGTTC CTGGTTGTCG GAGTGGTGAC CGGCCTCGGC ACCTTCCTGC AGATGTACAT 2450
Consensus TCAACTTCTC CATTCTGTTC CTGGTTGTCG GAGTGGTGAC CGGCCTCGGC ACCTTCCTGC AGATGTACAT
2,460 2,480 2,500 2,520
Apis mellifera ABCB1 X6 CDS GTTCGGGTTG GCGGGGGTGC GAATGACCAC GAGGATCAGG AAGATAACGT TCGCCGCGAT GCTGAAGCAG 2520
NCBI - Apis mellifera ABCB1 X6 GTTCGGGTTG GCGGGGGTGC GAATGACCAC GAGGATCAGG AAGATAACGT TCGCCGCGAT GCTGAAGCAG 2520
Consensus GTTCGGGTTG GCGGGGGTGC GAATGACCAC GAGGATCAGG AAGATAACGT TCGCCGCGAT GCTGAAGCAG

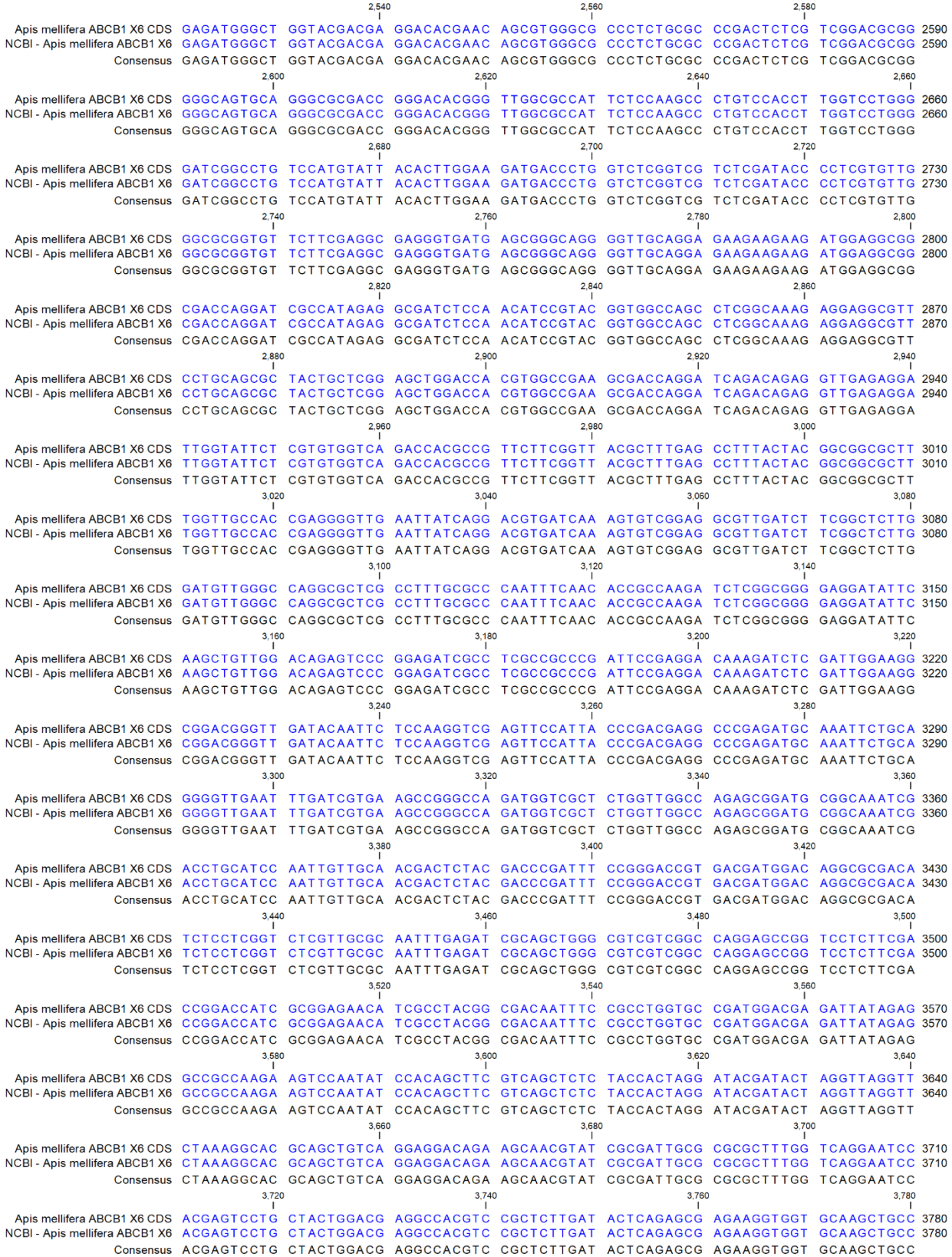
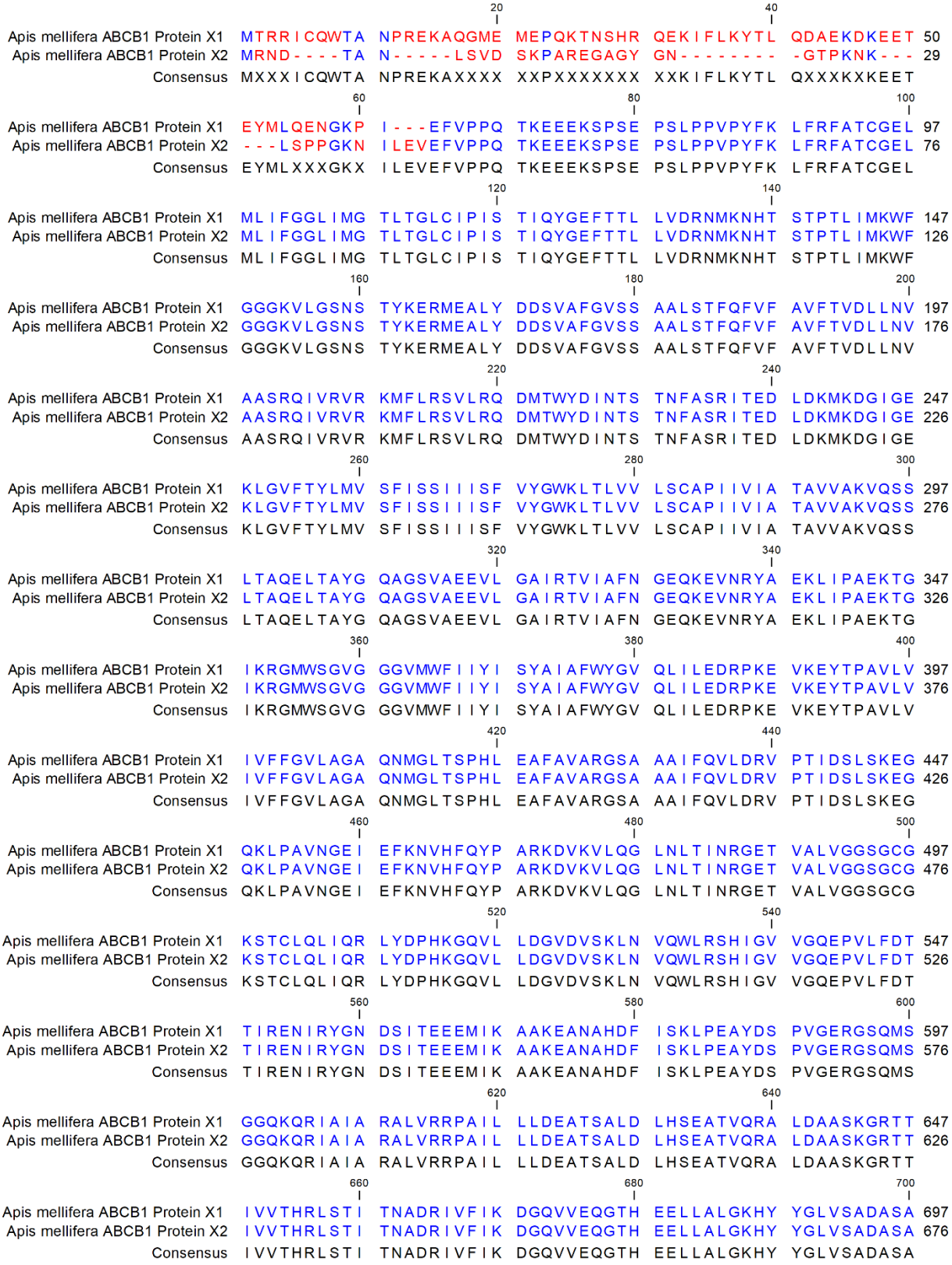

2,540 2,560 2,580
Apis mellifera ABCB1 X6 CDS GAGATGGGCT GGTACGACGA GGACACGAAC AGCGTGGGCG CCCTCTGCGC CCGACTCTCG TCGGACGCGG 2590
NCBI - Apis mellifera ABCB1 X6 GAGATGGGCT GGTACGACGA GGACACGAAC AGCGTGGGCG CCCTCTGCGC CCGACTCTCG TCGGACGCGG 2590
Consensus GAGATGGGCT GGTACGACGA GGACACGAAC AGCGTGGGCG CCCTCTGCGC CCGACTCTCG TCGGACGCGG
2,600 2,620 2,640 2,660
Apis mellifera ABCB1 X6 CDS GGGCAGTGCA GGGCGCGACC GGGACACGGG TTGGCGCCAT TCTCCAAGCC CTGTCCACCT TGGTCCTGGG 2660
NCBI - Apis mellifera ABCB1 X6 GGGCAGTGCA GGGCGCGACC GGGACACGGG TTGGCGCCAT TCTCCAAGCC CTGTCCACCT TGGTCCTGGG 2660
Consensus GGGCAGTGCA GGGCGCGACC GGGACACGGG TTGGCGCCAT TCTCCAAGCC CTGTCCACCT TGGTCCTGGG
2,680 2,700 2,720
Apis mellifera ABCB1 X6 CDS GATCGGCCTG TCCATGTATT ACACTTGGAA GATGACCCTG GTCTCGGTCG TCTCGATACC CCTCGTGTTG 2730
NCBI - Apis mellifera ABCB1 X6 GATCGGCCTG TCCATGTATT ACACTTGGAA GATGACCCTG GTCTCGGTCG TCTCGATACC CCTCGTGTTG 2730
Consensus GATCGGCCTG TCCATGTATT ACACTTGGAA GATGACCCTG GTCTCGGTCG TCTCGATACC CCTCGTGTTG
2,740 2,760 2,780 2,800
Apis mellifera ABCB1 X6 CDS GGCGCGGTGT TCTTCGAGGC GAGGGTGATG AGCGGGCAGG GGTTGCAGGA GAAGAAGAAG ATGGAGGCGG 2800
NCBI - Apis mellifera ABCB1 X6 GGCGCGGTGT TCTTCGAGGC GAGGGTGATG AGCGGGCAGG GGTTGCAGGA GAAGAAGAAG ATGGAGGCGG 2800
Consensus GGCGCGGTGT TCTTCGAGGC GAGGGTGATG AGCGGGCAGG GGTTGCAGGA GAAGAAGAAG ATGGAGGCGG
2,820 2,840 2,860
Apis mellifera ABCB1 X6 CDS CGACCAGGAT CGCCATAGAG GCGATCTCCA ACATCCGTAC GGTGGCCAGC CTCGGCAAAG AGGAGGCGTT 2870
NCBI - Apis mellifera ABCB1 X6 CGACCAGGAT CGCCATAGAG GCGATCTCCA ACATCCGTAC GGTGGCCAGC CTCGGCAAAG AGGAGGCGTT 2870
Consensus CGACCAGGAT CGCCATAGAG GCGATCTCCA ACATCCGTAC GGTGGCCAGC CTCGGCAAAG AGGAGGCGTT
2,880 2,900 2,920 2,940
Apis mellifera ABCB1 X6 CDS CCTGCAGCGC TACTGCTCGG AGCTGGACCA CGTGGCCGAA GCGACCAGGA TCAGACAGAG GTTGAGAGGA 2940
NCBI - Apis mellifera ABCB1 X6 CCTGCAGCGC TACTGCTCGG AGCTGGACCA CGTGGCCGAA GCGACCAGGA TCAGACAGAG GTTGAGAGGA 2940
Consensus CCTGCAGCGC TACTGCTCGG AGCTGGACCA CGTGGCCGAA GCGACCAGGA TCAGACAGAG GTTGAGAGGA
2,960 2,980 3,000
Apis mellifera ABCB1 X6 CDS TTGGTATTCT CGTGTGGTCA GACCACGCCG TTCTTCGGTT ACGCTTTGAG CCTTTACTAC GGCGGCGCTT 3010
NCBI - Apis mellifera ABCB1 X6 TTGGTATTCT CGTGTGGTCA GACCACGCCG TTCTTCGGTT ACGCTTTGAG CCTTTACTAC GGCGGCGCTT 3010
Consensus TTGGTATTCT CGTGTGGTCA GACCACGCCG TTCTTCGGTT ACGCTTTGAG CCTTTACTAC GGCGGCGCTT
3,020 3,040 3,060 3,080
Apis mellifera ABCB1 X6 CDS TGGTTGCCAC CGAGGGGTTG AATTATCAGG ACGTGATCAA AGTGTCGGAG GCGTTGATCT TCGGCTCTTG 3080
NCBI - Apis mellifera ABCB1 X6 TGGTTGCCAC CGAGGGGTTG AATTATCAGG ACGTGATCAA AGTGTCGGAG GCGTTGATCT TCGGCTCTTG 3080
Consensus TGGTTGCCAC CGAGGGGTTG AATTATCAGG ACGTGATCAA AGTGTCGGAG GCGTTGATCT TCGGCTCTTG
3,100 3,120 3,140
Apis mellifera ABCB1 X6 CDS GATGTTGGGC CAGGCGCTCG CCTTTGCGCC CAATTTCAAC ACCGCCAAGA TCTCGGCGGG GAGGATATTC 3150
NCBI - Apis mellifera ABCB1 X6 GATGTTGGGC CAGGCGCTCG CCTTTGCGCC CAATTTCAAC ACCGCCAAGA TCTCGGCGGG GAGGATATTC 3150
Consensus GATGTTGGGC CAGGCGCTCG CCTTTGCGCC CAATTTCAAC ACCGCCAAGA TCTCGGCGGG GAGGATATTC
3,160 3,180 3,200 3,220
Apis mellifera ABCB1 X6 CDS AAGCTGTTGG ACAGAGTCCC GGAGATCGCC TCGCCGCCCG ATTCCGAGGA CAAAGATCTC GATTGGAAGG 3220
NCBI - Apis mellifera ABCB1 X6 AAGCTGTTGG ACAGAGTCCC GGAGATCGCC TCGCCGCCCG ATTCCGAGGA CAAAGATCTC GATTGGAAGG 3220
Consensus AAGCTGTTGG ACAGAGTCCC GGAGATCGCC TCGCCGCCCG ATTCCGAGGA CAAAGATCTC GATTGGAAGG
3,240 3,260 3,280
Apis mellifera ABCB1 X6 CDS CGGACGGGTT GATACAATTC TCCAAGGTCG AGTTCCATTA CCCGACGAGG CCCGAGATGC AAATTCTGCA 3290
NCBI - Apis mellifera ABCB1 X6 CGGACGGGTT GATACAATTC TCCAAGGTCG AGTTCCATTA CCCGACGAGG CCCGAGATGC AAATTCTGCA 3290
Consensus CGGACGGGTT GATACAATTC TCCAAGGTCG AGTTCCATTA CCCGACGAGG CCCGAGATGC AAATTCTGCA
3,300 3,320 3,340 3,360
Apis mellifera ABCB1 X6 CDS GGGGTTGAAT TTGATCGTGA AGCCGGGCCA GATGGTCGCT CTGGTTGGCC AGAGCGGATG CGGCAAATCG 3360
NCBI - Apis mellifera ABCB1 X6 GGGGTTGAAT TTGATCGTGA AGCCGGGCCA GATGGTCGCT CTGGTTGGCC AGAGCGGATG CGGCAAATCG 3360
Consensus GGGGTTGAAT TTGATCGTGA AGCCGGGCCA GATGGTCGCT CTGGTTGGCC AGAGCGGATG CGGCAAATCG
3,380 3,400 3,420
Apis mellifera ABCB1 X6 CDS ACCTGCATCC AATTGTTGCA ACGACTCTAC GACCCGATTT CCGGGACCGT GACGATGGAC AGGCGCGACA 3430
NCBI - Apis mellifera ABCB1 X6 ACCTGCATCC AATTGTTGCA ACGACTCTAC GACCCGATTT CCGGGACCGT GACGATGGAC AGGCGCGACA 3430
Consensus ACCTGCATCC AATTGTTGCA ACGACTCTAC GACCCGATTT CCGGGACCGT GACGATGGAC AGGCGCGACA
3,440 3,460 3,480 3,500
Apis mellifera ABCB1 X6 CDS TCTCCTCGGT CTCGTTGCGC AATTTGAGAT CGCAGCTGGG CGTCGTCGGC CAGGAGCCGG TCCTCTTCGA 3500
NCBI - Apis mellifera ABCB1 X6 TCTCCTCGGT CTCGTTGCGC AATTTGAGAT CGCAGCTGGG CGTCGTCGGC CAGGAGCCGG TCCTCTTCGA 3500
Consensus TCTCCTCGGT CTCGTTGCGC AATTTGAGAT CGCAGCTGGG CGTCGTCGGC CAGGAGCCGG TCCTCTTCGA
3,520 3,540 3,560
Apis mellifera ABCB1 X6 CDS CCGGACCATC GCGGAGAACA TCGCCTACGG CGACAATTTC CGCCTGGTGC CGATGGACGA GATTATAGAG 3570
NCBI - Apis mellifera ABCB1 X6 CCGGACCATC GCGGAGAACA TCGCCTACGG CGACAATTTC CGCCTGGTGC CGATGGACGA GATTATAGAG 3570
Consensus CCGGACCATC GCGGAGAACA TCGCCTACGG CGACAATTTC CGCCTGGTGC CGATGGACGA GATTATAGAG
3,580 3,600 3,620 3,640
Apis mellifera ABCB1 X6 CDS GCCGCCAAGA AGTCCAATAT CCACAGCTTC GTCAGCTCTC TACCACTAGG ATACGATACT AGGTTAGGTT 3640
NCBI - Apis mellifera ABCB1 X6 GCCGCCAAGA AGTCCAATAT CCACAGCTTC GTCAGCTCTC TACCACTAGG ATACGATACT AGGTTAGGTT 3640
Consensus GCCGCCAAGA AGTCCAATAT CCACAGCTTC GTCAGCTCTC TACCACTAGG ATACGATACT AGGTTAGGTT
3,660 3,680 3,700
Apis mellifera ABCB1 X6 CDS CTAAAGGCAC GCAGCTGTCA GGAGGACAGA AGCAACGTAT CGCGATTGCG CGCGCTTTGG TCAGGAATCC 3710
NCBI - Apis mellifera ABCB1 X6 CTAAAGGCAC GCAGCTGTCA GGAGGACAGA AGCAACGTAT CGCGATTGCG CGCGCTTTGG TCAGGAATCC 3710
Consensus CTAAAGGCAC GCAGCTGTCA GGAGGACAGA AGCAACGTAT CGCGATTGCG CGCGCTTTGG TCAGGAATCC
3,720 3,740 3,760 3,780
Apis mellifera ABCB1 X6 CDS ACGAGTCCTG CTACTGGACG AGGCCACGTC CGCTCTTGAT ACTCAGAGCG AGAAGGTGGT GCAAGCTGCC 3780
NCBI - Apis mellifera ABCB1 X6 ACGAGTCCTG CTACTGGACG AGGCCACGTC CGCTCTTGAT ACTCAGAGCG AGAAGGTGGT GCAAGCTGCC 3780
Consensus ACGAGTCCTG CTACTGGACG AGGCCACGTC CGCTCTTGAT ACTCAGAGCG AGAAGGTGGT GCAAGCTGCC

Figure A11: Alignment of fully cloned *Apis mellifera* ABCB1 X6 gene with *Apis mellifera* ABCB1 X6 gene pulled from NCBI. Both sequences are identical aside from three SNPs that do not result in any amino acid changes.

Apis mellifera ABCB1 Protein X1 and X2



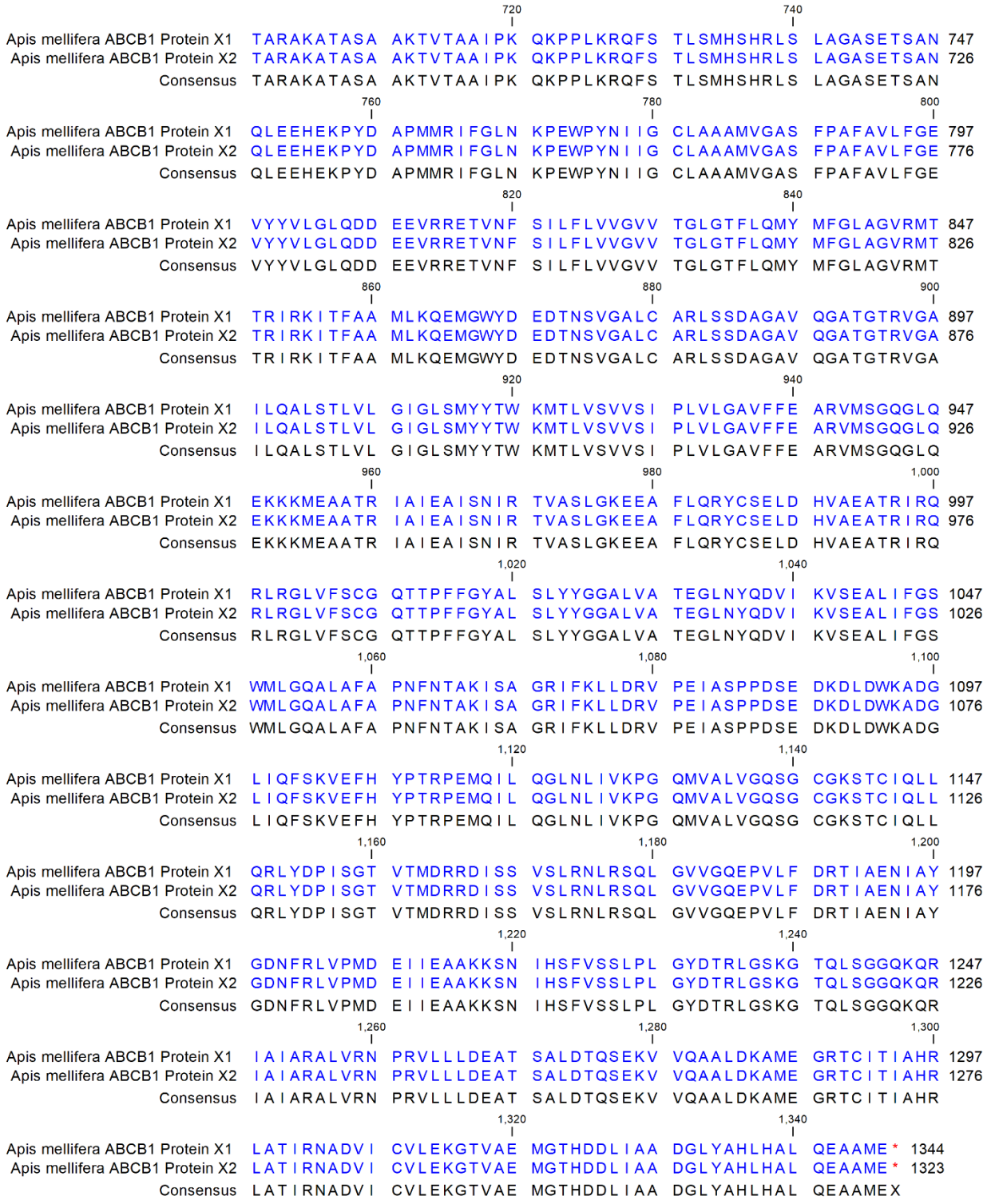


Figure A12: Alignment of two fully cloned *Apis mellifera* ABCB1 genes translated to protein isoforms X1 and X2. Cloned gene variant X1 translated to protein isoform X1 and cloned gene variant X6 translated to protein isoform X2.

Apis mellifera ABCC1 X1


2,000 2,020 2,040 2,060
Apis Mellifera ABCC1 X1 CDS GGATCTGGAA AAAGCTCTCT TTTATCAGCT CTTCTTGGAG AAATGGAGAA AATAAATGGC AGAGTTAATA CAAAAGGTTC TATTGCATAT 2049
NCBI - Apis mellifera ABCC1 X1 GGATCTGGAA AAAGCTCTCT TTTATCAGCT CTTCTTGGAG AAATGGAGAA AATAAATGGC AGAGTTAATA CAAAAGGTTC TATTGCATAT 2070
Consensus GGATCTGGAA AAAGCTCTCT TTTATCAGCT CTTCTTGGAG AAATGGAGAA AATAAATGGC AGAGTTAATA CAAAAGGTTC TATTGCATAT
2,080 2,100 2,120 2,140 2,160
Apis Mellifera ABCC1 X1 CDS GTATCTCAAC AAGCATGGAT TCAAAATGCA TCATTACAAG ATAATGTTTT ATTTGGAAAA TCATTGCACA AAAATTTATA CAATCGTGTA 2139
NCBI - Apis mellifera ABCC1 X1 GTATCTCAAC AAGCATGGAT TCAAAATGCA TCATTACAAG ATAATGTTTT ATTTGGAAAA TCATTGCACA AAAATTTATA CAATCGTGTA 2160
Consensus GTATCTCAAC AAGCATGGAT TCAAAATGCA TCATTACAAG ATAATGTTTT ATTTGGAAAA TCATTGCACA AAAATTTATA CAATCGTGTA
2,180 2,200 2,220 2,240
Apis Mellifera ABCC1 X1 CDS ATTGAAGCAT GTGCATTAAC TCCAGATTTA AAAGTGTTGC CTGCAGGTGA TCAAACTGAA ATTGGAGAAA AAGGAATAAA TTTATCTGGT 2229
NCBI - Apis mellifera ABCC1 X1 ATTGAAGCAT GTGCATTAAC TCCAGATTTA AAAGTGTTGC CTGCAGGTGA TCAAACTGAA ATTGGAGAAA AAGGAATAAA TTTATCTGGT 2250
Consensus ATTGAAGCAT GTGCATTAAC TCCAGATTTA AAAGTGTTGC CTGCAGGTGA TCAAACTGAA ATTGGAGAAA AAGGAATAAA TTTATCTGGT
2,260 2,280 2,300 2,320 2,340
Apis Mellifera ABCC1 X1 CDS GGACAAAAAC AAAGAGTATC ATTGGCCAGA GCAGTATACA ATGATTCCGA TATTTATTTT TTGGATGATC CATTAAGTGC AGTAGATTCA 2319
NCBI - Apis mellifera ABCC1 X1 GGACAAAAAC AAAGAGTATC ATTGGCCAGA GCAGTATACA ATGATTCCGA TATTTATTTT TTGGATGATC CATTAAGTGC AGTAGATTCA 2340
Consensus GGACAAAAAC AAAGAGTATC ATTGGCCAGA GCAGTATACA ATGATTCCGA TATTTATTTT TTGGATGATC CATTAAGTGC AGTAGATTCA
2,360 2,380 2,400 2,420
Apis Mellifera ABCC1 X1 CDS CATGTTGGAA AACATATATT TGAAAATGTA ATTGGCTCTA GTGGTTTGCT TAAGAAGAAA ACAAGAATAC TCGTCACACA TGGTATCACT 2409
NCBI - Apis mellifera ABCC1 X1 CATGTTGGAA AACATATATT TGAAAATGTA ATTGGCTCTA GTGGTTTGCT TAAGAAGAAA ACAAGAATAC TCGTCACACA TGGTATCACT 2430
Consensus CATGTTGGAA AACATATATT TGAAAATGTA ATTGGCTCTA GTGGTTTGCT TAAGAAGAAA ACAAGAATAC TCGTCACACA TGGTATCACT
2,440 2,460 2,480 2,500 2,520
Apis Mellifera ABCC1 X1 CDS TATTTGCCAG AAGTAGATAA CATTATTGTT CTTAAAGATG GTGAAATAAC AGAAGTTGGA ACTTACAAAC AACTTTTAGA GAAAAGGGGA 2499
NCBI - Apis mellifera ABCC1 X1 TATTTGCCAG AAGTAGATAA CATTATTGTT CTTAAAGATG GTGAAATAAC AGAAGTTGGA ACTTACAAAC AACTTTTAGA GAAAAGGGGA 2520
Consensus TATTTGCCAG AAGTAGATAA CATTATTGTT CTTAAAGATG GTGAAATAAC AGAAGTTGGA ACTTACAAAC AACTTTTAGA GAAAAGGGGA
2,540 2,560 2,580 2,600
Apis Mellifera ABCC1 X1 CDS GCCTTTTCTG AGTTTTTAGT GCAACATCTT CAGGAAGT-- -------ACA TGCTGATGGT GAATCAGAAG CAGATCTACA TGAAATTAAA 2580
NCBI - Apis mellifera ABCC1 X1 GCCTTTTCTG AGTTTTTAGT GCAACATCTT CAGGAAGTTG GAAATCTACA TGCTGATGGT GAATCAGAAG CAGATCTACA TGAAATTAAA 2610
Consensus GCCTTTTCTG AGTTTTTAGT GCAACATCTT CAGGAAGTTG GAAATCTACA TGCTGATGGT GAATCAGAAG CAGATCTACA TGAAATTAAA
2,620 2,640 2,660 2,680 2,700
Apis Mellifera ABCC1 X1 CDS CAACATTTGG AATCTACAAT TGGATCAAAT GAATTACAAC AGAAATTAAC AAGAGGTAAA TCAAGAATGT CAGAAAGTCA AAGTGAATCT 2670
NCBI - Apis mellifera ABCC1 X1 CAACATTTGG AATCTACAAT TGGATCAAAT GAATTACAAC AGAAATTAAC AAGAGGTAAA TCAAGAATGT CAGAAAGTCA AAGTGAATCT 2700
Consensus CAACATTTGG AATCTACAAT TGGATCAAAT GAATTACAAC AGAAATTAAC AAGAGGTAAA TCAAGAATGT CAGAAAGTCA AAGTGAATCT
2,720 2,740 2,760 2,780
Apis Mellifera ABCC1 X1 CDS GGTTCCATAG CTGATAGAAA ATCTTTAAAT GGTTCATTAA AAAGACAATA TTCTACAAGT AGTCAACAAT CTGGTACTTA TGAAAATAGT 2760
NCBI - Apis mellifera ABCC1 X1 GGTTCCATAG CTGATAGAAA ATCTTTAAAT GGTTCATTAA AAAGACAATA TTCTACAAGT AGTCAACAAT CTGGTACTTA TGAAAATAGT 2790
Consensus GGTTCCATAG CTGATAGAAA ATCTTTAAAT GGTTCATTAA AAAGACAATA TTCTACAAGT AGTCAACAAT CTGGTACTTA TGAAAATAGT
2,800 2,820 2,840 2,860 2,880
Apis Mellifera ABCC1 X1 CDS AATATAAAAG AAGCAAAATT ATTATCTCCA AAATCAGGAG GAAAATTAAT AGAAGTAGAA AAAACTGAAA CTGGAAGTGT TAAGTGGCGA 2850
NCBI - Apis mellifera ABCC1 X1 AATATAAAAG AAGCAAAATT ATTATCTCCA AAATCAGGAG GAAAATTAAT AGAAGTAGAA AAAACTGAAA CTGGAAGTGT TAAGTGGCGA 2880
Consensus AATATAAAAG AAGCAAAATT ATTATCTCCA AAATCAGGAG GAAAATTAAT AGAAGTAGAA AAAACTGAAA CTGGAAGTGT TAAGTGGCGA
2,900 2,920 2,940 2,960
Apis Mellifera ABCC1 X1 CDS GTCTATTCTC ACTATTTTAA ATCCATTGGT TGGTTTTTAT CAATATCAAC TATTATAATG AACGCTATTT TTCAAGGATT TAGTATTGGT 2940
NCBI - Apis mellifera ABCC1 X1 GTCTATTCTC ACTATTTTAA ATCCATTGGT TGGTTTTTAT CAATATCAAC TATTATAATG AACGCTATTT TTCAAGGATT TAGTATTGGT 2970
Consensus GTCTATTCTC ACTATTTTAA ATCCATTGGT TGGTTTTTAT CAATATCAAC TATTATAATG AACGCTATTT TTCAAGGATT TAGTATTGGT
2,980 3,000 3,020 3,040 3,060
Apis Mellifera ABCC1 X1 CDS TCAAATACTT GGCTTAGTAT GTGGTCAGAT GATAATTTAA CAGATGTTAA TAATACGGTT GATCACATTA AGCAAAACAT GTATCTTGGA 3030
NCBI - Apis mellifera ABCC1 X1 TCAAATACTT GGCTTAGTAT GTGGTCAGAT GATAATTTAA CAGATGTTAA TAATACGGTT GATCACATTA AGCAAAACAT GTATCTTGGA 3060
Consensus TCAAATACTT GGCTTAGTAT GTGGTCAGAT GATAATTTAA CAGATGTTAA TAATACGGTT GATCACATTA AGCAAAACAT GTATCTTGGA
3,080 3,100 3,120 3,140
Apis Mellifera ABCC1 X1 CDS GTATATGGTG GACTTGGTCT TGGCCAAGGC ATGACAGTGC TTGGAGGGGC ATTATTCTTG GCAAAAGGAA CAATACGCGC TTCCGTGCAT 3120
NCBI - Apis mellifera ABCC1 X1 GTATATGGTG GACTTGGTCT TGGCCAAGGC ATGACAGTGC TTGGAGGGGC ATTATTCTTG GCAAAAGGAA CAATACGCGC TTCCGTGCAT 3150
Consensus GTATATGGTG GACTTGGTCT TGGCCAAGGC ATGACAGTGC TTGGAGGGGC ATTATTCTTG GCAAAAGGAA CAATACGCGC TTCCGTGCAT
3,160 3,180 3,200 3,220 3,240
Apis Mellifera ABCC1 X1 CDS CTCTTCGAGA GTACGTTGCA ACGTGTTCTC CGGAATCCAA TGTCATTCTT TGACCAAACT CCAACTGGTC GAATTCTTAA TCGACTCTCT 3210
NCBI - Apis mellifera ABCC1 X1 CTCTTCGAGA GTACGTTGCA ACGTGTTCTC CGGAATCCAA TGTCATTCTT TGACCAAACT CCAACTGGTC GAATTCTTAA TCGACTCTCT 3240
Consensus CTCTTCGAGA GTACGTTGCA ACGTGTTCTC CGGAATCCAA TGTCATTCTT TGACCAAACT CCAACTGGTC GAATTCTTAA TCGACTCTCT
3,260 3,280 3,300 3,320
Apis Mellifera ABCC1 X1 CDS AAAGATACTG ATGTCATTGA TAATACGCTG CCATCCATAC TGCGTTCTTG GATTACTTGC CTCTTTGGGG TTATAGCCAC TTTAGTGGTT 3300
NCBI - Apis mellifera ABCC1 X1 AAAGATACTG ATGTCATTGA TAATACGCTG CCATCCATAC TGCGTTCTTG GATTACTTGC CTCTTTGGGG TTATAGCCAC TTTAGTGGTT 3330
Consensus AAAGATACTG ATGTCATTGA TAATACGCTG CCATCCATAC TGCGTTCTTG GATTACTTGC CTCTTTGGGG TTATAGCCAC TTTAGTGGTT
3,340 3,360 3,380 3,400 3,420
Apis Mellifera ABCC1 X1 CDS ATAAGTTTTA GTACACCAAT ATTTATTTCA GTCATAATAC CAATAAGTGT AATATATTAT TTCGTTCAAC GGTTATATGT TGCATCTTCA 3390
NCBI - Apis mellifera ABCC1 X1 ATAAGTTTTA GTACACCAAT ATTTATTTCA GTCATAATAC CAATAAGTGT AATATATTAT TTCGTTCAAC GGTTATATGT TGCATCTTCA 3420
Consensus ATAAGTTTTA GTACACCAAT ATTTATTTCA GTCATAATAC CAATAAGTGT AATATATTAT TTCGTTCAAC GGTTATATGT TGCATCTTCA
3,440 3,460 3,480 3,500
Apis Mellifera ABCC1 X1 CDS AGACAGCTAA AACGTTTAGA ATCTGTTTCA AGATCTCCTA TATATTCGCA TTTCAGTGAA ACAGTTTCTG GAGCACAAAT GATTAGAGCA 3480
NCBI - Apis mellifera ABCC1 X1 AGACAGCTAA AACGTTTAGA ATCTGTTTCA AGATCTCCTA TATATTCGCA TTTCAGTGAA ACAGTTTCTG GAGCACAAAT GATTAGAGCA 3510
Consensus AGACAGCTAA AACGTTTAGA ATCTGTTTCA AGATCTCCTA TATATTCGCA TTTCAGTGAA ACAGTTTCTG GAGCACAAAT GATTAGAGCA
3,520 3,540 3,560 3,580 3,600
Apis Mellifera ABCC1 X1 CDS TTTGGAGTAC AAGAACGATT TATTAATGAA TCCGAAAGTA AAGTAGATTT TAATCAAGTA TGTTATTATC CTAGTATAAT TGCAAACAGA 3570
NCBI - Apis mellifera ABCC1 X1 TTTGGAGTAC AAGAACGATT TATTAATGAA TCCGAAAGTA AAGTAGATTT TAATCAAGTA TGTTATTATC CTAGTATAAT TGCAAACAGA 3600
Consensus TTTGGAGTAC AAGAACGATT TATTAATGAA TCCGAAAGTA AAGTAGATTT TAATCAAGTA TGTTATTATC CTAGTATAAT TGCAAACAGA
3,620 3,640 3,660 3,680
Apis Mellifera ABCC1 X1 CDS TGGTTAGCTG TACGTTTAGA AATGGTTGGA AATTTAATTA TTTTTTTTGC TGCATTGTTT GCTGTATTAA ATAAAGACAC TGTAAGCTCT 3660
NCBI - Apis mellifera ABCC1 X1 TGGTTAGCTG TACGTTTAGA AATGGTTGGA AATTTAATTA TTTTTTTTGC TGCATTGTTT GCTGTATTAA ATAAAGACAC TGTAAGCTCT 3690
Consensus TGGTTAGCTG TACGTTTAGA AATGGTTGGA AATTTAATTA TTTTTTTTGC TGCATTGTTT GCTGTATTAA ATAAAGACAC TGTAAGCTCT
3,700 3,720 3,740 3,760 3,780
Apis Mellifera ABCC1 X1 CDS GGTTTAGTTG GATTATCTGT TAGTTATGCA TTACAAGTTA CTCAAACATT GAATTGGTTA GTACGAATGA CTTCTGATGT TGAAACTAAC 3750
NCBI - Apis mellifera ABCC1 X1 GGTTTAGTTG GATTATCTGT TAGTTATGCA TTACAAGTTA CTCAAACATT GAATTGGTTA GTACGAATGA CTTCTGATGT TGAAACTAAC 3780
Consensus GGTTTAGTTG GATTATCTGT TAGTTATGCA TTACAAGTTA CTCAAACATT GAATTGGTTA GTACGAATGA CTTCTGATGT TGAAACTAAC
3,800 3,820 3,840 3,860
Apis Mellifera ABCC1 X1 CDS ATTGTAGCTG TAGAAAGAAT AAAAGAATAT GGAGAAACCC CTCAAGAAGC ATCATGGAAA AATCCAGATT ATATACCACC TAAAGAATGG 3840
NCBI - Apis mellifera ABCC1 X1 ATTGTAGCTG TAGAAAGAAT AAAAGAATAT GGAGAAACCC CTCAAGAAGC ATCATGGAAA AATCCAGATT ATATACCACC TAAAGAATGG 3870
Consensus ATTGTAGCTG TAGAAAGAAT AAAAGAATAT GGAGAAACCC CTCAAGAAGC ATCATGGAAA AATCCAGATT ATATACCACC TAAAGAATGG
3,880 3,900 3,920 3,940 3,960
Apis Mellifera ABCC1 X1 CDS CCTGTACAAG GACGAGTAGA ATTTAAAGAT TATAAAGTTC GTTATAGGGA AGACTTAGAA CTTGTACTTC GTGGATTATC GTTTTCTATT 3930
NCBI - Apis mellifera ABCC1 X1 CCTGTACAAG GACGAGTAGA ATTTAAAGAT TATAAAGTTC GTTATAGGGA AGACTTAGAA CTTGTACTTC GTGGATTATC GTTTTCTATT 3960
Consensus CCTGTACAAG GACGAGTAGA ATTTAAAGAT TATAAAGTTC GTTATAGGGA AGACTTAGAA CTTGTACTTC GTGGATTATC GTTTTCTATT
3,980 4,000 4,020 4,040
Apis Mellifera ABCC1 X1 CDS AAGGGAGGAG AAAAAGTTGG TATTGTTGGT AGAACTGGTG CTGGAAAATC ATCTTTAACA TTGGCTTTAT TCAGAATAAT AGAAGCAGCT 4020
NCBI - Apis mellifera ABCC1 X1 AAGGGAGGAG AAAAAGTTGG TATTGTTGGT AGAACTGGTG CTGGAAAATC ATCTTTAACA TTGGCTTTAT TCAGAATAAT AGAAGCAGCT 4050
Consensus AAGGGAGGAG AAAAAGTTGG TATTGTTGGT AGAACTGGTG CTGGAAAATC ATCTTTAACA TTGGCTTTAT TCAGAATAAT AGAAGCAGCT

Figure A13: Alignment of fully cloned *Apis mellifera* ABCC1 X1 gene with *Apis mellifera* ABCC1 X1 gene pulled from NCBI. Both sequences are identical aside from one SNP that does not result in any amino acid changes, a 20bp deletion at 783 bases, and a 9bp deletion at 2549 bases.

Appendix 2: Western Blot Optimization

2.1. Extraction Methods

One of the first variables you can change with a western blot is how you extract the proteins from your tissue or culture samples. I tested five different methods of tissue homogenization: liquid nitrogen, glass homogenizer, tube homogenizer, bead mill, and sonicator. Separately, I also tested two different protease inhibitor cocktails from Sigma (Cat#MSSAFE-1VL) and Pierce (Cat#A32961).

I would not recommend liquid nitrogen as the primary form of tissue homogenization. It was hard to transfer the ground tissue from the mortar into a clean tube leading to quite a bit of lost protein. The tissue is also homogenized before adding RIPA buffer or protease inhibitors so there is a possibility of degradation while grinding the tissues. The liquid nitrogen samples showed a lot of unspecific binding at 70, 50, and ~40 kDa. The extra bands we see in Figure A14 could be due to degraded proteins from excess mechanical force from the liquid nitrogen extraction.

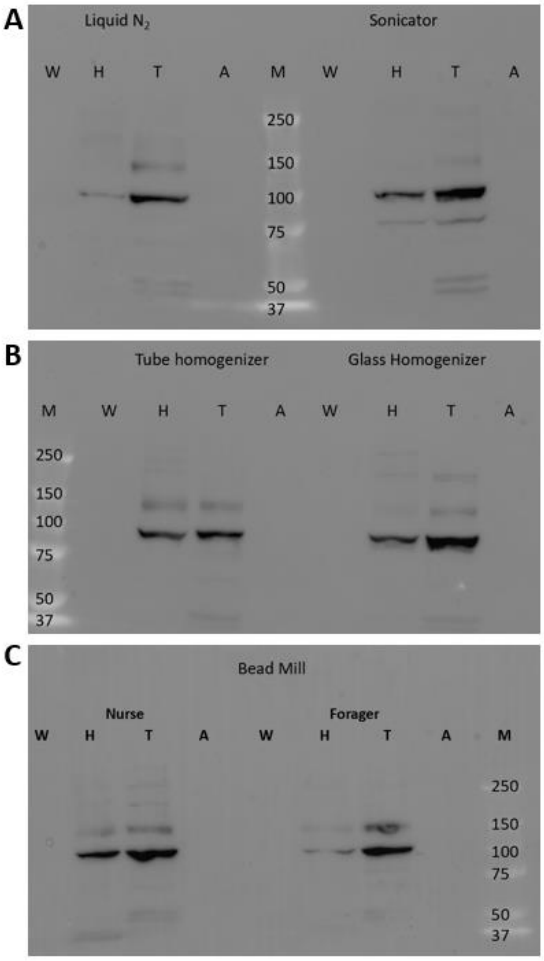
Sonication was also too much force for western blot protein extraction. I sonicated the samples in 1.5mL tubes for 3-5 seconds at 30% amplitude. The hard exoskeleton was hard to break through, so I had to do several sonications, holding the samples on ice in between to prevent protein degradation. Because of the high speed of the sonicator tip, the samples can easily overheat so it is important to only do short bursts and then hold samples on ice to keep them from degrading. Although the signal was stronger with sonication, it also causes more pronounced unspecific binding at 75, 50, and ~40 kDa (Figure A14). The brighter unspecific binding bands show that sonication causes more degradation than any of the other homogenization methods. I would not recommend sonication as a western blot tissue homogenization method.

The plastic homogenization set that come with a little plastic pestle and a 1.5mL tube will be referred to as the tube homogenizers. These are a good choice for smaller samples but if you try to homogenize a whole bee, it will overflow. However, the western blot for the tube homogenizers shows very nice, clean bands (Figure A14). We still see some light degradation bands, but they are less pronounced than in the sonicator samples. Although *Am*-ABCB1 should be at ~147 kDa we see the brightest band at ~100 kDa. With the tube homogenizer samples, we see the strongest 147 kDa bands of all the homogenization test samples.

The glass homogenizer still uses a pestle to manually grind tissue in a tube, but the glass set up is much larger and better equipped to handle larger sample sizes. With the bee the glass homogenizer still shows low degradation bands but there are more aggregate bands at 200 and 250 kDa (Figure A14). With the fish samples, the glass homogenizers showed the cleaner, more consistent bands when compared to the bead mill samples (Figure A15).

The bead mill, which I used for most of the western blots I tested, showed mixed results. With the fish samples, although the samples still showed bands at ~150 kDa, but they were inconsistent across the samples (Figure A15). With the bees, the bead mill shows the cleanest bands with the fewest unspecific binding bands (Figure A14).

I also compared old tissues with newer tissues. With the Tuna samples from Figure A15, there is a difference between the “new” samples we got from Dr. Amro Hamdoun (SIO/UCSD) and the older samples that were stored in the freezer. Looking at just the glass homogenizer set, the new sample had a stronger signal than either of the older samples. Although it is an unfair comparison because they are difference species, we can also look at the older Tuna samples with the fresh Sturgeon samples that were collected within days of running the western blot. You can see stronger ABCB1 bands and fewer degradation bands.



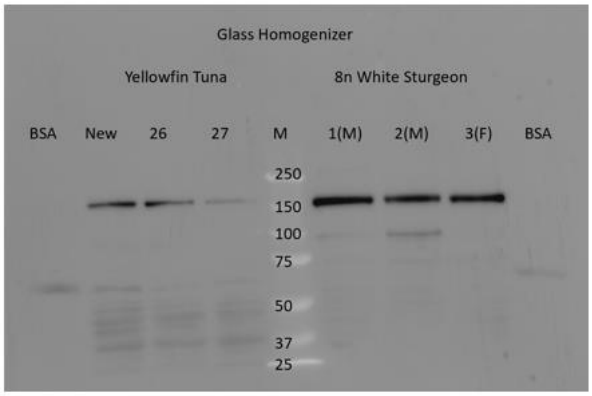
Parameters	Left of Marker	Right of Marker
Extraction Method	Liquid Nitrogen	Sonicator
Protein Content	40 ug	40 ug
Loading Buffer	Laemmli, 0.05% BME (Cat#1610737)	Laemmli, 0.05% BME (Cat#1610737)
Incubation	37C for 45min	37C for 45min
Gel Type	Hand-Cast 4%/7.5%	Hand-Cast 4%/7.5%
Running Buffer	BioRad (Cat#1610772)	BioRad (Cat#1610772)
Membrane	.2 BioRad PVDF (MeOH)	.2 BioRad PVDF (MeOH)
Transfer Buffer	BioRad+ 20% MeOH	BioRad+ 20% MeOH
Blocking	5% BSA, 1 Hr	5% BSA, 1 Hr
Primary	1:2000 C219 Mouse Monoclonal (Cat#517310)	1:2000 C219 Mouse Monoclonal (Cat#517310)
Secondary	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity (Cat# 1705060)	BioRad Clarity (Cat# 1705060)
Imager	BioRad ChemiDoc	BioRad ChemiDoc

Parameters	Left of Marker	Right of Marker
Extraction Method	Tube Homogenizer	Glass Homogenizer
Protein Content	40 ug	40 ug
Loading Buffer	Laemmli, 0.05% BME (Cat#1610737)	Laemmli, 0.05% BME (Cat#1610737)
Incubation	37C for 45min	37C for 45min
Gel Type	Hand-Cast 4%/7.5%	Hand-Cast 4%/7.5%
Running Buffer	BioRad (Cat#1610772)	BioRad (Cat#1610772)
Membrane	.2 BioRad PVDF (MeOH)	.2 BioRad PVDF (MeOH)
Transfer Buffer	BioRad+ 20% MeOH	BioRad+ 20% MeOH
Blocking	5% BSA, 1 Hr	5% BSA, 1 Hr
Primary	1:2000 C219 Mouse Monoclonal (Cat#517310)	1:2000 C219 Mouse Monoclonal (Cat#517310)
Secondary	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity (Cat# 1705060)	BioRad Clarity (Cat# 1705060)
Imager	BioRad ChemiDoc	BioRad ChemiDoc

Parameters	Whole Gel
Extraction Method	Bead Mill
Protein Content	40 ug
Loading Buffer	Laemmli, 0.05% BME (Cat#1610737)
Incubation	37C for 45min
Gel Type	Hand-Cast 4%/7.5%
Running Buffer	BioRad (Cat#1610772)
Membrane	.2 BioRad PVDF (MeOH)
Transfer Buffer	BioRad+ 20% MeOH
Blocking	5% BSA, 1 Hr
Primary	1:2000 C219 Mouse Monoclonal (Cat#517310)
Secondary	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity (Cat# 1705060)
Imager	BioRad ChemiDoc

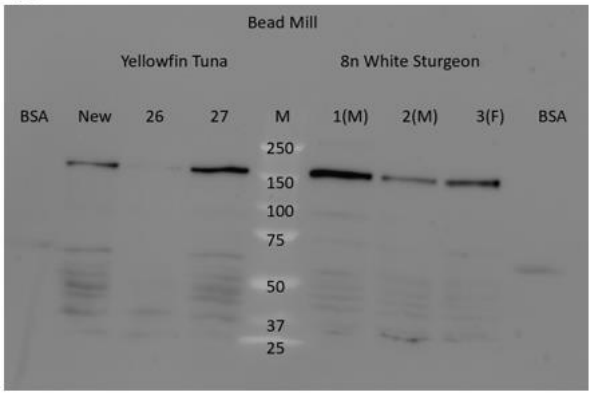
Figure A14: Honeybee western blots performed with five different protein extraction methods. [A] Western blot of liquid nitrogen and sonicator samples. Western blot parameters to the right. **[B]** Western blot of tube and glass homogenized samples. Parameters to the right. **[C]** Western blot of mead mill samples with parameters to the right. Incubation refers to the heating time and temperature to denature the proteins prior to loading on the gels. All gels were run for 30 minutes at 60V then 45 minutes at 160V. Marker is All Blue Precision Plus Protein from BioRad (Cat # 1610373).

A



Parameters	Tuna Liver	Sturgeon Liver
Extraction Method	Glass Homogenizer	Glass Homogenizer
Protein Content	20 ug	20 ug
Loading Buffer	Laemmli, 0.05% BME (Cat#1610737)	Laemmli, 0.05% BME (Cat#1610737)
Incubation	37C for 45min	37C for 45min
Gel Type	Hand-Cast 4%/7.5%	Hand-Cast 4%/7.5%
Running Buffer	BioRad (Cat#1610772)	BioRad (Cat#1610772)
Membrane	.2 BioRad PVDF (MeOH)	.2 BioRad PVDF (MeOH)
Transfer Buffer	BioRad+ 20% MeOH	BioRad+ 20% MeOH
Blocking	5% BSA, 1 Hr	5% BSA, 1 Hr
Primary	1:2000 C219 Mouse Monoclonal (Cat#517310)	1:2000 C219 Mouse Monoclonal (Cat#517310)
Secondary	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity (Cat# 1705060)	BioRad Clarity (Cat# 1705060)
Imager	BioRad ChemiDoc	BioRad ChemiDoc

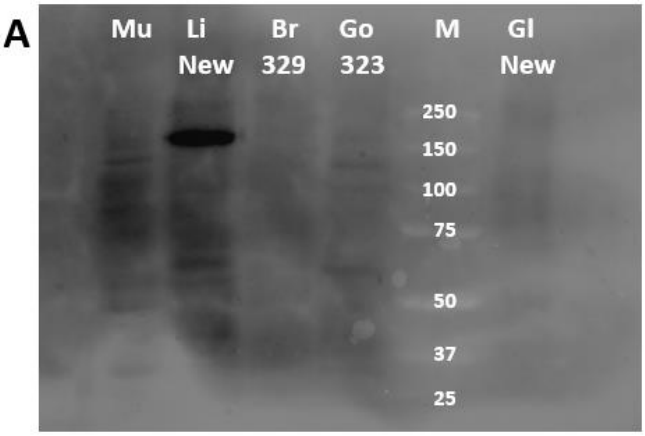
B



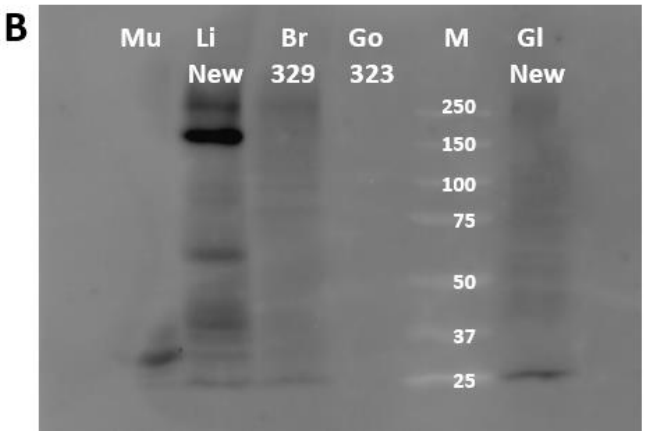
Parameters	Tuna Liver	Sturgeon Liver
Extraction Method	Bead Mill	Bead Mill
Protein Content	20 ug	20 ug
Loading Buffer	Laemmli, 0.05% BME (Cat#1610737)	Laemmli, 0.05% BME (Cat#1610737)
Incubation	37C for 45min	37C for 45min
Gel Type	Hand-Cast 4%/7.5%	Hand-Cast 4%/7.5%
Running Buffer	BioRad (Cat#1610772)	BioRad (Cat#1610772)
Membrane	.2 BioRad PVDF (MeOH)	.2 BioRad PVDF (MeOH)
Transfer Buffer	BioRad+ 20% MeOH	BioRad+ 20% MeOH
Blocking	5% BSA, 1 Hr	5% BSA, 1 Hr
Primary	1:2000 C219 Mouse Monoclonal (Cat#517310)	1:2000 C219 Mouse Monoclonal (Cat#517310)
Secondary	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity (Cat# 1705060)	BioRad Clarity (Cat# 1705060)
Imager	BioRad ChemiDoc	BioRad ChemiDoc

Figure A15: Fish western blots performed with two different protein extraction methods. [A] Western blot of Tuna and Sturgeon liver from glass homogenizer. Western blot parameters to the right. **[B]** Western blot of Tuna and Sturgeon liver homogenized with bead mill. Parameters to the right. Incubation refers to the heating time and temperature to denature the proteins prior to loading on the gels. All gels were run for 30 minutes at 60V then 45 minutes at 160V. Marker is All Blue Precision Plus Protein from BioRad (Cat # 1610373).

Another parameter to be tested while extracting proteins is the protease/phosphatase inhibitor cocktail added to the RIPA buffer during or after tissue homogenization. I did not see a difference between the western blots for the two different protease inhibitors (see Figure A16), but there was a small difference in the BCA total protein concentration. There was more protein recovered from the extraction using Pierce protease inhibitor cocktail (see Table A5).



Parameters	Pierce (Cat# A329161)
Extraction Method	Bead Mill
Protein Content	20 ug
Loading Buffer	Laemmli (Cat#1610737)
Incubation	70C for 10min
Gel Type	Hand-Cast 4%/7.5%
Running Buffer	BioRad (Cat#1610772)
Membrane	.2 BioRad PVDF (MeOH)
Transfer Buffer	BioRad+ 20% MeOH
Blocking	5% BSA, 1 Hr
Primary	1:2000 C219 Mouse Monoclonal (Cat#517310)
Secondary	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity (Cat# 1705060)
Imager	BioRad ChemiDoc



Parameters	Sigma (Cat# MSSAFE-1VL)
Extraction Method	Bead Mill
Protein Content	20 ug
Loading Buffer	Laemmli (Cat#1610737)
Incubation	70C for 10min
Gel Type	Hand-Cast 4%/7.5%
Running Buffer	BioRad (Cat#1610772)
Membrane	.2 BioRad PVDF (MeOH)
Transfer Buffer	BioRad+ 20% MeOH
Blocking	5% BSA, 1 Hr
Primary	1:2000 C219 Mouse Monoclonal (Cat#517310)
Secondary	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity (Cat# 1705060)
Imager	BioRad ChemiDoc

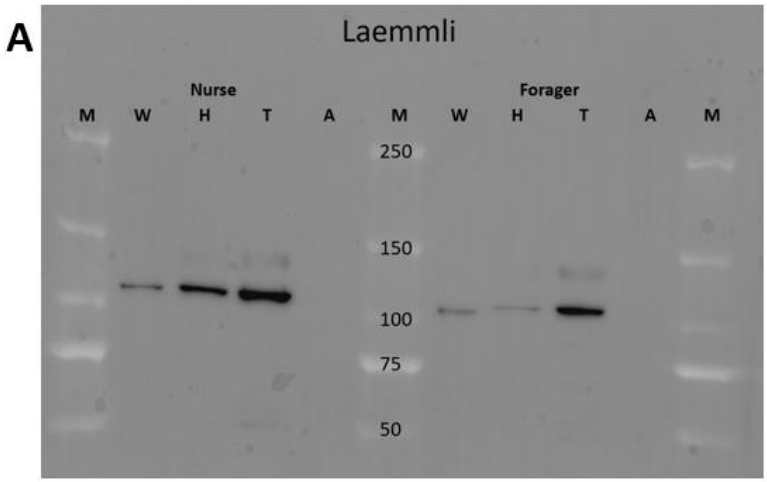
Figure A16: Tuna western blots performed with two different protease inhibitor cocktails. [A] Western blot of Tuna with Pierce protease inhibitor cocktail. Western blot parameters to the right. **[B]** Western blot of Tuna with Sigma protease inhibitor cocktail. Parameters to the right. Incubation refers to the heating time and temperature to denature the proteins prior to loading on the gels. All gels were run for 30 minutes at 60V then 45 minutes at 160V. Marker is All Blue Precision Plus Protein from BioRad (Cat # 1610373).

Table A6: BCA results for Pierce and Sigma Protease inhibitor cocktail protein extractions. Samples were diluted 1:50 with water prior to assay with the Pierce BCA kit in order to stay in range of the standard curve.

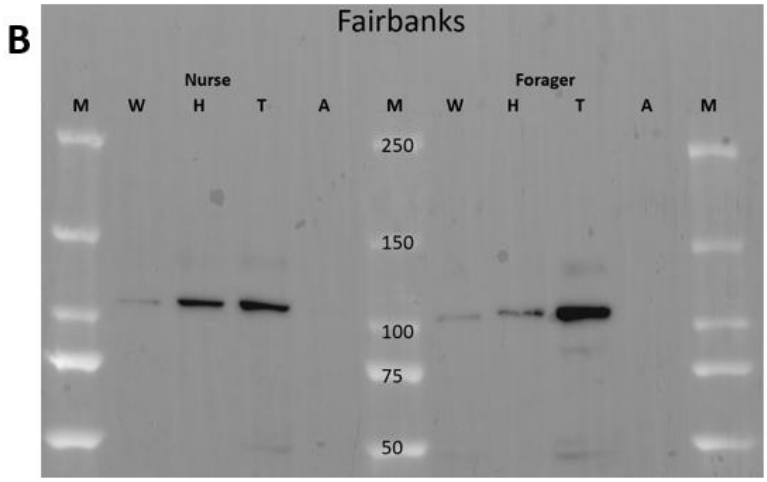
Sample	Pierce Protein Concentration	Sigma Protein Concentration
	ug/ml	ug/ml
Muscle New	943.067	692.546
Liver New	823.596	887.685
Liver 26	525.502	589.577
Liver 27	1359.016	928.292
Brain 329	489.22	254.842
Brain 330	245.876	191.832
Gonad 323	609.104	724.143
Gonad 328	671.797	253.299
Gill New	676.371	296.992
Gill 27	709.18	299.368
Gill 28	561.302	266.968

2.2. Sample Preparation: Protein Dilutions and Loading buffers

Homogenization methods were not the only variables you can change with samples. You can also change the loading buffer. The western blot kit from BioRad came with 2x Laemmli buffer (Cat# 1610737). I also tested Fairbanks buffer to see if it would provide better conditions for ABCB1 which is an insoluble membrane protein. Western blots done with Laemmli, and Fairbanks buffer (Fairbanks, Steck, and Wallach 1971) showed that there is no real difference between the two (Figure 17). You could use whichever buffer you want, and it would make no difference.



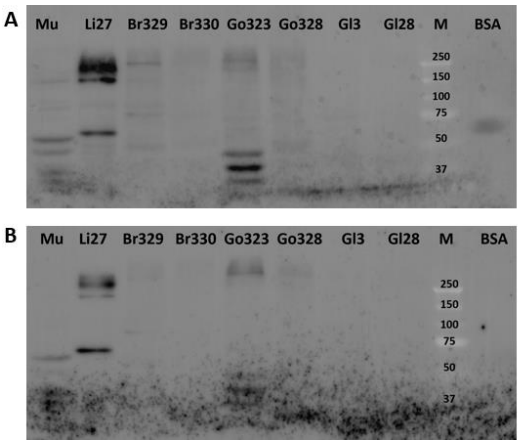
Parameters	Laemmli
Extraction Method	Bead Mill
Protein Content	20 ug
Loading Buffer	Laemmli (Cat#1610737)
Incubation	70C for 10min
Gel Type	TruPAGE 4-12% Gradient (Cat#PCG2011-10EA)
Running Buffer	TruPAGE Buffer (Cat#PCG3001-4L)
Membrane	.45µm Immobilon-P (Sigma)
Transfer Buffer	Tricine TruPAGE + 20% MeOH
Blocking	5% BSA, 1 Hr
Primary	1:2000 C219 Mouse Monoclonal (Cat#517310)
Secondary	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity (Cat# 1705060)
Imager	BioRad ChemiDoc



Parameters	Fairbanks
Extraction Method	Bead Mill
Protein Content	20 ug
Loading Buffer	Fairbanks
Incubation	70C for 10min
Gel Type	TruPAGE 4-12% Gradient (Cat#PCG2011-10EA)
Running Buffer	TruPAGE Buffer (Cat#PCG3001-4L)
Membrane	.45µm Immobilon-P (Sigma)
Transfer Buffer	Tricine TruPAGE + 20% MeOH
Blocking	5% BSA, 1 Hr
Primary	1:2000 C219 Mouse Monoclonal (Cat#517310)
Secondary	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity (Cat# 1705060)
Imager	BioRad ChemiDoc

Figure A17: Honeybee western blots performed with two different loading buffers. [A] Honeybee western blot of samples prepared with Laemmli buffer. Western blot parameters to the right. **[B]** Honeybee western blot of samples prepared with Fairbanks buffer. Western blot parameters to the right. Incubation refers to the heating time and temperature to denature the proteins prior to loading on the gels. All gels were run for 30 minutes at 60V then 45 minutes at 160V. Marker is All Blue Precision Plus Protein from BioRad (Cat # 1610373).

Another variable you can change at the sample preparation level is the amount of total protein diluted by the loading buffer. Generally, many guides and protocols say to load a total of 20µg of protein into each well if you are working with a total protein lysate. When I started testing western blots, I loaded 20µg of each sample and that worked fine but some samples were weaker than others. Liver tissue, for example, has a high concentration of ABCB1 so liver samples usually had a very strong band. Other tissues like the brain and the bees were less concentrated and had weaker bands. To accommodate for this, I tested sample dilutions with higher protein content. Although I did test different protein concentrations, the final western blots did not show any large differences (see Figure A18). There are spots toward the bottom of the blot that might be cleaved horseradish peroxidase from degraded secondary antibody.



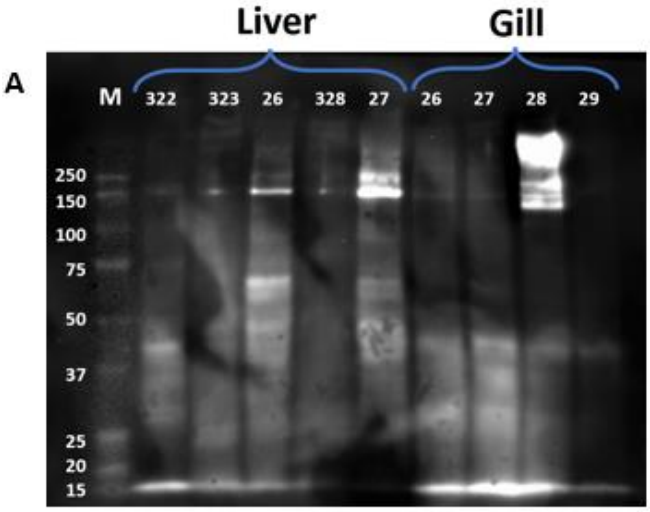
Parameters	Top (3X)	Bottom (4X)
Extraction Method	Glass Homogenizer	Glass Homogenizer
Protein Content	60 ug (Liver 20ug)	80 ug (Liver 20ug)
Loading Buffer	Laemmli, 0.05% BME (Cat#1610737)	Laemmli, 0.05% BME (Cat#1610737)
Incubation	37C for 45min	37C for 45min
Gel Type	Hand-Cast 4%/7.5%	Hand-Cast 4%/7.5%
Running Buffer	BioRad (Cat#1610772)	BioRad (Cat#1610772)
Membrane	.2 BioRad PVDF (MeOH)	.2 BioRad PVDF (MeOH)
Transfer Buffer	BioRad+ 20% MeOH	BioRad+ 20% MeOH
Blocking	5% BSA, 1 Hr	5% BSA, 1 Hr
Primary	1:2000 C219 Mouse Monoclonal (Cat#517310)	1:2000 C219 Mouse Monoclonal (Cat#517310)
Secondary	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity (Cat# 1705060)	BioRad Clarity (Cat# 1705060)
Imager	BioRad ChemiDoc	BioRad ChemiDoc

Figure A18: Tuna western blots performed with two different protein concentrations. [A] Tuna western blot with sample concentrations 3X normal (except liver which stayed at 20µg/mL). Western blot parameters to the right. **[B]** Tuna western blot with sample concentrations 4X normal (except liver which stayed at 20µg/mL). Western blot parameters to the right. Incubation refers to the heating time and temperature to denature the proteins prior to loading on the gels. All gels were run for 30 minutes at 60V then 45 minutes at 160V. Marker is All Blue Precision Plus Protein from BioRad (Cat # 1610373).

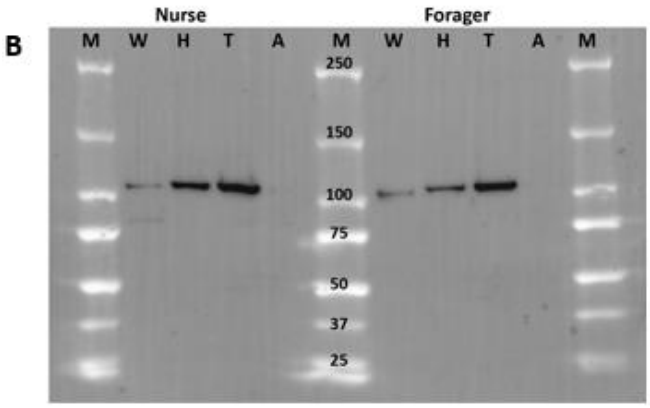
2.3. PAGE Comparison: Gradient Gels

For the most part, I used hand-cast gels for all my testing because it is cheaper and more available. I also tested some store-bought gradient gels. Although a bit pricier than just making gels yourself, they offer a cleaner, more spaced-out gel that would look good for publications. I tested both the BioRad 4-8% gradient gel and the Sigma TruPAGE 4-8% gradient gels. Note, this was not a direct comparison because these were two separate western blots with different samples. Looking at the gel, the samples migrated very similarly (see Figure A19).

Going forward, I suggest purchasing the BioRad gradient gels for publication level western blots. BioRad would be more cost effective because you can use the same Tris/Glycine/SDS buffer we already have in the lab. With the Sigma TruPAGE gradient gels, you would also need to purchase the TruPAGE buffers to go with it.



Parameters	Top: BioRad Gradient
Extraction Method	Bead Mill
Protein Content	25 ug
Loading Buffer	Laemmli (Cat#1610737)
Incubation	70C for 10 Minutes
Gel Type	BioRad 4-12% Gradient (Cat#4561085)
Running Buffer	BioRad Tris/Glycine/SDS Buffer (Cat#1610772)
Membrane	.2µm BioRad PVDF (MeOH)
Transfer Buffer	BioRad+ 20% MeOH
Blocking	None
Primary	1:500 (Used) C219 Mouse Monoclonal (Cat#MA1-26528)
Secondary	1:3000 Biorad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity (Cat# 1705060)
Imager	Thermo My ECL



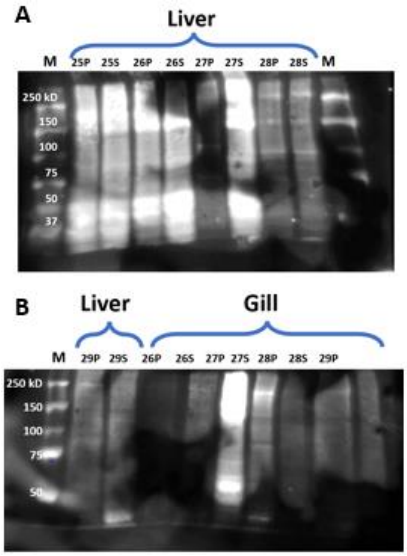
Parameters	Bottom
Extraction Method	Bead Mill
Protein Content	25ug
Loading Buffer	Laemmli (Cat#1610737)
Incubation	70C for 10 Minutes
Gel Type	TruPAGE 4-12% Gradient (Cat#PCG2011-10EA)
Running Buffer	Tricine TruPAGE Buffer (Cat#PCG3001-4L)
Membrane	.45µm Immobilon-P (Sigma)
Transfer Buffer	Tricine TruPAGE + 20% MeOH
Blocking	5% BSA, 1 Hr
Primary	1:2000 C219 Mouse Monoclonal (Cat#517310)
Secondary	1:3000 BioRad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity (Cat# 1705060)
Imager	BioRad ChemiDoc

Figure A19: Western blots Run on two different gradient gels. [A] Tuna western blot run on BioRad gradient gel. Western blot parameters to the right. **[B]** Honeybee western blot run on Sigma TruPAGE gradient gel. Western blot parameters to the right. Incubation refers to the heating time and temperature to denature the proteins prior to loading on the gels. All gels were run for 30 minutes at 60V then 45 minutes at 160V. Marker is All Blue Precision Plus Protein from BioRad (Cat # 1610373).

2.4. Antibodies and Dilutions

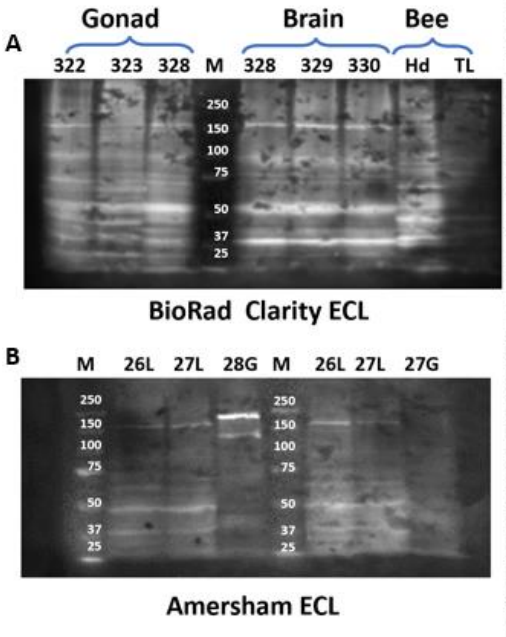
When I first started western blot on bees, we did not know if the C219 antibody would work. Luckily, the C219 epitope is available, and I was able to locate two sites on the *Am*-ABCB1 amino acid sequence were C219 would bind (see Figure 8). C219 seems to bind to a very highly conserved sequence of ABCB1 because, even though C219 is listed to have Specific reactivity to human, mouse, rat, dog, and primate, we have seen C219 bind to fish and bees.

Although we know that C219 works for our fish and bees, I had to determine the correct antibody dilution. I started out with a 1:500 dilution of primary and a 1:3000 dilution of secondary. This turned out to be too high a dilution, especially with the Clarity Max ECL solution meant for low protein content. This led to a very overblown image (see Figure A20). Seeing as the dilutions were so high, I saved the dilutions in the freezer and used the primary again with no issue, but I did make a new secondary 1:5000 dilution (see Figure A19a). The next dilution set I tried was a 1:2000 primary with a 1:5000 (see Figure A21). *Note, these blots were imaged without plastic film because the plastic was absorbing too much of the light emission.* This still looked too bright with a lot of non-specific binding. From here, I tried a different approach and changed the antibody dilutions one at a time. I tried one blot with a 1:2000 primary dilution with a 1:5000 secondary and another blot with a 1:1000 primary dilution with a 1:10000 secondary dilution (See Figure A22). I did see a difference between the two so for the next test, I tried a 1:2000 primary dilution with a 1:10000 secondary dilution (see Figure A19b). This worked out the best and used the least stock antibodies, so I kept the 1:2000 primary and 1:10000 secondary dilution ratio for the rest of my western blots.



Parameters	Top: Clarity Max	Bottom: Clarity
Extraction Method	Bead Mill	Bead Mill
Protein Content	25 ug	25ug
Loading Buffer	Laemmli (Cat#1610737)	Laemmli (Cat#1610737)
Incubation	70C for 10 Minutes	70C for 10 Minutes
Gel Type	Hand-Cast 4%/7.5%	Hand-Cast 4%/7.5%
Running Buffer	BioRad (Cat#1610772)	BioRad BioRad (Cat#1610772)
Membrane	.2 BioRad PVDF (MeOH)	.2 BioRad PVDF (MeOH)
Transfer Buffer	BioRad+ 20% MeOH	BioRad+ 20% MeOH
Blocking	None	None
Primary	1:500 C219 Mouse Monoclonal (Cat#MA1-26528)	1:500 C219 Mouse Monoclonal (Cat#MA1-26528)
Secondary	1:3000 Biorad goat anti-mouse + HRP (Cat#1706516)	1:3000 Biorad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity Max (Cat# 1705062)	BioRad Clarity (Cat# 1705060)
Imager	Thermo My ECL	Thermo My ECL

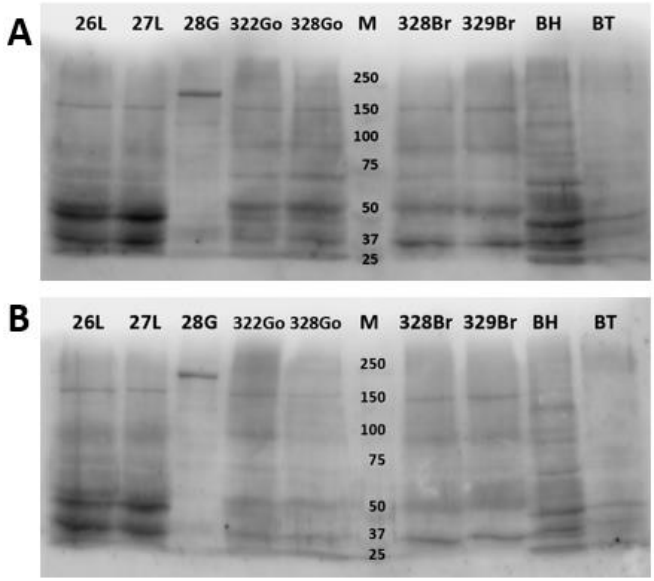
Figure A20: Western blots developed with two different ECL solutions. [A] Tuna western blot developed with BioRad Clarity Max. Western blot parameters to the right. **[B]** Tuna western blot developed with BioRad Clarity ECL. Western blot parameters to the right. Samples labeled S are sample supernatants and P are resuspended sample pellets. Incubation refers to the heating time and temperature to denature the proteins prior to loading on the gels. All gels were run for 30 minutes at 60V then 45 minutes at 160V. Marker is All Blue Precision Plus Protein from BioRad (Cat # 1610373).



Parameters	Top: BioRad Clarity ECL
Extraction Method	Bead Mill
Protein Content	25 ug
Loading Buffer	Laemmli (Cat#1610737)
Incubation	70C for 10 Minutes
Gel Type	Hand-Cast 4%/7.5%
Running Buffer	BioRad (Cat#1610772)
Membrane	.2 BioRad PVDF (MeOH)
Transfer Buffer	BioRad+ 20% MeOH
Blocking	5% BSA, 1 Hr
Primary	1:2000 C219 Mouse Monoclonal (Cat#517310)
Secondary	1:5000 BioRad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity (Cat# 1705060)
Imager	Thermo My ECL (No Plastic)

Parameters	Bottom: Amersham Select ECL
Extraction Method	Bead Mill
Protein Content	25ug
Loading Buffer	Fairbanks
Incubation	70C for 10 Minutes
Gel Type	Hand-Cast 4%/7.5%
Running Buffer	BioRad (Cat#1610772)
Membrane	.2 BioRad PVDF (MeOH)
Transfer Buffer	BioRad+ 20% MeOH
Blocking	5% BSA, 1 Hr
Primary	1:2000 C219 Mouse Monoclonal (Cat#517310)
Secondary	1:5000 BioRad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity (Cat# 1705060)
Imager	Thermo My ECL (No Plastic)

Figure A21: Western blots developed with two different ECL solutions. [A] Tuna western blot developed with BioRad Clarity ECL. Western blot parameters to the right. **[B]** Tuna western blot developed with Amersham Select ECL. Samples labed G are Gill. Western blot parameters to the right. Incubation refers to the heating time and temperature to denature the proteins prior to loading on the gels. All gels were run for 30 minutes at 60V then 45 minutes at 160V. Marker is All Blue Precision Plus Protein from BioRad (Cat # 1610373).



Parameters	Top	Bottom
Extraction Method	Bead Mill	Bead Mill
Protein Content	25 ug	25ug
Loading Buffer	Laemmli (Cat#1610737)	Fairbanks
Incubation	70C for 10 Minutes	70C for 10 Minutes
Gel Type	Hand-Cast 4%/7.5%	Hand-Cast 4%/7.5%
Running Buffer	BioRad (Cat#1610772)	BioRad (Cat#1610772)
Membrane	.2 BioRad PVDF (MeOH)	.2 BioRad PVDF (MeOH)
Transfer Buffer	BioRad+ 20% MeOH	BioRad+ 20% MeOH
Blocking	5% BSA, 1 Hr	5% BSA, 1 Hr
Primary	1:2000 C219 Mouse Monoclonal (Cat#517310)	1:1000 C219 Mouse Monoclonal (Cat#517310)
Secondary	1:5000 BioRad goat anti-mouse + HRP (Cat#1706516)	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity (Cat# 1705060)	BioRad Clarity (Cat# 1705060)
Imager	BioRad ChemiDoc	BioRad ChemiDoc

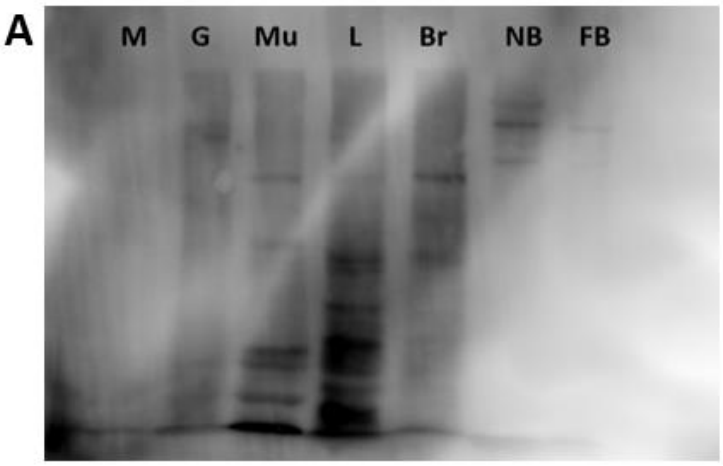
Figure A22: Western blots probed with different antibody dilutions. [A] Tuna western blot probed with 1:2000 primary and 1:5000 secondary. Western blot parameters to the right. **[B]** Tuna western blot probed with 1:1000 primary and 1:10,000 secondary. Western blot parameters to the right. Incubation refers to the heating time and temperature to denature the proteins prior to loading on the gels. All gels were run for 30 minutes at 60V then 45 minutes at 160V. Marker is All Blue Precision Plus Protein from BioRad (Cat # 1610373).

Because C219 has published data showing the antibody epitope, I had no trouble determining if it would work on the bees. Trying to do a western blot for honeybee ABCC1, on the other hand, did not go as smoothly. At the current state, I have not been able to find a commercially available ABCC1 antibody that works with honeybees or fish *in silico*. Many of the antibodies I found online did not list their epitopes for a variety of reasons from the epitope being proprietary knowledge to the manufacturer not knowing the actual epitope altogether. The antibodies that did list the ABCC1 epitopes were, unfortunately, incompatible with our samples. Going forward, we would have to create a construct for bee and fish ABCC1 ourselves if we want to western blot it.

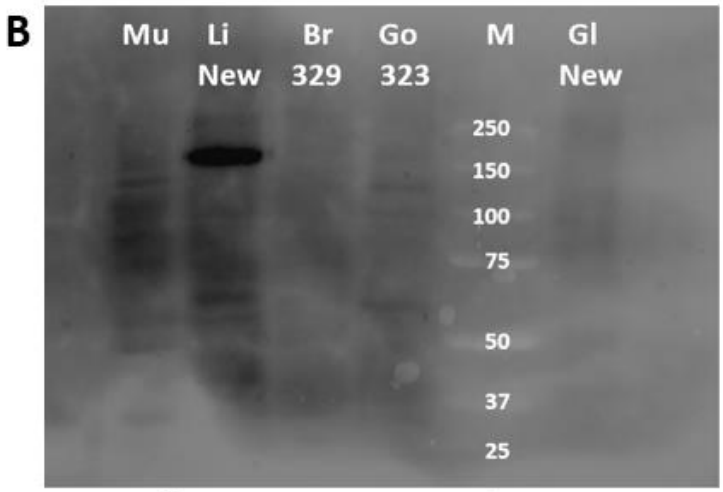
2.5. Chemiluminescence (ECL) Solutions

When we first got the western blot system, we received a few different ECL solutions to try with it: BioRad Clarity (Cat#1705060), BioRad Clarity Max (Cat#1705062), and Amersham ECL Select (Cat#45-000-999). Clarity Max, I only tested once (See figure A20). As it turns out, the Clarity Max solution is made for blots with very low protein content because the Clarity Max solution create a very right image (See Figure A21). Between the Clarity and Amersham ECL solutions, the clarity produced a brighter image with a lot of non-specific binding while the Amersham solution showed a cleaning western blot with bright target bands and lighter, yet still visible, non-specific bands (See Figure A22). Dr. Eleonora Cremonini, who has been a huge help with western blots, said that the Amersham solution might look better because this was imaged on a Thermo machine and suggested that I try imaging the BioRad Clarity solution on a BioRad Machine (see Figure A22). This turned out a lot a better and seeing as we have a BioRad ChemiDoc in the lab now, I would suggest continuing to use the BioRad Clarity ECL solution moving forward.

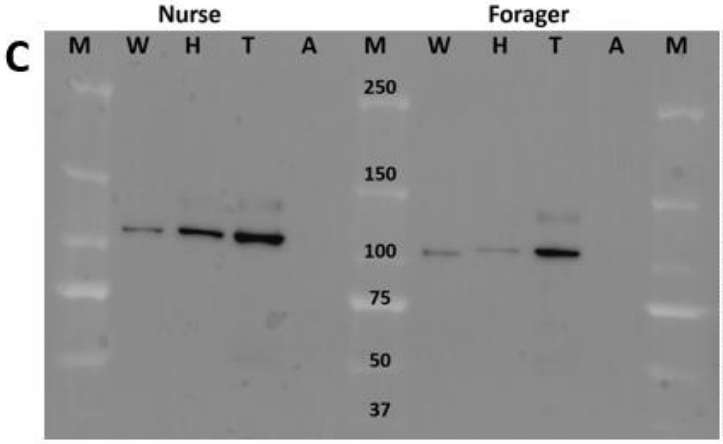
A huge note I would like to add about ECL solutions: check the expiration date. There was a good amount of time where I was doing back-to-back western blots and they all looked terrible (See Figure A23). It turned out the problem the whole time was that the ECL solution had expired. As soon as I got new ECL Solution, the images came out so much better. The solution expired in June, and I had been using it well through September with no good results before I realized the solution had degraded. ECL Solution lasts about a year on the shelf at room-temp so please keep track of those dates, so you do not lose any blots.



Parameters	Top
Extraction Method	Bead Mill
Protein Content	25 ug
Loading Buffer	Laemmli (Cat#1610737)
Incubation	70C for 10 Minutes
Gel Type	Hand-Cast 4%/7.5%
Running Buffer	BioRad (Cat#1610772)
Membrane	.2 BioRad PVDF (MeOH)
Transfer Buffer	BioRad+ 20% MeOH
Blocking	5% BSA, 1 Hr
Primary	1:2000 C219 Mouse Monoclonal (Cat#MA1-26528)
Secondary	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity (Cat# 1705060)
Imager	BioRad ChemiDoc



Parameters	Middle
Extraction Method	Bead Mill
Protein Content	25 ug
Loading Buffer	Laemmli (Cat#1610737)
Incubation	70C for 10 Minutes
Gel Type	Hand-Cast 4%/7.5%
Running Buffer	BioRad (Cat#1610772)
Membrane	.2 BioRad PVDF (MeOH)
Transfer Buffer	BioRad+ 20% MeOH
Blocking	5% BSA, 1 Hr
Primary	1:2000 C219 Mouse Monoclonal (Cat#517310)
Secondary	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity (Cat# 1705060)
Imager	BioRad ChemiDoc



Parameters	Bottom
Extraction Method	Bead Mill
Protein Content	25ug
Loading Buffer	Laemmli (Cat#1610737)
Incubation	70C for 10 Minutes
Gel Type	TruPAGE 4-12% Gradient (Cat#PCG2011-10EA)
Running Buffer	TruPAGE Buffer (Cat#PCG3001-4L)
Membrane	.45µm Immobilon-P (Sigma)
Transfer Buffer	Tricine TruPAGE + 20% MeOH
Blocking	5% BSA, 1 Hr
Primary	1:2000 C219 Mouse Monoclonal (Cat#517310)
Secondary	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity (Cat# 1705060)
Imager	BioRad ChemiDoc

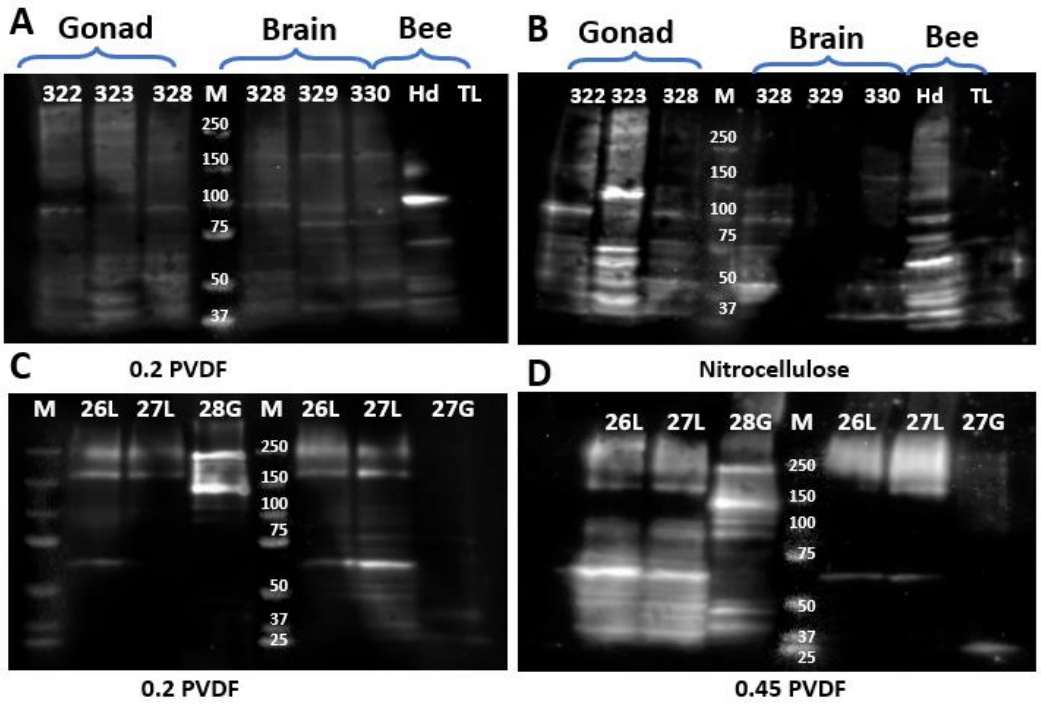
Figure A23: Western blots developed with expired and fresh ECL solution. [A] Tuna western blot developed with ECL one month past expiration. Western blot parameters to the right. **[B]** Tuna western blot developed with ECL 3 months past expiration. Western blot parameters to the right. **[C]** Honeybee western blot developed with new ECL. Western blot parameters to the right. Incubation refers to the heating time and temperature to denature the proteins prior to loading on the gels. All gels were run for 30 minutes at 60V then 45 minutes at 160V. Marker is All Blue Precision Plus Protein from BioRad (Cat # 1610373).

2.6. Membranes

The actual blotting membrane is another variable that you can change. I tested different materials (nitrocellulose vs. PVDF), different pore sizes (0.2µm vs 0.45µm), and different proprietary membranes as well (Immobilon-P, Immobilon-E, and Immobilon-P^{SQ}). Overall, the best membrane I found, and the one I use most often, is the 0.2µm PVDF. The nitrocellulose membrane shows a lot of non-specific binding and has a very splotchy background (see Figure A24b and A25a). It would be good for playing around with, but I would not use it if given the choice. Between 0.2µm vs 0.45µm pore size, it depends on personal preference. 0.2µm has a smaller pore size and would bind more protein, but if you are working with larger proteins, 0.45µm might be better so that the smaller non-target proteins can pass through them membrane without binding. Visually, I see no real difference between the 0.2µm vs 0.45µm (See Figure A24.

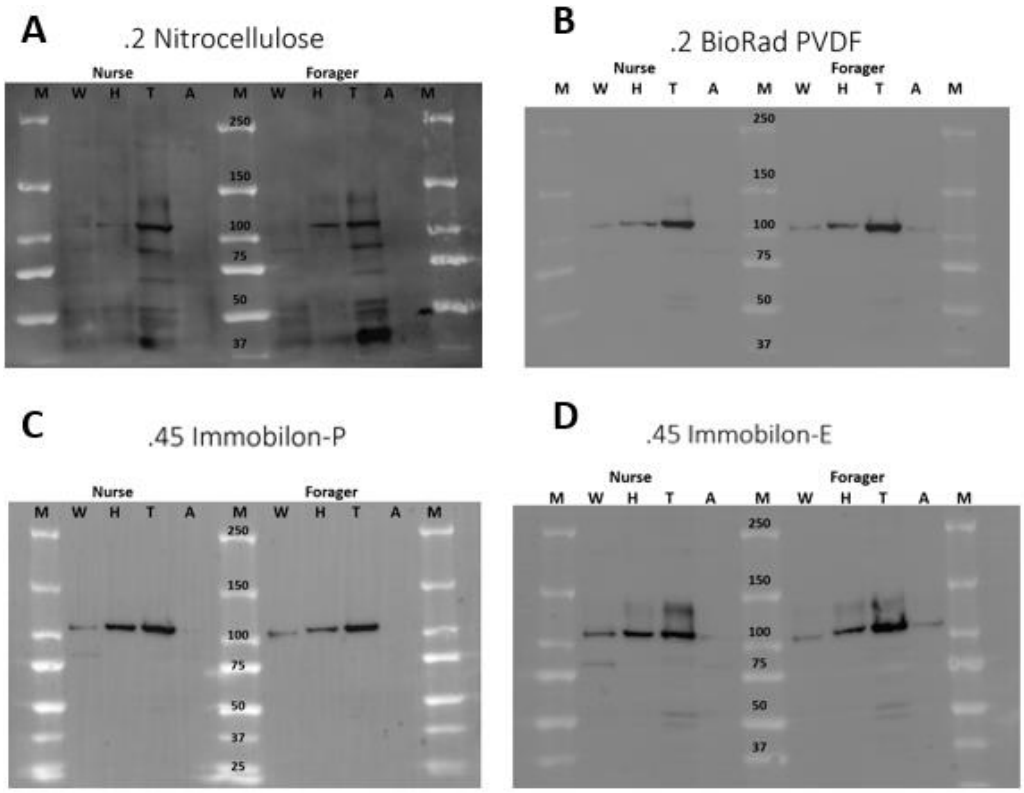
For the proprietary membranes from Sigma, I was given Immobilon-P, Immobilon-E, and Immobilon-P^{SQ}, all 0.45µm PVDF. The best one I found was the Immobilon-P, which is Sigma's basic PVDF membrane. The Immobilon-P came out the cleanest (see figure A25c). The Immobilon-E, which is the membrane that does not need to be activated with Methanol, showed a lot of aggregates and non-specific binding (see Figure A25d). The Immobilon-P^{SQ}, designed with more texture to catch smaller proteins, did not perform well for my purposes (see Figure 23a). However, I did test the Immobilon-P^{SQ} with bad ECL so the P^{SQ} might deserve another try.

Overall, I think the BioRad 0.22µm PVDF membrane works best with the 0.45µm Sigma Immobilon-P PVDF as a close second.



Parameters	0.2 PVDF	0.2 Nitrocellulose	0.2 PVDF	0.45 PVDF
Extraction Method	Bead Mill	Bead Mill	Bead Mill	Bead Mill
Protein Content	25 ug	25ug	25 ug	25ug
Loading Buffer	Laemmli	Laemmli	Laemmli	Laemmli
Incubation	70C for 10 Minutes	70C for 10 Minutes	70C for 10 Minutes	70C for 10 Minutes
Gel Type	Hand-Cast (4/7.5%)	Hand-Cast (4/7.5%)	Hand-Cast (4/7.5%)	Hand-Cast (4/7.5%)
Running Buffer	BioRad (Cat#1610772)	BioRad (Cat#1610772)	BioRad (Cat#1610772)	BioRad (Cat#1610772)
Membrane	.2µm BioRad PVDF (MeOH)	.2µm BioRad PVDF (MeOH)	.2µm BioRad PVDF (MeOH)	.2µm BioRad PVDF (MeOH)
Transfer Buffer	BioRad+ 20% MeOH	BioRad+ 20% MeOH	BioRad+ 20% MeOH	BioRad+ 20% MeOH
Blocking	None	None	None	None
Primary	1:200 C219 Mouse Monoclonal (Cat#MA1-26528)	1:200 C219 Mouse Monoclonal (Cat#MA1-26528)	1:200 C219 Mouse Monoclonal (Cat#MA1-26528)	1:200 C219 Mouse Monoclonal (Cat#MA1-26528)
Secondary	1:5000 BioRad goat anti-mouse + HRP (Cat#1706516)	1:5000 BioRad goat anti-mouse + HRP (Cat#1706516)	1:5000 BioRad goat anti-mouse + HRP (Cat#1706516)	1:5000 BioRad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity (Cat# 1705060)	BioRad Clarity (Cat# 1705060)	BioRad Clarity (Cat# 1705060)	BioRad Clarity (Cat# 1705060)
Imager	Thermo My ECL	Thermo My ECL	Thermo My ECL	Thermo My ECL

Figure A24: Western blots performed on four different membranes. [A] Tuna and bee blotted on 0.2 PVDF. Western blot parameters below. **[B]** Tuna and bee blotted on 0.2 nitrocellulose. Western blot parameters below. **[C]** Tuna blotted on 0.2 PVDF. Western blot parameters below. **[D]** Tuna blotted on 0.45 PVDF. Western blot parameters below. Incubation refers to the heating time and temperature to denature the proteins prior to loading on the gels. All gels were run for 30 minutes at 60V then 45 minutes at 160V. Marker is All Blue Precision Plus Protein from BioRad (Cat # 1610373).

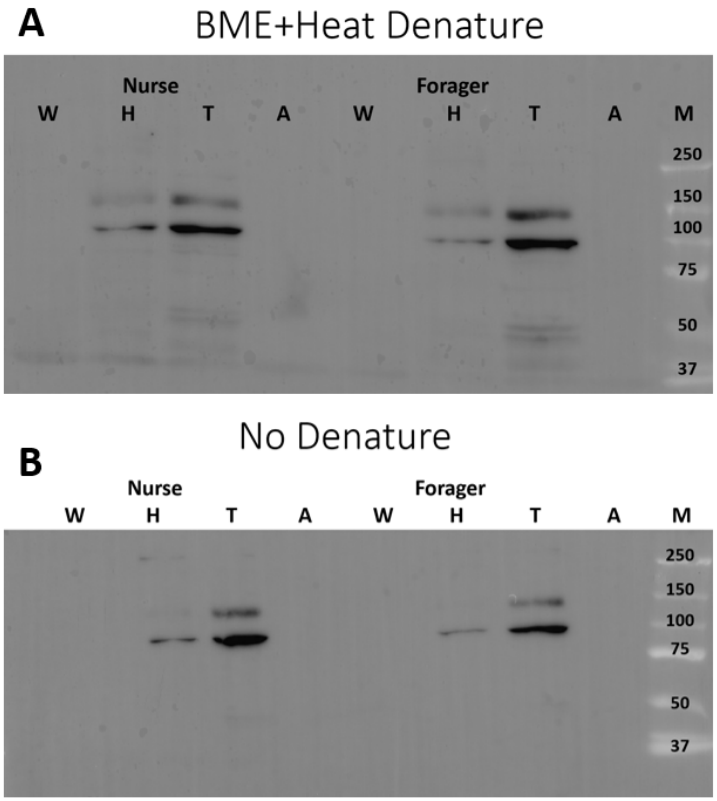


Parameters	.2 Nitrocellulose	.2 BioRad PVDF	.45 Immobilon-P	.45 Immobilon-E
Extraction Method	Bead Mill	Bead Mill	Bead Mill	Bead Mill
Protein Content	40 ug	40 ug	40 ug	40 ug
Loading Buffer	Laemmli (Cat#1610737)	Laemmli (Cat#1610737)	Laemmli (Cat#1610737)	Laemmli (Cat#1610737)
Incubation	70C for 10 Minutes	70C for 10 Minutes	70C for 10 Minutes	70C for 10 Minutes
Gel Type	TruPAGE 4-12% Gradient (Cat#PCG2011-10EA)	TruPAGE 4-12% Gradient (Cat#PCG2011-10EA)	TruPAGE 4-12% Gradient (Cat#PCG2011-10EA)	TruPAGE 4-12% Gradient (Cat#PCG2011-10EA)
Running Buffer	TruPAGE Buffer (Cat#PCG3001-4L)	TruPAGE Buffer (Cat#PCG3001-4L)	TruPAGE Buffer (Cat#PCG3001-4L)	TruPAGE Buffer (Cat#PCG3001-4L)
Membrane	.45µm Immobilon-P (Sigma)	.45µm Immobilon-P (Sigma)	.45µm Immobilon-P (Sigma)	.45µm Immobilon-P (Sigma)
Transfer Buffer	Tricine TruPAGE + 20% MeOH	Tricine TruPAGE + 20% MeOH	Tricine TruPAGE + 20% MeOH	Tricine TruPAGE + 20% MeOH
Blocking	5% BSA, 1 Hr	5% BSA, 1 Hr	5% BSA, 1 Hr	5% BSA, 1 Hr
Primary	1:2000 C219 Mouse Monoclonal (Cat#517310)	1:2000 C219 Mouse Monoclonal (Cat#517310)	1:2000 C219 Mouse Monoclonal (Cat#517310)	1:2000 C219 Mouse Monoclonal (Cat#517310)
Secondary	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity (Cat# 1705060)	BioRad Clarity (Cat# 1705060)	BioRad Clarity (Cat# 1705060)	BioRad Clarity (Cat# 1705060)
Imager	BioRad ChemiDoc	BioRad ChemiDoc	BioRad ChemiDoc	BioRad ChemiDoc

Figure A25: Western blots performed on four different membranes. [A] Honeybee blotted on 0.2 Nitrocellulose. Western blot parameters below. **[B]** Honeybee blotted on 0.2 PVDF. Western blot parameters below. **[C]** Honeybee blotted on 0.45 Immobilon-P. Western blot parameters below. **[D]** Honeybee blotted on 0.45 Immobilon-E. Western blot parameters below. Incubation refers to the heating time and temperature to denature the proteins prior to loading on the gels. All gels were run for 30 minutes at 60V then 45 minutes at 160V. Marker is All Blue Precision Plus Protein from BioRad (Cat # 1610373).

2.7. Chemical and Heat Denaturation

As seen in Figure A26, the *Am*-ABCB1 shows up at ~100 kDa. However, the theoretical weight should be ~147 kDa. This is a natural protein derived from live bees, so we expected some differences between the theoretical and actual value, but we expected the protein to be larger, not smaller. In live animals, the protein could go through post-translational modification that would make the protein larger. Our problem is that the protein we have is significantly smaller. Maybe this is due to degradation and pieces of the protein were cleaved off.

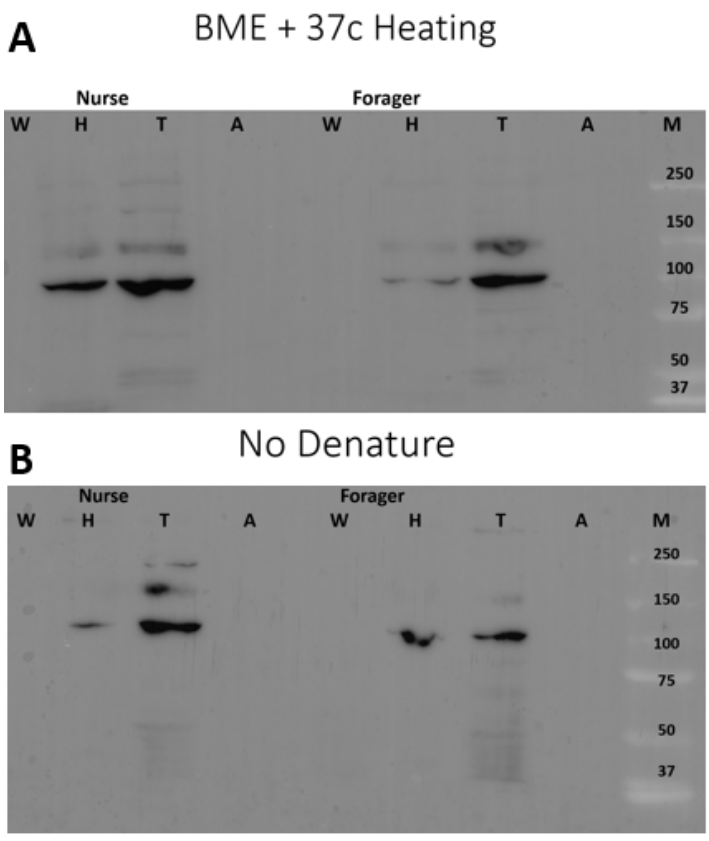


Parameters	BME + Heat Denature
Extraction Method	Bead Mill
Protein Content	40 ug
Loading Buffer	Laemmli, 0.05% BME (Cat#1610737)
Incubation	70C for 10 Minutes
Gel Type	Hand-Cast 4%/7.5%
Running Buffer	BioRad (Cat#1610772)
Membrane	.2 BioRad PVDF (MeOH)
Transfer Buffer	BioRad+ 20% MeOH
Blocking	5% BSA, 1 Hr
Primary	1:2000 C219 Mouse Monoclonal (Cat#517310)
Secondary	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity (Cat# 1705060)
Imager	BioRad ChemiDoc

Parameters	No Denature
Extraction Method	Bead Mill
Protein Content	40 ug
Loading Buffer	Laemmli (Cat#1610737)
Incubation	None
Gel Type	Hand-Cast 4%/7.5%
Running Buffer	BioRad (Cat#1610772)
Membrane	.2 BioRad PVDF (MeOH)
Transfer Buffer	BioRad+ 20% MeOH
Blocking	5% BSA, 1 Hr
Primary	1:2000 C219 Mouse Monoclonal (Cat#517310)
Secondary	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity (Cat# 1705060)
Imager	BioRad ChemiDoc

Figure A26: Western blots with two different sample preparation methods. [A] Honeybee western blot with samples denatured with heat and BME. Western blot parameters to the right. **[B]** Honeybee western blot with no denatured samples. Western blot parameters to the right. Incubation refers to the heating time and temperature to denature the proteins prior to loading on the gels. All gels were run for 30 minutes at 60V then 45 minutes at 160V. Marker is All Blue Precision Plus Protein from BioRad (Cat # 1610373).

ResearchGate (https://www.researchgate.net) suggested changing the denaturation steps to minimize degradation, so I ran a few tests with heating and BME. First, I tested a western with heat (70ºC for 10 minutes) and BME denaturation against a western blot with no heat and no BME denaturation. Although the un-denatured blot shows no degradation pattern, the denatured blot had more bands at the correct size (see Figure A26). For membrane proteins, ResearchGate suggested heating at lower temperature for longer to prevent protein aggregate from forming so I also tested a blot with BME and heat (37ºC for 45 minutes) denaturation against another blot with no denaturation (see Figure A27).



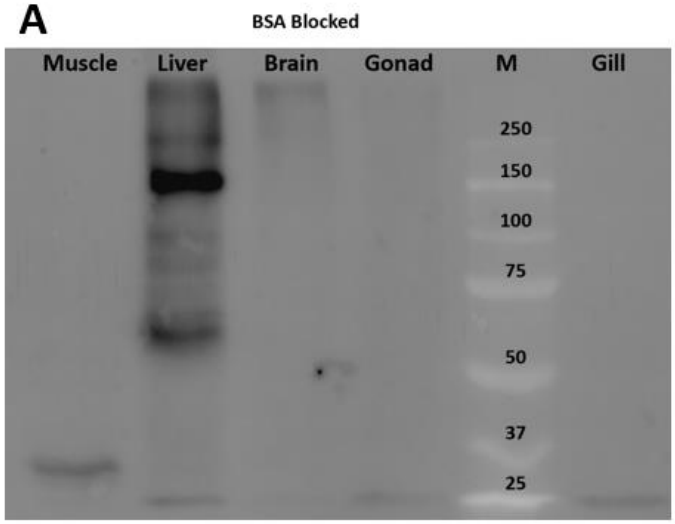
Parameters	BME + Heat Denature
Extraction Method	Bead Mill
Protein Content	40 ug
Loading Buffer	Laemmli, 0.05% BME (Cat#1610737)
Incubation	37C for 45 Minutes
Gel Type	Hand-Cast 4%/7.5%
Running Buffer	BioRad (Cat#1610772)
Membrane	.2 BioRad PVDF (MeOH)
Transfer Buffer	BioRad+ 20% MeOH
Blocking	5% BSA, 1 Hr
Primary	1:2000 C219 Mouse Monoclonal (Cat#517310)
Secondary	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity (Cat# 1705060)
Imager	BioRad ChemiDoc

Parameters	No Denature
Extraction Method	Bead Mill
Protein Content	40 ug
Loading Buffer	Laemmli (Cat#1610737)
Incubation	None
Gel Type	Hand-Cast 4%/7.5%
Running Buffer	BioRad (Cat#1610772)
Membrane	.2 BioRad PVDF (MeOH)
Transfer Buffer	BioRad+ 20% MeOH
Blocking	5% BSA, 1 Hr
Primary	1:2000 C219 Mouse Monoclonal (Cat#517310)
Secondary	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity (Cat# 1705060)
Imager	BioRad ChemiDoc

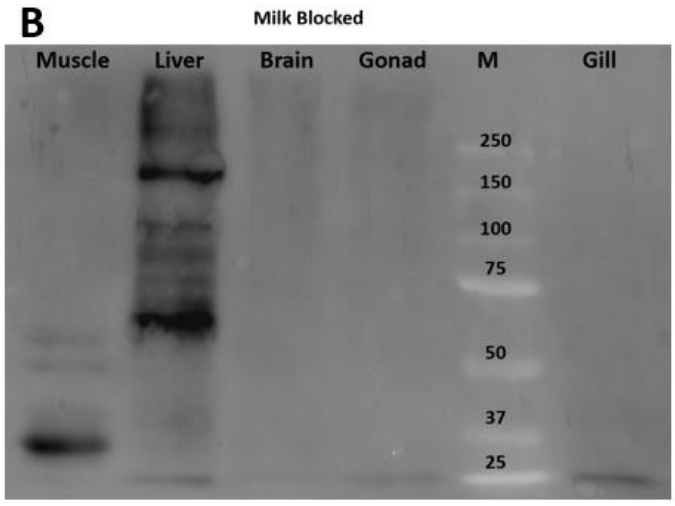
Figure A27: Western blots with two different sample preparation methods. [A] Honeybee western blot with samples denatured with heat and BME. Western blot parameters to the right. **[B]** Honeybee western blot with no denatured samples. Western blot parameters to the right. Incubation refers to the heating time and temperature to denature the proteins prior to loading on the gels. All gels were run for 30 minutes at 60V then 45 minutes at 160V. Marker is All Blue Precision Plus Protein from BioRad (Cat # 1610373)

2.8. Blocking

Blocking is an important step to prevent non-specific binding down the line. There are two conventional ways to block a western blot, skim milk and BSA (see Figure A28). If blocking with milk, make sure it is skim milk and not whole milk because whole milk has proteins that could cause non-specific binding. Overall, BSA provides a clearer image, but BSA is also more expensive. Milk works fine as a cheap and easy way to block your western blots.



Parameters	BSA
Extraction Method	Glass Homogenizer
Protein Content	20 ug
Loading Buffer	Laemmli, 0.05% BME (Cat#1610737)
Incubation	37C for 45min
Gel Type	Hand-Cast 4%/7.5%
Running Buffer	BioRad (Cat#1610772)
Membrane	.2 BioRad PVDF (MeOH)
Transfer Buffer	BioRad+ 20% MeOH
Blocking	5% BSA, 1 Hr
Primary	1:2000 C219 Mouse Monoclonal (Cat#517310)
Secondary	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity (Cat# 1705060)
Imager	BioRad ChemiDoc



Parameters	Milk
Extraction Method	Glass Homogenizer
Protein Content	20 ug
Loading Buffer	Laemmli, 0.05% BME (Cat#1610737)
Incubation	37C for 45min
Gel Type	Hand-Cast 4%/7.5%
Running Buffer	BioRad (Cat#1610772)
Membrane	.2 BioRad PVDF (MeOH)
Transfer Buffer	BioRad+ 20% MeOH
Blocking	5% Milk, 1 Hr
Primary	1:2000 C219 Mouse Monoclonal (Cat#517310)
Secondary	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity (Cat# 1705060)
Imager	BioRad ChemiDoc

Figure A28: Western blots blocked with two different blocking buffers. [A] Honeybee western blot blocked with BSA. Western blot parameters to the right. **[B]** Honeybee western blot blocked with milk. Western blot parameters to the right. Incubation refers to the heating time and temperature to denature the proteins prior to loading on the gels. All gels were run for 30 minutes at 60V then 45 minutes at 160V. Marker is All Blue Precision Plus Protein from BioRad (Cat # 1610373).

2.9. *Ponceau Staining*

Ponceau staining the membrane, along with Coomassie staining the gel, helps to ensure total protein transfer. Ponceau also helps to visualize a BSA control if you ran one. Ponceau is a relatively easy step to add to a protocol. After blotting onto the membrane, rinse the membrane and stain in Ponceau (0.01% Ponceau S, 5% Glacial Acetic Acid) for 1-2 minutes. Destain by rinsing quickly with clean water. Be careful not to destain too much because Ponceau is easily washed off. Just destain until the background is light pink then dry on the benchtop between two sheets of filter paper before imaging. This is a good place to stop and store the membrane if you do not have time to finish the protocol (see section 2.10). After imaging, reactive the membrane with methanol if PVDF or TBST if nitrocellulose and continue with the rest of the protocol. No need to destain completely because the TBST from the blocking buffer will fully destain the membrane.

An important note with Ponceau stain, you must keep track of how old the staining solution is because Ponceau no longer works after 6 months (see Figure A29). Ponceau can be continuously reused like Coomassie but after 6 months it will no longer stain your proteins so just toss it and make a fresh batch.

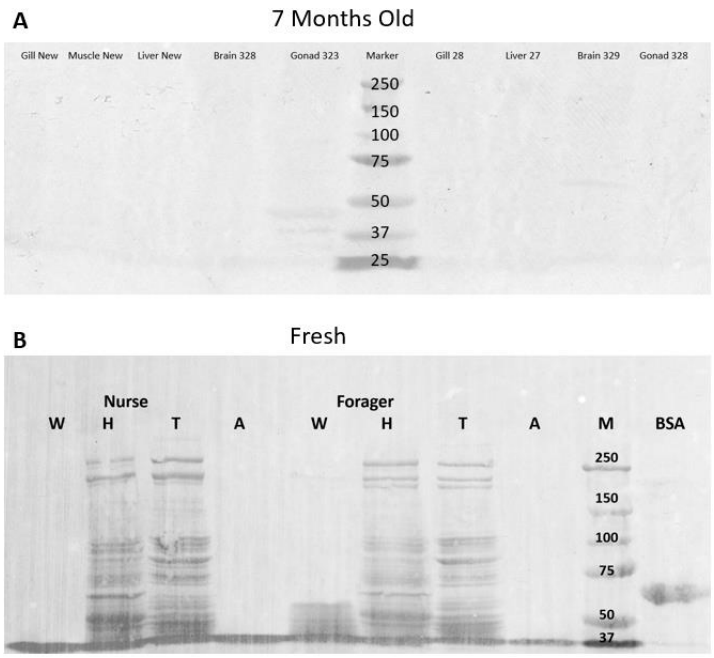


Figure A29: Western blot membrane Ponceau stains. [A] Western blot stained with ponceau that was 7 months old. **[B]** Western blot stained with fresh ponceau stain.

2.10. Western Blot Storage, Stripping, and Re-Probing

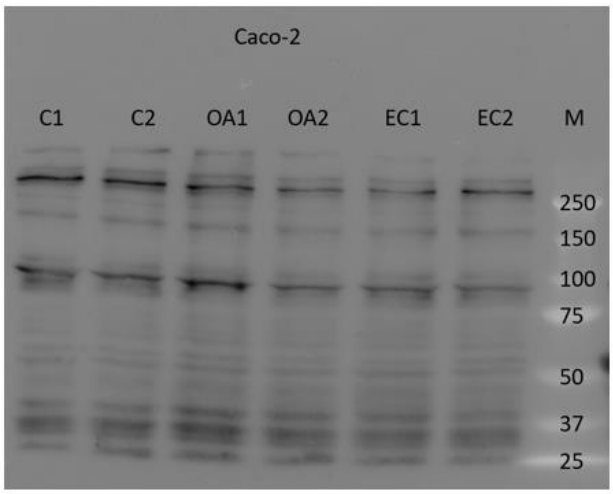
Western Blots can be reused. This could save you several days-worth of work if you just want to re-probe the same samples. After you image your blot the first time, you can strip with a mild (15g glycine, 1g SDS, 10mL Tween 20, pH 2.2, water to 1L), dry between two filter papers on the benchtop, then seal in plastic and store in the freezer for up to a year. Note, always strip before you dry because after you dry, the proteins and antibodies will be permanently bound to the membrane. You will not be able to strip as effectively after you've dried the membrane.

To re-probe, thaw the membrane and reactive in methanol if working with PVDF or TBST if working with nitrocellulose. Once the membrane is reactivated, you can incubate in primary and secondary like normal then image.

Western blot storage can also give you more flexibility with your protocol. If you ran the gel and the blotted it onto the membrane already but you do not have time to probe or image, you can dry the membrane between two filter papers on the benchtop then seal it in plastic and store it in the freezer for up to a year. Then, when you're ready to probe, just thaw the membrane and reactive in methanol or TBST depending on the membrane type and continue with the protocol like usual.

2.11. Side Projects

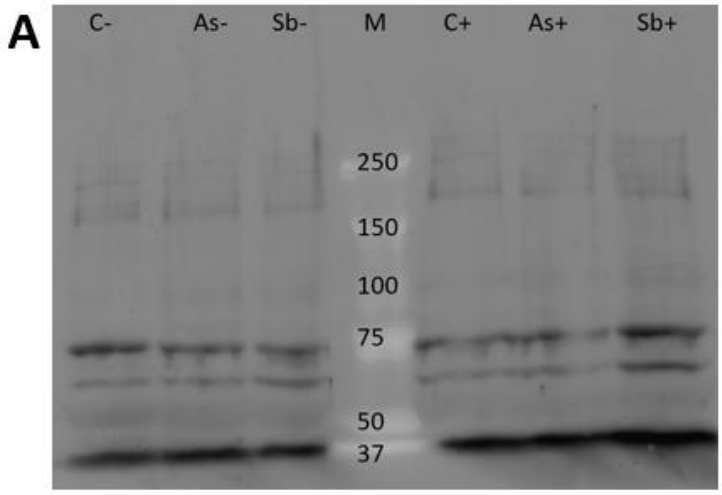
I did a few western blots on the side. The first was the CaCo-2 cells from Dr. Patricia Orteiza (UC Davis) and Eleonora. The CaCo-2 western blot confirms the presence of ABCB1 in the intestinal cells (see Figure A30). I also ran some skin cell western blots for a collaboration with Dr. Robert Rice (UC Davis). The skin western blot did not show promise for a collaboration on ABCC2, but the ABCB1 western confirmed the presence of ABCB1 and further testing can go forward (See Figure A31). The last little side western I did was the Sturgeons. As seen in Figure A15 from section 2.1, sturgeons do have ABCB1 and they can be probed with C219.



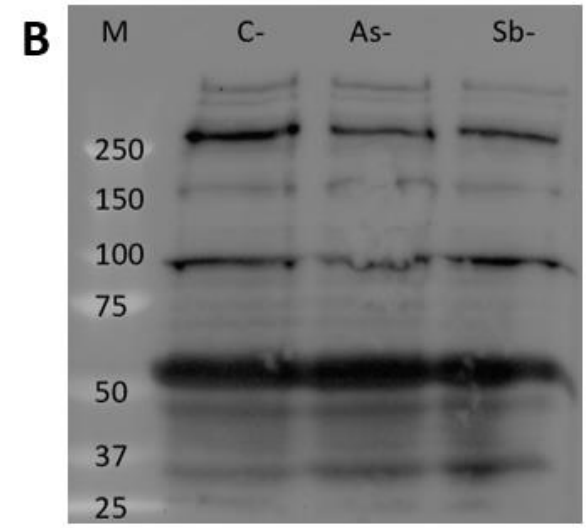
Parameters	CaC0-2 ABCB1
Extraction Method	Bead Mill*
Protein Content	30ug*
Loading Buffer	Laemmli*
Incubation	95C for 5 Minutes*
Gel Type	Hand-Cast 4%/7.5%
Running Buffer	BioRad (Cat#1610772)
Membrane	.2 BioRad PVDF (MeOH)
Transfer Buffer	BioRad+ 20% MeOH
Blocking	5% BSA, 1 Hr
Primary	1:2000 C219 Mouse Monoclonal (Cat#517310)
Secondary	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity (Cat# 1705060)
Imager	BioRad ChemiDoc

*Prepared by Eleonora

Figure A30: CaCo-2 Cell western blot probed with C219 antibody. Samples prepared by Eleonora. C1=control 1, C2=Control 2, OA1=Oleic Acid 1, OA2=Oleic Acid 2, EC1= Epicatechin 1, EC2= Epicatechin 2. Incubation refers to the heating time and temperature to denature the proteins prior to loading on the gels. All gels were run for 30 minutes at 60V then 45 minutes at 160V. Marker is All Blue Precision Plus Protein from BioRad (Cat # 1610373).



Parameters	Skin ABCC2
Extraction Method	Bead Mill
Protein Content	20 ug
Loading Buffer	Laemmli, 0.05% BME (Cat#1610737)
Incubation	37C for 45min
Gel Type	Hand-Cast 4%/7.5%
Running Buffer	BioRad (Cat#1610772)
Membrane	.2 BioRad PVDF (MeOH)
Transfer Buffer	BioRad+ 20% MeOH
Blocking	5% BSA, 1 Hr
Primary	1:2000 C219 Mouse Monoclonal (Cat#517310)
Secondary	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity (Cat# 1705060)
Imager	BioRad ChemiDoc



Parameters	Skin ABCB1
Extraction Method	Bead Mill
Protein Content	20 ug
Loading Buffer	Laemmli, 0.05% BME (Cat#1610737)
Incubation	37C for 45min
Gel Type	Hand-Cast 4%/7.5%
Running Buffer	BioRad (Cat#1610772)
Membrane	.2 BioRad PVDF (MeOH)
Transfer Buffer	BioRad+ 20% MeOH
Blocking	5% BSA, 1 Hr
Primary	1:2000 C219 Mouse Monoclonal (Cat#517310)
Secondary	1:10,000 BioRad goat anti-mouse + HRP (Cat#1706516)
ECL Solution	BioRad Clarity (Cat# 1705060)
Imager	BioRad ChemiDoc

Figure A31: Skin Cell western blot probed with anti-ABCC2 and C219. Samples from Dr. Rice. C=control, As=Arsenic, Sb=Antimony. +/- denotes the presence or absence of EGF. Incubation refers to the heating time and temperature to denature the proteins prior to loading on the gels. All gels were run for 30 minutes at 60V then 45 minutes at 160V. Marker is All Blue Precision Plus Protein from BioRad (Cat # 1610373).

Appendix 3: qPCR

3.1. Absolute vs Relative

There are two methods of setting up and analyzing a qPCR. Relative qPCR uses a housekeeping gene to normalize the data and quantify the unknown samples. Absolute qPCR uses a standard curve of known DNA concentrations to quantify the unknown samples and can give a more quantified answer. Where Relative qPCR can only give you fold-change, absolute can give you actual amounts DNA of the samples.

Relative qPCR is a very easy way to run an analysis. The housekeeping gene is used to normalize the unknowns and set a common baseline so you can compare the unknown samples more accurately. You can run more samples at a time with relative qPCR because they can all use the same housekeeping gene for normalization of the whole plate. With absolute qPCR, each primer pair must have its own standard curve.

3.2. cDNA vs Plasmid Standard

There are 2 Types of Absolute qPCR: cDNA standard that can give you a readout of DNA concentration and Plasmid standard that can give you a readout of total copy number. With the cDNA standard, the setup is a bit different from a relative qPCR. Like previously state, for each primer pair you want to test, you must have a standard. You must also know the exact concentrations of your cDNA standard so you can make a standard curve to compare you unknown to later. The standard should have a dilution pattern (for example, each standard sample is a 1:10 dilution). You should also use the higher concentration of cDNA you have because if your cDNA is not concentrated enough, your unknown samples might not fall on the standard curve.

The set up for an absolute qPCR with a plasmid standard is like setup with a cDNA standard. Each primer pair must have its own standard and Plasmid standards should have a dilution pattern. The standard uses Plasmids with the target gene already in it. Plasmids can be used to calculate copy number of your target gene using the equation:

$$Number\ of\ Copies = \frac{DNA\ Conentration\ (ng)\times 6.022\times 10^{23}}{Lenth\ of\ DNA\ (bp)\times 10^{9}\times 650}.$$

Plasmid standards can give you a more accurate value for your target gene in the unknown samples. The known DNA concentration will be used to create the standard curve graph (see Figure A33). When using a plasmid standard for your qPCR, you can know the exact number of copies of your genes in the standard so you can calculate the exact number of copies of your genes in your unknown sample.

3.3. Difference in Data between Relative and Absolute

With different methods of quantification, you get different data. When running a relative qPCR, you can only get fold-changes of gene expression relative to the control. For the Honeybees, I had GAPDH as a reference to normalize the data, then I set the fragment that is present in both X1 and X6 as my control. From here I was able to get a fold-change for just X1 vs X1+X6. I used these fold-change numbers to calculate the estimated percent of X1 and X2 isoform that are present in different parts of the honeybee body (see Figure A32a). The relative qPCR showed that there was a higher amount of X6 gene variant in all parts of the bee expect in forager abdomens. Higher X6 in forager abdomen could mean that the X2 protein isoform of ABCB1 could be more associated with gut protection of xenobiotics because the foragers are the ones that are most exposed to external toxins.

With an absolute qPCR, you can get a more quantified value for your samples because you will have a standard curve to compare to your unknown samples. The bee qPCR I ran with a cDNA standard gave me actual concentrations of each gene variant based on the concentration of the standard curve (See Figure A32c). I used these concentrations to calculate the percentages of each variant in each honeybee body part (See Figure A32b). The results from the absolute qPCR were similar to the relative qPCR, showing that there is more X6 variant in all parts of the bee body than the X1 variant. A difference between the relative and absolute values were the forager abdomen numbers. Relative showed that X1 was significantly more abundant in the forager abdomen, but the absolute qPCR showed that the values are no different from nurse bees. This absolute qPCR showed that there was no difference in expression between nurse and forager bees despite their different job descriptions.

The absolute plasmid qPCR was meant to be a tiebreaker and give the most accurate values. However, the percentage values for this qPCR (See Figure 32d) showed extremely low, almost non-existent, values for the X1 isoform. This data corroborates the idea that nurse and foragers have no difference in expression and that the X2 isoform (or the X6 gene variant) is most abundant in the bee. I am skeptical about the copy numbers, however (see figure A32e). I would do at least one more absolute plasmid qPCR to be certain of the values.

A

Relative qPCR				
	Nurse		Forager	
	X1	X2	X1	X2
Whole	4%	96%	63%	37%
Head	8%	92%	8%	92%
Thorax	10%	90%	8%	92%
Abdomen	27%	73%	85%	15%

B

Absolute qPCR - cDNA Standard				
	Nurse		Forager	
	X1	X2	X1	X2
Whole	14%	86%	25%	75%
Head	7%	93%	10%	90%
Thorax	8%	92%	6%	94%
Abdomen	33%	67%	27%	73%

C

Absolute qPCR - cDNA Standard				
	Nurse		Forager	
	X1	X2	X1	X2
Whole	2901 ng/µL	18241 ng/µL	9050 ng/µL	27772 ng/µL
Head	182 ng/µL	2343 ng/µL	287 ng/µL	2460 ng/µL
Thorax	611 ng/µL	7043 ng/µL	1162 ng/µL	17923 ng/µL
Abdomen	8412 ng/µL	16985 ng/µL	4824 ng/µL	12842 ng/µL

D

Absolute qPCR - Plasmid Standard				
	Nurse		Forager	
	X1	X2	X1	X2
Whole	2.81%	97.19%	1.48%	98.52%
Head	0.44%	99.56%	0.04%	99.96%
Thorax	0.44%	99.56%	0.07%	99.93%
Abdomen	6.35%	93.65%	0.69%	99.31%

E

Absolute qPCR - Plasmid Standard				
	Nurse		Forager	
	X1	X2	X1	X2
Whole	34.5 C/µL	1194.1 C/µL	223.1 C/µL	14818.7 C/µL
Head	0.5 C/µL	101.9 C/µL	0.8 C/µL	2113.5 C/µL
Thorax	2.9 C/µL	653.5 C/µL	7.0 C/µL	10592.1 C/µL
Abdomen	184.2 C/µL	2717.2 C/µL	83.8 C/µL	11987.1 C/µL

Figure A32: Ratios of *Am*-ABCB1 isoforms in nurse and forager bee sections using relative qPCR. **[A]** The results of the relative qPCR show the X2 isoform is dominant in all parts of the nurse bee anatomy whereas in the forager bee, X2 is only dominant in the Head and Thorax. Similar abundance is seen the head and thorax of both nurse and forager, but inverse proportions are seen in abdomen of nurse and foragers. These numbers are reflected in the differences between nurse and forager whole. Relative qPCR was normalized to a primer pair that bound to both isoforms and GAPDH was used as a reference gene. **[B]** The results of the absolute qPCR using a cDNA standard show higher levels of X2 isoform in all samples and similar isoform abundance in both nurse and forager bees. **[C]** Results of the Absolute qPCR with cDNA standard shown as DNA concentration. **[D]** The results of the Absolute qPCR with the plasmid standard show much lower levels of the X1 variant overall. **[E]** Results of the Absolute qPCR with Plasmid standards shown as copy number per uL of DNA. The values represent the mean of duplicate measurements using cDNA prepared from 1 bee per whole sample and 2-3 bees per dissected sample.

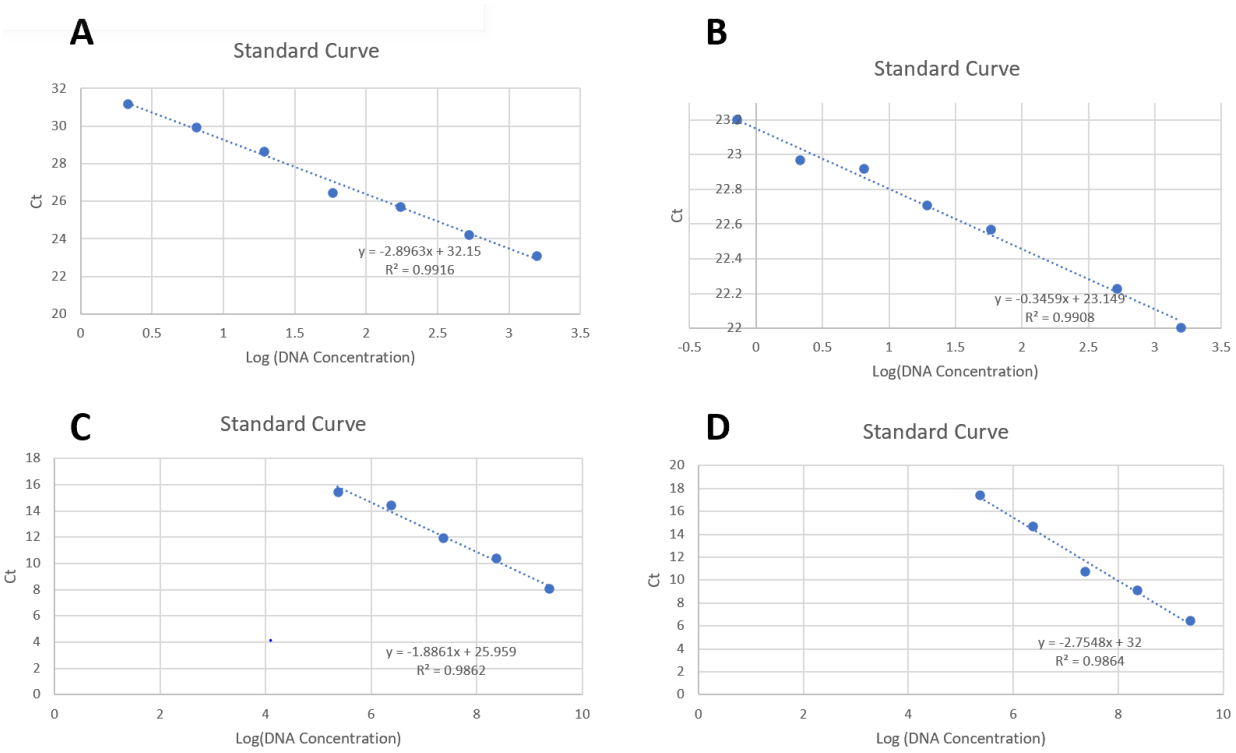


Figure A33: Standard curve charts for the absolute qPCR. [A] Standard cDNA curve for the X1 only fragment primer pair. **[B]** Standard cDNA curve for the X1+X6 fragment primer pair. **[C]** Standard plasmid curve for the X1 only fragment primer pair. **[D]** Standard plasmid curve for the X1+X6 fragment primer pair.

3.4. qPCR Primer Design Optimization

For qPCR, you only need fragment between 80-200. The best way I have found for making primers for qPCR (and general cloning) is https://primer3plus.com/cgi-bin/dev/primer3plus.cgi. You just copy in your sequence, change a few parameters (See Figures A33-A37), and the program will return as many primer pairs as you want. Changing these parameters will ensure you get the right size and optimized primers.

Once you have your primers, I usually copy and paste them into an excel spreadsheet similar to Table A7. Here you will also copy in the Tm and the fragment length from Primer3plus.

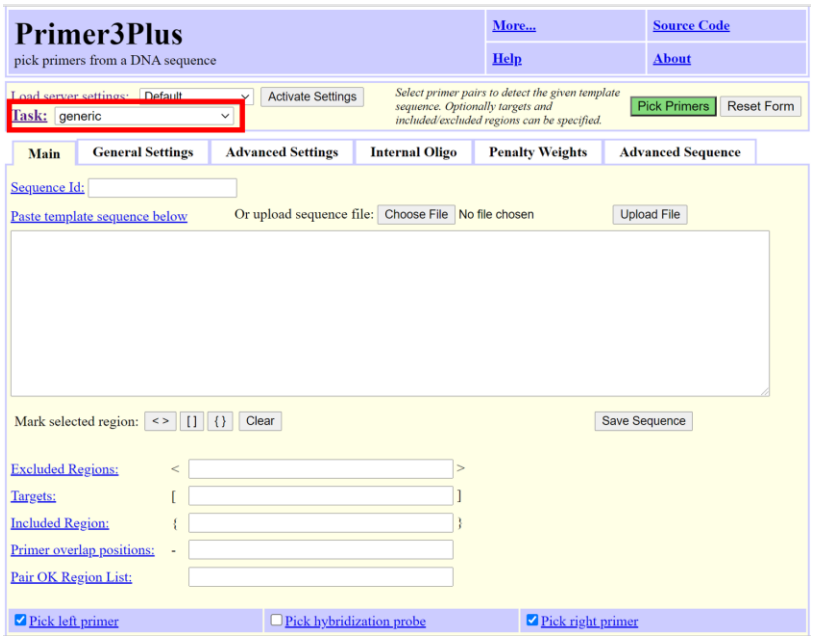


Figure A34: Parameters for "Main" tab of Primer3Plus. For designing qPCR primer pairs, leave the task as "generic." If making cloning primers, change task to "sequencing."

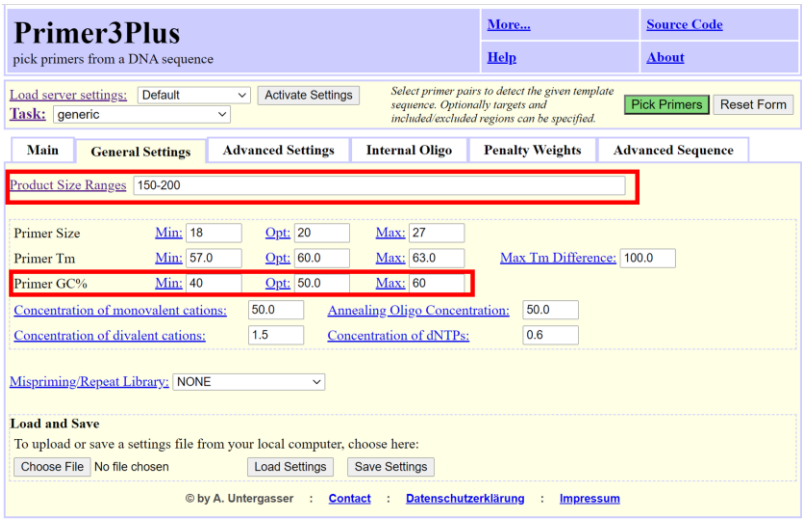


Figure A35: Parameters for "General Settings" tab of Primer3Plus. For designing qPCR primer pairs, change the product size range to your desired size, usually 80-150bp or 150-200bp. If making cloning primers, change desired size range to 600-800. For any primer design change Primer GC% to have a minimum of 40% and a maximum of 60%.

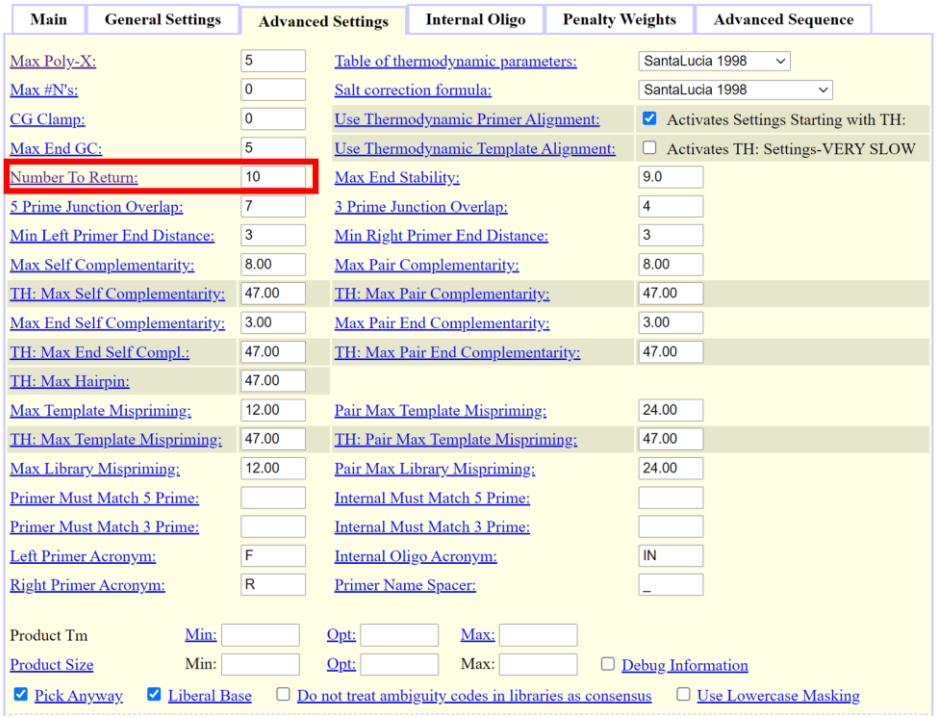


Figure A36: Parameters for "Advanced Settings" tab of Primer3Plus. The only thing you need to change on this tab is the number to return. Default is 5 but you should set it to 10 or 20 to maximize number of primers.

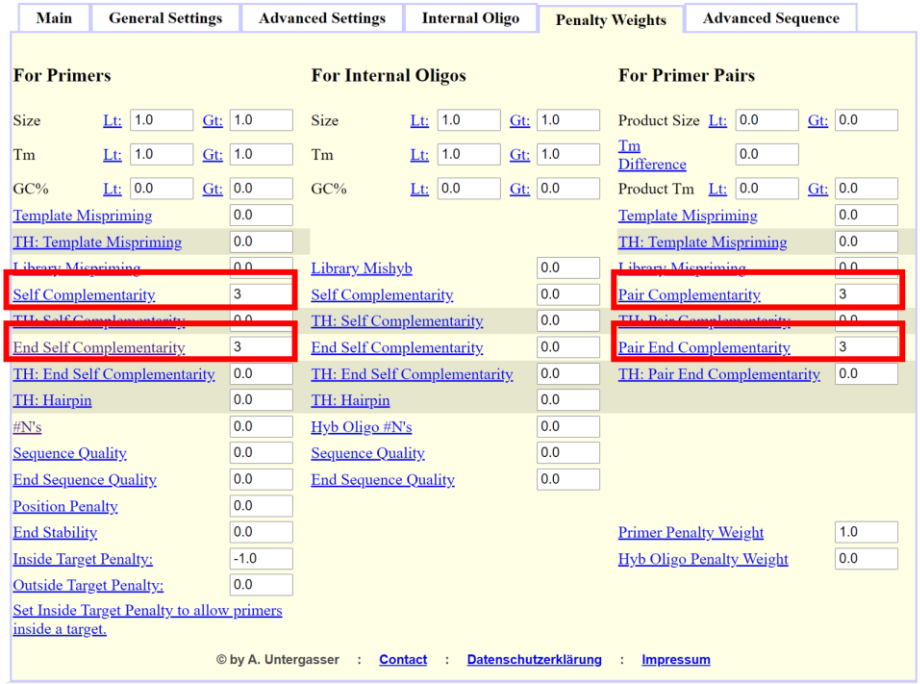


Figure A37: Parameters for "Penalty Weights" tab of Primer3Plus. Increasing the penalty weights for self and pair complementarity will decrease the change of Primer3Plus giving you primer pairs that would create dimers. Change all highlighted fields to 3.

The next step is to further optimize your primers by taking the primer pairs that Primer3Plus gave you and running them through another software called Beacon Designer (https://www.premierbiosoft.com/qOligo/Oligo.jsp?PID=1). Beacon designer will give you penalty values for each primer pair. Make sure to change the assay type to SYBR green (see Figure A38. The values will represent self-dimers, cross dimers, and hairpin binding. You will want to sum up all the penalty values for each primer pair and choose the primers that have to lowest penalty weight

Table A7: Template excel sheet for primer design optimization. This is where you will fill in the primers, melting temperature (Tm), fragment length (bp), penalty values for the self-dimerization of the forward primer, hairpin binding of the forward primer, cross dimerization of the primer pair, self-dimerization of the reverse primer, and hairpin binding of the reverse primer. There is also a template on Box.com under Lab Protocols>Molecular Biology>PCR>Primer3Plus + Beacon Designer.

	Forward	Tm	Reverse	Tm	Length	Fw Self	Fw Hairpin	Cross	Rv Self	Rv Hairpin	Sum
GAPDH	ACATCAAGAAGGTTGTGAAAGCTG	65	ATCAAAGATGGAGGAGTGAGAATC	64	119	-3	-1	-2.1	-0.9	-0.9	-7.9
ADCY5_1	GATCCAGGTGACGGCTGATC	68	TCATCATCTCTCCTTTGCCTTTGAC	67	101	-2	-2	-2.4	-0.9	-0.9	-8.2
ADCY5_2	CGGATGATGGACCAGATGAAATAC	65	ACCGGGCCAATGTTTAGACC	68	83	-1.5	0	-2.4	-4.4	0	-8.3

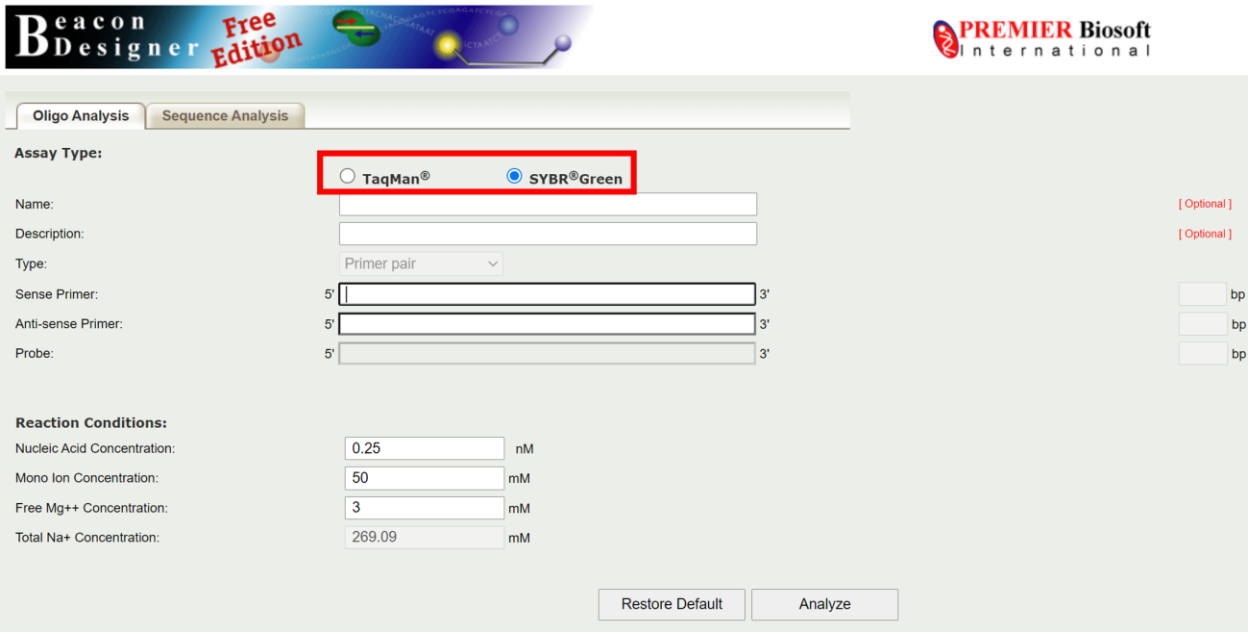


Figure A38: Home Page for Beacon Designer. Beacon designer is used to determine the penalty values for the primer pairs designed on Primer3Plus. Make sure to change the assay type from TaqMan to SYBR Green.

3.5. Guppy Project

A small side project I did with the high school summer Environmental Toxicology class was the guppy project. We wanted to see if there were any difference in expression between guppies that live in wastewater treatment plants (WWTP) and guppies from a pet store. The idea is that the WWTP fish are exposed to so many toxins in the wastewater and should have much higher transporter expression than pet store guppies that have only lived in filtered aquariums. The students and I designed primers (Table A8) and produced qPCR data. Then the students analyzed all the data to come to several conclusion:

- Group 1 found that female WWTP guppies expressed the most ABCB1 out of all the guppies most likely due to the added pressure of carrying offspring. Interestingly, they also found that the WWTP males had half the ABCB1 expression as pet shop males. The

reason for this is unknown but might be due to chemicals in the water causing down-regulation of ABCB1 in the WWTP males.

- Group 2 found that WWTP guppies had higher ABCC1 expression than pet shop guppies (both male and female).
- Group 3 found that WWTP guppies overall had higher expression of ABCC2, and females had almost 2-fold higher expression than males.
- Group 4 found that WWTP guppies had a 5-fold increase in ABCG2 expression compared to pet shop guppies. Additionally, they found that the female guppies had a 1.6-fold increase from the male guppies.

Table A8: qPCR primers designed for Guppy ABCB1, ABCC1, ABCC2, and ABCG2. I created the primers on the right and the students created the primers on the left.

Name	Sequence
Guppy_ABCB1_1F	CACCACCATCACCGAGAACA
Guppy_ABCB1_1R	TCTGTCCTCCGCTCATCTGA
Guppy_ABCB1_3F	ACAATCAGGAACGCCGACAT
Guppy_ABCB1_3R	GGACTTTTCTCGCCTGGTGA
Guppy_ABCB1_18F	TGACAGCCACAAAAACAGCG
Guppy_ABCB1_18R	TCCGAACAGAACCGCAGAAA
Guppy_ABCC1_19F	CGACCTGGACATCTTACCCG
Guppy_ABCC1_19R	ACATGAGCGTCAACAGCAGA
Guppy_ABCC1_16F	AGTTCGGCCCGTACTTCTTG
Guppy_ABCC1_16R	AGGCAGGAGAGGAGGAACAT
Guppy_ABCC1_1F	CTGCGTCTACCTGTGGTTGT
Guppy_ABCC1_1R	CCATGTGGTGCTGGATGTCT
Guppy_ABCC2_3F	TGCTCTTGGAATGGCTCAGG
Guppy_ABCC2_3R	GCGGTTGAGGATTCTCCCAA
Guppy_ABCC2_18F	CTTGAGAGCGACATTGTGCG
Guppy_ABCC2_18R	CAGGAGTGCCGACAAGAGAG
Guppy_ABCC2_19F	ATCATCGCCCGTGAGTGAAG
Guppy_ABCC2_19R	ATAGCCAGAGGAAGCCCAGA
Guppy_ABCG2x2_1F	GAGGGGAGAGGAAAAGGTGC
Guppy_ABCG2x2_1R	CGTGGTTGGTGGATGGAGAA
Guppy_ABCG2x2_3F	ACCTGCGTCTCAACCCAAAA
Guppy_ABCG2x2_3R	GCACCTTTTCCTCTCCCCTC
Guppy_ABCG2x2_15F	GTGTCAGGAGGGGAGAGGAA
Guppy_ABCG2x2_15R	ATGGAGTAGCGTGGTTGGTG

Name	Sequence
gpy_MDR1_GSP18F	ATGGGGAAGAAAAGCGAG
gpy_MDR1_118R	GGCTCCTTCGGCTTCTTCTC
gpy_MDR1_638F	TCGCAGCTTTCATCATCGGT
gpy_MDR1_754R	GCACTCTGCTCTTTGGTCGT
gpy_MDR1_1150F	AACCCAACCATCGACAGCTA
gpy_MDR1_1282R	GTCTGTCCACTGCTCACACT
gpy_MDR1_GSP19R	TCACTCTGTCCCGTGGCCC
gpy_MRP1_579F	TGACCAACCACCGCTTTTCT
gpy_MRP1_735R	GGGAGCAGTCATCAGCGTTT
gpy_MRP1_3118F	CGCTCACCCATGTCCTTCTT
gpy_MRP1_3246R	TGACACAGGAACCCAACACA
gpy_MRP1_3582F	ATTTGCTGCCCTGTTTGCTG
gpy_MRP1_3747R	CCTCTTTCCCTGTGCTGGAG
gpy_MRP1_GSP18F	ATGGGGCTGGAAAGGTTC
gpy_MRP1_GSP19R	TTAGACCAGTCCAGCATCT
gpy_MRP2_332F	CGAACCCAGTCCTCTTTGCT
gpy_MRP2_463R	AGGAGGGTCTGGAAAGGGAA
gpy_MRP2_3704F	GAAACTCCATTGACAGCGGC
gpy_MRP2_3816R	TCACTCACTCTTTCTACGGCT
gpy_MRP2_3303F	CAACCGATTTGCCAAGGACA
gpy_MRP2_3392R	TCACAGCCAGAGGAATGATGA
gpy_MRP2_GSP20F	ATGTGTGCTGCGCCTCTGGA
gpy_MRP2_X_GSP21R	TTAAAGAGATGTTAGGTTTTC
gpy_G2b_109F	TGCCGAAAGATAGGTCCTGA
gpy_G2b_265R	TTGCCGTTGACTTGGACTGT
gpy_G2bX1_1699F	ACCATAGTCCCAGGAGAGGT
gpy_G2bX1_1825R	CATCGGTTGATTCGCAGCAG
gpy_G2bX2_1423F	GCCTCTGATCACCTCACACC
gpy_G2bX2_GSP20R	TTAGATGCAGTGAACATTTT
gpy_G2b_GSP18F	ATGACCAAGGGCGCGCAG
gpy_G2bX1_GSP20R	TCACTTCCATCGGTTGATTC
gpy_GAPDH_F	ACATCAAGAAGGTTGTGAAAGCTG
gpy_GAPDH_R	ATCAAAGATGGAGGAGTGAGAATC
gpy_adcy5_1F	GATCCAGGTGACGGCTGATC
gpy_adcy5_1R	TCATCATCTCTCCTTTGCCTTTGAC
gpy_adcy5_2F	ACCGGGCCAATGTTTAGACC
gpy_adcy5_2R	ACCGGGCCAATGTTTAGACC

Appendix 4: Metadata Tables

Table A9: Crops ranked by value from global, EU, US, and CA. Global values from http://www.fao.org/faostat/en/#data/QC, click Bulk Downloads and you will get an Excel sheet of all the UN crop data. EU data from https://ec.europa.eu/eurostat/databrowser/view/APRI_AP_CRPOUTA__custom_1280440/default/table?lang=en, select all the vegetable products then drag the whole box to the row display. US data from https://quickstats.nass.usda.gov/#55E53AAF-5548-3FF3-B453-3871ACDB8043, Select Census for program, crops for sector, Field crops/fruits & tree nuts/vegetables for group, select all for commodity, and sales for category. Ca data from https://www.cdfa.ca.gov/Statistics/, scroll to the bottom and download the latest report.

Rank	Region	Item	Value	Unit	Year
1	Global	Apples	$50,872,898,000.00	USD	2018
2	Global	Cucumbers And Gherkins	$39,207,918,000.00	USD	2018
3	Global	Rapeseed	$36,015,812,000.00	USD	2018
4	Global	Watermelons	$31,883,956,000.00	USD	2018
5	Global	Mangoes, Mangosteens, Guavas	$29,527,015,000.00	USD	2018
6	Global	Pears	$21,373,186,000.00	USD	2018
7	Global	Sunflower Seed	$19,680,890,000.00	USD	2018
8	Global	Carrots And Turnips	$15,017,583,000.00	USD	2018
9	Global	Cauliflowers And Broccoli	$14,590,331,000.00	USD	2018
10	Global	Almonds, With Shell	$13,100,183,000.00	USD	2018
11	Global	Pumpkins, Squash and Gourds	$8,358,031,000.00	USD	2018
12	Global	Peaches And Nectarines	$7,239,505,000.00	USD	2018
13	Global	Cocoa, Beans	$6,876,004,000.00	USD	2018
14	Global	Avocados	$5,811,886,000.00	USD	2018
15	European Union	Rape And Turnip Rape Seed	$4,973,385,245.90	USD	2019
16	California	Almonds	$4,901,000,000.00	USD	2019
17	Global	Cantaloupes	$4,859,745,000.00	USD	2018
18	Global	Cherries	$3,651,768,000.00	USD	2018
19	Global	Plums And Sloes	$3,506,388,000.00	USD	2018
20	European Union	Apples	$3,466,245,901.64	USD	2019
21	European Union	Sunflower	$2,596,786,885.25	USD	2019
22	Global	Kiwi Fruit	$2,506,613,000.00	USD	2018
23	Global	Apricots	$2,467,866,000.00	USD	2018
24	Global	Blueberries	$1,897,757,000.00	USD	2018
25	Global	Raspberries	$1,896,061,000.00	USD	2018
26	European Union	Pears	$1,329,319,672.13	USD	2019
27	European Union	Peaches	$1,278,737,704.92	USD	2019
28	Global	Cranberries	$960,203,000.00	USD	2018
29	Global	Cherries, Sour	$933,148,000.00	USD	2018
30	European Union	Cauliflower	$929,327,868.85	USD	2019
31	United States	Apples	$474,703,090.00	USD	2019
32	Global	Buckwheat	$302,289,000.00	USD	2018
33	United States	Blueberries	$205,226,500.00	USD	2019
34	California	Raspberries And Blackberries	$162,000,000.00	USD	2019
35	United States	Carrots	$131,807,473.00	USD	2019
36	California	Prunes	$126,000,000.00	USD	2019
37	California	Cauliflowers	$125,000,000.00	USD	2019
38	United States	Onions	$112,709,590.00	USD	2019
39	United States	Broccoli	$109,437,988.00	USD	2019
40	United States	Raspberries	$91,989,511.00	USD	2019
41	United States	Cauliflower	$68,289,453.00	USD	2019
42	United States	Blackberries	$49,695,678.00	USD	2019
43	United States	Peaches	$36,411,251.00	USD	2019
44	United States	Cherries, Sweet	$32,977,147.00	USD	2019
45	United States	Almonds	$32,975,908.00	USD	2019
46	United States	Avocados	$27,587,005.00	USD	2019
47	United States	Pears	$26,168,583.00	USD	2019
48	United States	Watermelon	$16,108,439.00	USD	2019
49	Global	Vanilla	$11,325,000.00	USD	2018
50	United States	Plums	$11,161,047.00	USD	2019

Table A10: Metadata of pesticide mixtures tested on bees. These are all published data on honeybee mortality when exposed to pesticide combinations.

Compound 1	Class	Compound 2	Class	Compound 3	Class	Assay	Outcome	Reference
Imidacloprid	Neonicotinoid	Acephate	Organophosphate			Mortality	high	Zhu et al - Synergistic toxicity and physiological... (2017)
Verapamil	P-gp Inhibitor	Acetamiprid	Neonicotinoid			Mortality	High	Hawthorne and Dively - Killing them with kindness (2011)
coumaphos	Organophosphate	amitraz	Formamidine			Mortality	med	Johnson et al. Acaride, fungicide, and drug... (2013)
fenpyroximate	Phenyl pyrazole	amitraz	Formamidine			Mortality	med	Johnson et al. Acaride, fungicide, and drug... (2013)
Tau-fluvalinate	Pyrethroid	amitraz	Formamidine			Mortality	med	Johnson et al. Acaride, fungicide, and drug... (2013)
Chlorantranilip role	Carboxamide	Boscalid	carboxamide	pyraclostrobin	Carbamate	Mortality	low	Wade et al - Combined Toxicity of insecticides... (2019)
diflubenzuron	Insect Growth Regulator	Boscalid	carboxamide	pyraclostrobin	Carbamate	Mortality	High	Wade et al - Combined Toxicity of insecticides... (2019)
Tau-fluvalinate	Pyrethroid	chlorothalon il	chloronitrile			Mortality	med	Johnson et al. Acaride, fungicide, and drug... (2013)
thymol	Bio-Pesticide	chlorothalon il	chloronitrile			Mortality	low	Johnson et al. Acaride, fungicide, and drug... (2013)
Imidacloprid	Neonicotinoid	clothianidin	Neonicotinoid			Mortality	low	Zhu et al - Synergistic toxicity and physiological... (2017)
Neonicatinoid	Neonicotinoid	clothianidin	Neonicotinoid			Mortality	low	Williamson and Willis - Exposure to neonicotinoids... (2014)
fenpyroximate	Phenyl pyrazole	coumaphos	Organophosphate			Mortality	med	Johnson et al. Acaride, fungicide, and drug... (2013)
Tau-fluvalinate	Pyrethroid	coumaphos	Organophosphate			Mortality	High	Johnson et al. Acaride, fungicide, and drug... (2013)
thymol	Bio-Pesticide	coumaphos	Organophosphate			Mortality	med	Johnson et al. Acaride, fungicide, and drug... (2013)
Verapamil	P-gp Inhibitor	Coumaphos	Organophosphate			Mortality	med	Hawthorne and Dively - Killing them with kindness (2011)
coumaphos	Organophosphate	DEF	carboxylesterase inhibitor			Mortality	med	Johnson et al. Acaride, fungicide, and drug... (2013)
fenpyroximate	Phenyl pyrazole	DEF	carboxylesterase inhibitor			Mortality	med	Johnson et al. Acaride, fungicide, and drug... (2013)
Tau-fluvalinate	Pyrethroid	DEF	carboxylesterase inhibitor			Mortality	med	Johnson et al. Acaride, fungicide, and drug... (2013)
Neonicatinoid	Neonicotinoid	dinotefuran	Neonicotinoid			Mortality	low	Williamson and Willis - Exposure to neonicotinoids... (2014)
coumaphos	Organophosphate	fenpyroxima te	Phenyl pyrazole			Mortality	med	Johnson et al. Acaride, fungicide, and drug... (2013)
Tau-fluvalinate	Pyrethroid	fenpyroxima te	Phenyl pyrazole			Mortality	med	Johnson et al. Acaride, fungicide, and drug... (2013)
Verapamil	P-gp Inhibitor	Fluvalinate	Pyrethroid			Mortality	med	Hawthorne and Dively - Killing them with kindness (2011)
Ivermectin	Avermectin	fumagillin	Antibiotic			Mortality	high	Guseman et al - Multi-drug resistance transporters... (2016)
Verapamil	P-gp Inhibitor	Imidacloprid	Neonicotinoid			Mortality	High	Hawthorne and Dively - Killing them with kindness (2011)
Chlorantranilip role	Carboxamide	iprodione	dicarboximide			Mortality	med	Wade et al - Combined Toxicity of insecticides... (2019)
diflubenzuron	Insect Growth Regulator	iprodione	dicarboximide			Mortality	High	Wade et al - Combined Toxicity of insecticides... (2019)
Methoxyfenozi de	Insect Growth Regulator	iprodione	dicarboximide			Mortality	med	Wade et al - Combined Toxicity of insecticides... (2019)
amitraz	Formamidine	oxalic acid	Pyrethroid			Mortality	Better	Johnson et al. Acaride, fungicide, and drug... (2013)
fenpyroximate	Phenyl pyrazole	oxalic acid	Pyrethroid			Mortality	med	Johnson et al. Acaride, fungicide, and drug... (2013)
Tau-fluvalinate	Pyrethroid	oxalic acid	Pyrethroid			Mortality	med	Johnson et al. Acaride, fungicide, and drug... (2013)
thymol	Bio-Pesticide	oxalic acid	Pyrethroid			Mortality	low	Johnson et al. Acaride, fungicide, and drug... (2013)
Imidacloprid	Neonicotinoid	oxamyl	carbamate			Mortality	high	Zhu et al - Synergistic toxicity and physiological... (2017)
coumaphos	Organophosphate	Oxytetracycl ine	Tetracycline Antibiotic			Mortality	High	Hawthorne and Dively - Killing them with kindness (2011)
Fluvalinate	Pyrethroid	Oxytetracycl ine	Tetracycline Antibiotic			Mortality	med	Hawthorne and Dively - Killing them with kindness (2011)
coumaphos	Organophosphate	PBO	Pesticide Synergist			Mortality	med	Johnson et al. Acaride, fungicide, and drug... (2013)
fenpyroximate	Phenyl pyrazole	PBO	Pesticide Synergist			Mortality	High	Johnson et al. Acaride, fungicide, and drug... (2013)
Tau-fluvalinate	Pyrethroid	PBO	Pesticide Synergist			Mortality	High	Johnson et al. Acaride, fungicide, and drug... (2013)
Ivermectin	Avermectin	Pristine	Carbamate + Carboxamide			Mortality	high	Guseman et al - Multi-drug resistance transporters... (2016)
coumaphos	Organophosphate	prochloraz	Imidazole			Mortality	High	Johnson et al. Acaride, fungicide, and drug... (2013)

fenpyroximate	Phenyl pyrazole	prochloraz	Imidazole			Mortality	High	Johnson et al. Acaride, fungicide, and drug… (2013)
Tau-fluvalinate	Pyrethroid	prochloraz	Imidazole			Mortality	High	Johnson et al. Acaride, fungicide, and drug… (2013)
Chlorantranilip role	Carboxamide	propiconazo le	Triazole			Mortality	High	Wade et al - Combined Toxicity of insecticides… (2019)
diflubenzuron	Insect Growth Regulator	propiconazo le	Triazole			Mortality	High	Wade et al - Combined Toxicity of insecticides… (2019)
fenpyroximate	Phenyl pyrazole	pyraclostrob in	Carbamate			Mortality	med	Johnson et al. Acaride, fungicide, and drug… (2013)
Tau-fluvalinate	Pyrethroid	pyraclostrob in	Carbamate	Boscalid	carboxami de	Mortality	med	Johnson et al. Acaride, fungicide, and drug… (2013)
Tau-fluvalinate	Pyrethroid	pyraclostrob in	Carbamate			Mortality	med	Johnson et al. Acaride, fungicide, and drug… (2013)
Ivermectin	Avermectin	Quercetin	Flavinoid			Mortality	high	Guseman et al - Multi-drug resistance transporters… (2016)
Imidacloprid	Neonicotinoid	sulfoxaflor	sulfoximines			Mortality	med	Zhu et al - Synergistic toxicity and physiological… (2017)
coumaphos	Organophosphate	Tau-Fluvalinate	Pyrethroid			Mortality	med	Johnson et al. Acaride, fungicide, and drug… (2013)
thymol	Bio-Pesticide	Tau-Fluvalinate	Pyrethroid			Mortality	med	Johnson et al. Acaride, fungicide, and drug… (2013)
Imidacloprid	Neonicotinoid	tetraconazol e	Triazole			Mortality	med	Zhu et al - Synergistic toxicity and physiological… (2017)
Verapamil	P-gp Inhibitor	Thiacloprid	Neonicotinoid			Mortality	High	Hawthorne and Dively - Killing them with kindness (2011)
Neonicatinoid	Neonicotinoid	thiamethoxa m	Neonicotinoid			Mortality	high	Williamson and Willis - Exposure to neonicotinoids… (2014)
Tau-fluvalinate	Pyrethroid	thymol	Bio-Pesticide			Mortality	low	Johnson et al. Acaride, fungicide, and drug… (2013)
Ivermectin	Avermectin	verapamil	P-gp Inhibitor			Mortality	high	Guseman et al - Multi-drug resistance transporters… (2016)

Table A11: Table of published pesticide residue data in honey, nectar, pollen, wax, and bees.

Compound	Honey	Nectar	Pollen	Wax	Bee	Reference
3-keto-carbofuran			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
3-OH-carbofuran			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
5-OH-Imidacloprid			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Acephate			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Acetamiprid			x			Codling et al - Concentrations of neonicotinoids… (2016)
Alachlor			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Atrazine			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Azinphos-methyl			x	x		Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Azinphos-methyl				x		Chauzat & Faucon - Pesticide Residues in beeswax… (2007)
Azinphos-methyl			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Azoxystrobin			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Bendiocarb			x	x		Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Bentazon			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Boscalid			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Bromacil			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Carbaryl			x	x		Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Carbaryl			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Carbendazim			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Carbofuran			x	x		Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Carbofuran			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Chlorantraniliprole			x			Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Chlorpyrifos			x	x		Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Chlorpyrifos				x		Chauzat & Faucon - Pesticide Residues in beeswax… (2007)
Chlorpyrifos			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Clothianidin			x			Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Clothianidin	x		x		x	Codling et al - Concentrations of neonicotinoids… (2016)
Clothianidin			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Coumaphos			x	x		Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Coumaphos				x		Chauzat & Faucon - Pesticide Residues in beeswax… (2007)
Coumaphos			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Coumaphos Oxon			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Coumaphos oxon			x	x		Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Cyfluthrin				x		Chauzat & Faucon - Pesticide Residues in beeswax… (2007)
Cypermethrin				x		Chauzat & Faucon - Pesticide Residues in beeswax… (2007)
Cyproconazole			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Cyprodinil			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Deltamethrin				x		Chauzat & Faucon - Pesticide Residues in beeswax… (2007)
Diazinon			x	x		Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Diazinon			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Dichlorvos			x	x		Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Dichlorvos			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Difenoconazole			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Diflubenzuron			x	x		Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Dimethoate			x	x		Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Dimethoate			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Dimethomorph			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Dinotefuran		x	x			Dively - Insecticides residues in pollen and nectar… (2012)
Dinotefuran			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Diphenylamine			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Dithiopyr			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Diuron			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Endosulfan				x		Chauzat & Faucon - Pesticide Residues in beeswax… (2007)
Fenbuconazole			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Fenhexamid			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Fenitrothion				x		Chauzat & Faucon - Pesticide Residues in beeswax… (2007)
Fenpropathrin			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Fenpyroximate			x	x		Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Fenthion				x		Chauzat & Faucon - Pesticide Residues in beeswax… (2007)
Fenthion			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Fipronil			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Fluvalinate			x	x		Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Fluvalinate			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Imazalil			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Imidacloprid		x	x			Dively - Insecticides residues in pollen and nectar… (2012)
Imidacloprid	x		x			Codling et al - Concentrations of neonicotinoids… (2016)
Imidacloprid			x			Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Imidacloprid			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Imidacloprid 5-Hydroxy			x			Codling et al - Concentrations of neonicotinoids… (2016)

Imidacloprid olefin			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Imidacloprid olefin			x			Codling et al - Concentrations of neonicotinoids… (2016)
Imidacloprid urea			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Imidacloprid, Olefin			x			Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Imidacloprid, urea			x			Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Indoxacarb			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Lindane				x		Chauzat & Faucon - Pesticide Residues in beeswax… (2007)
Malathion			x	x		Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Malathion				x		Chauzat & Faucon - Pesticide Residues in beeswax… (2007)
Malathion			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Metalaxyl			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Methamidophos			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Methidathion				x		Chauzat & Faucon - Pesticide Residues in beeswax… (2007)
Methiocarb			x			Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Methiocarb			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Methomyl			x			Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Methomyl			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Methoxyfenozide			x			Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Metolachlor			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Mevinphos				x		Chauzat & Faucon - Pesticide Residues in beeswax… (2007)
Myclobutanil			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Napropamide			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
OH-Carbofuran (carbofuran)			x	x		Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Omethoate (methoate)			x			Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Oxadiazon			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
oxamyl		x	x			Dively - Insecticides residues in pollen and nectar… (2012)
Oxyflourfen			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Parathion				x		Chauzat & Faucon - Pesticide Residues in beeswax… (2007)
Parathion-methyl				x		Chauzat & Faucon - Pesticide Residues in beeswax… (2007)
Pendimethalin			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Phorate			x			Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Phorate Sulfoxide (phorate)			x			Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Phosmet			x	x		Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Phosmet			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Pinoxaden			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Piperonyl butoxide				x		Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Pirimicarb			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Procymidone				x		Chauzat & Faucon - Pesticide Residues in beeswax… (2007)
Procymidone			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Prodiamine			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Propargite				x		Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Propiconazole			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Propoxur			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Propyzamide			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Pyraclostrobin			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Pyridaben				x		Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Pyrimethanil			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Resmethrin				x		Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Rotenone			x	x		Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Simazine			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Spinetoram			x			Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Spiridoclofen			x	x		Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Sulfometuron- methyl			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Tau-fluvalinate				x		Chauzat & Faucon - Pesticide Residues in beeswax… (2007)
Thiabendazole			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Thiacloprid	x		x			Codling et al - Concentrations of neonicotinoids… (2016)
Thiacloprid			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Thiamethoxam			x	x		Ostiguy et al - Honey Bee Exposure to Pesticides (2019)
Thiamethoxam		x	x			Dively - Insecticides residues in pollen and nectar… (2012)
Thiamethoxam			x			Codling et al - Concentrations of neonicotinoids… (2016)
Thiamethoxam			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Thiophanate-methyl			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Trichlorfon			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Trifloxystrobin			x			Stoner & Eitzer - Using a Hazard Quotient to evaluate… (2013)
Vinclozolin				x		Chauzat & Faucon - Pesticide Residues in beeswax… (2007)

Table A12: Table of pesticides we ordered for the ATPase assay. CA top 100 denotes compounds of the 2018 top 100 pesticides by pound used in California according to the California Department of Pesticide Regulation (https://www.cdpr.ca.gov/docs/pur/pur18rep/top_100_sites_lbs_2018.htm). Binary Tested are compounds that were previously shown *in vivo* to cause increased mortality in combinations (Johnson et al. 2013; Guseman et al. 2016; Zhu et al. 2017; Wade et al. 2019). Crop pesticides are compound listed by the UC Agriculture & Natural Resources Integrated Pest Management (ANR IPM) program database (https://www2.ipm.ucanr.edu/agriculture/).

	Pesticide	Class	Binary Tested	CA Top 100	Crop Pesticide
Bee Med	Amitraz	Formamidine	x		
Bee Med	Oxytetracycline	Tetracycline Antibiotic	x		
Bee Med	Coumaphos	Organophosphate	x		
Bee Med	Fluvalinate	Synthetic pyrethroid	x		
Insecticide	Permethrin	Synthetic pyrethroid		x	
Insecticide	Diazinon	Organophosphate			x
Insecticide	Imidacloprid	Neonicotinoid	x	x	x
Insecticide	Acetamiprid	Neonicotinoid		x	x
Insecticide	Ivermectin	Abamectin	x		
Herbicide	Glyphosate	Glycine		x	x
Herbicide	Paraquat	Bipyridylium		x	x
Fungicide	Chlorothalonil	Chloronitriles	x	x	x
Fungicide	Propiconazole	Triazole	x	x	x

Table A13: Summary table for Aquaculture fish. Listed are the scientific and common names, the protein name and isoform as it appears in NCBI, protein length, accession number, gene ID, refseq status, whether the protein has been published, and the source of the protein (i.e., KEGG, BLAST, NCBI). Red font denotes something weird or wrong. *Chanos chanos* ABCB1-Like is a low quality protein, according to NCBI.

Scientific name	Common Name	Protein	Isoform	Length (AA)	Accession	Gene ID	RefSeq Status	Bibliography	Source
Lates calcarifer	Barramundi	MDR1		1287	XP_018541648.1	108889588	Predicted	genone/transcriptome only	NCBI Orthologs ABCB4
Oreochromis niloticus	Nile Tilapia	MDR1	X1	1273	XP_019220038.1	100534453	Model	Yes	NCBI Orthologs ABCB4
Oreochromis niloticus	Nile Tilapia	MDR1	X2	1272	XP_019220039.1	100534453	Model	Yes	NCBI Orthologs ABCB4
Oncorhynchus mykiss	Rainbow Trout	ABCB1	X1	1279	XP_036794808.1	100136278	Model	Yes	NCBI Orthologs ABCB4
Oncorhynchus mykiss	Rainbow Trout	ABCB1	X2	1159	XP_036794815.1	100136278	Model	Yes	NCBI Orthologs ABCB4
Oncorhynchus mykiss	Rainbow Trout	ABCB1	X1	1341	XP_036821525.1	100653442	Model	Yes	NCBI Orthologs ABCB5
Oncorhynchus mykiss	Rainbow Trout	ABCB1	X2	1340	XP_036821532.1	100653442	Model	Yes	NCBI Orthologs ABCB5
Oncorhynchus mykiss	Rainbow Trout	ABCB1	X3	1340	XP_036821542.1	100653442	Model	Yes	NCBI Orthologs ABCB5
Oncorhynchus mykiss	Rainbow Trout	ABCB1	X4	1336	XP_036821552.1	100653442	Model	Yes	NCBI Orthologs ABCB5
Oncorhynchus kisutch	Coho Salmon	ABCB1	Like X1	1335	XP_031667777.1	109883394	Provisonal	Transcriptome Only	NCBI Orthologs ABCB5
Oncorhynchus kisutch	Coho Salmon	ABCB1	Like X2	1334	XP_031667778.1	109883394	Provisonal	Transcriptome Only	NCBI Orthologs ABCB5
Oncorhynchus kisutch	Coho Salmon	ABCB1	Like X3	1334	XP_031667779.1	109883394	Provisonal	Transcriptome Only	NCBI Orthologs ABCB5
Oncorhynchus kisutch	Coho Salmon	ABCB1	Like X4	1330	XP_031667780.1	109883394	Provisonal	Transcriptome Only	NCBI Orthologs ABCB5
Oncorhynchus kisutch	Coho Salmon	ABCB1	Like X5	1329	XP_031667781.1	109883394	Provisonal	Transcriptome Only	NCBI Orthologs ABCB5
Oncorhynchus kisutch	Coho Salmon	ABCB1	Like X6	1329	XP_031667782.1	109883394	Provisonal	Transcriptome Only	NCBI Orthologs ABCB5
Oncorhynchus kisutch	Coho Salmon	ABCB1	Like X7	1297	XP_031667783.1	109883394	Provisonal	Transcriptome Only	NCBI Orthologs ABCB5
Oncorhynchus nerka	Sockeye Salmon	MDR1	Like X1	1278	XP_029544656.1	115146736	Model	none	NCBI Orthologs ABCB4
Oncorhynchus nerka	Sockeye Salmon	MDR1	Like X2	1156	XP_029544672.1	115146736	Model	none	NCBI Orthologs ABCB4
Ictalurus punctatus	Channel Catfish	MDR1		1335	XP_017308977.1	108256530	Model	annotation only	NCBI Orthologs ABCB4
Ictalurus punctatus	Channel Catfish	MDR1	Like	1344	XP_017326849.1	108267330	Model	none	NCBI Orthologs ABCB5
Gadus morhua	Atlantic Cod	ABCB1	Like	1254	XP_030226791.1	115554272	Model	Genome only	NCBI Orthologs ABCB4
Micropterus salmoides	Largemouth Bass	ABCB4	X1	1291	XP_038567410.1	119897634	Model	None	NCBI Gene search (ABCB4)
Micropterus salmoides	Largemouth Bass	ABCB4	X2	1287	XP_038567411.1	119897634	Model	None	NCBI Gene search (ABCB4)
Salmo trutta	Brown Trout	MDR1	Like X1	1287	XP_029592305.1	115176414	Model	None	BLAST
Salmo trutta	Brown Trout	MDR1	Like X2	1281	XP_029592306.1	115176414	Model	None	BLAST
Salvelinus namaycush	Lake Trout	ABCB1	Like X1	1287	XP_038832727.1	120031179	Model	None	NCBI Gene search (ABCB1)
Salvelinus namaycush	Lake Trout	ABCB1	Like X2	1281	XP_038832728.1	120031179	Model	None	NCBI Gene search (ABCB1)
Salvelinus namaycush	Lake Trout	ABCB1	Like X3	1159	XP_038832729.1	120031179	Model	None	NCBI Gene search (ABCB1)
Salvelinus namaycush	Lake Trout	ABCB1	Like X4	988	XP_038832730.1	120031179	Model	None	NCBI Gene search (ABCB1)
Salvelinus namaycush	Lake Trout	ABCB1	Like X5	295	XP_038832731.1	120031179	Model	None	NCBI Gene search (ABCB1)
Seriola dumerili	greater amberjack	MDR1		1293	XP_022621891.1	111237185	Model	Genome only	NCBI Gene search (ABCB4)
Anabas testudineus	climbing perch	ABCB4		1293	XP_026226619.1	113169439	Model	None	NCBI Gene search (ABCB4)
Sparus aurata	gilthead seabream	ABCB1		1297	XP_030250179.1	115567592	Model	None	NCBI Gene search (ABCB4)
Morone saxatilis	striped sea-bass	ABCB4		1286	XP_035527748.1	118335533	Model	Transcriptome Only	NCBI Gene search (ABCB4)
Hippoglossus hippoglossus	Atlantic halibut	ABCB4		1301	XP_034467969.1	117777366	Model	None	NCBI Gene search (ABCB4)
Oreochromis aureus	blue tilapia	ABCB4		1272	XP_039476119.1	116331357	Model	None	NCBI Gene search (ABCB4)
Oreochromis aureus	blue tilapia	ABCB5		161	XP_039475459.1	120442646	Model	None	NCBI Gene search (ABCB5)
Hippoglossus stenolepis	Pacific halibut	ABCB4		1301	XP_035019498.1	118113676	Model	None	NCBI Gene search (ABCB4)
Paralichthys olivaceus	Japanese flounder	MDR1		986	XP_019948186.1	109633038	Model	None	NCBI Gene search (ABCB4)
Paralichthys olivaceus	Japanese flounder	ABCB4	Like	168	XP_019948880.1	109633458	Model	None	NCBI Gene search (ABCB4)
Esox lucius	northern pike	ABCB4	X1	1285	XP_028971452.2	105029249	Model	Genome only	NCBI Gene search (ABCB4)
Esox lucius	northern pike	ABCB4	X2	1280	XP_034144508.1	105029249	Model	Genome only	NCBI Gene search (ABCB4)
Esox lucius	northern pike	ABCB4	X3	1279	XP_034144509.1	105029249	Model	Genome only	NCBI Gene search (ABCB4)
Esox lucius	northern pike	ABCB4	X4	1236	XP_034144510.1	105029249	Model	Genome only	NCBI Gene search (ABCB4)
Esox lucius	northern pike	ABCB4	X5	1032	XP_034144511.1	105029249	Model	Genome only	NCBI Gene search (ABCB4)
Esox lucius	northern pike	ABCB1		1333	XP_010882867.1	105018828	Model	None	NCBI Gene search (ABCB5)
Chanos chanos	milkfish	ABCB1	Like	1283	XP_030639389.1	115820075	Model	None	NCBI Gene search (ABCB4)
Chanos chanos [Low Quality]	milkfish	ABCB1	Like	1351	XP_030639135.1	115819773	Model	None	NCBI Gene search (ABCB5)
Pangasianodon hypophthalmus	striped catfish	ABCB4	X1	1277	XP_026780001.2	113532684	Model	None	NCBI Gene search (ABCB4)
Pangasianodon hypophthalmus	striped catfish	ABCB4	X2	1040	XP_026780002.2	113532684	Model	None	NCBI Gene search (ABCB4)
Pangasianodon hypophthalmus	striped catfish	ABCB1	X1	1337	XP_034163847.1	113542464	Model	None	NCBI Gene search (ABCB5)
Pangasianodon hypophthalmus	striped catfish	ABCB1	X2	1336	XP_034163851.1	113542464	Model	None	NCBI Gene search (ABCB5)
Clupea harengus	Atlantic herring	ABCB1	X1	1290	XP_031432464.1	105891787	Model	None	NCBI Gene search (ABCB4)
Clupea harengus	Atlantic herring	ABCB1	X2	1277	XP_031432466.1	105891787	Model	None	NCBI Gene search (ABCB4)
Clupea harengus	Atlantic herring	ABCB1		1260	XP_031432823.1	105906308	Model	None	NCBI Gene search (ABCB5)
Oncorhynchus tshawytscha	Chinook salmon	MDR1	Like	817	XP_024241191.1	112222699	Model	None	NCBI Gene search (ABCB4)

Table A14: Summary table for model vertebrates (green), model fish (red) and other fish. Listed are the scientific and common names, the protein name and isoform as it appears in NCBI, protein length, accession number, gene ID, refseq status, whether the protein has been published, and the source of the protein (i.e., KEGG, BLAST, NCBI). Red font denotes something weird or wrong. *Danio* ABCB4 isoform 2 seems truncated and should not be used. *Gambusia affinis* P-gp was a direct submit and did not have a gene ID or any RefSeq information.

Scientific name	Common Name	Protein	Isoform	Length (AA)	Accession	Gene ID	RefSeq Status	Bibliography	Note
Homo Sapiens	Human	ABCB1	1	1350	NP_001335874.1	5243	Reviewed	yes	Weird new one, NCBI Orthologs ABCB1
Homo Sapiens	Human	ABCB1	2	1280	NP_001335875.1	5243	Reviewed	yes	In the Chang Paper, KEGG ABCB1 lists IF2, NCBI Orthologs ABCB1
Homo Sapiens	Human	ABCC1		1531	NP_004987.2	4363	Reviewed	yes	KEGG ABCB1
Oryctolagus cuniculus	Rabbit	ABCB1		1279	NP_001075628.1	100008914	Provisional	yes	KEGG ABCB1, NCBI Orthologs ABCB1
Mus musculus	Mouse	ABCB1a		1276	NP_035206.2	18671	Validated	yes	KEGG ABCB1
Mus musculus	Mouse	ABCB1b		1270	NP_035205.1	18669	Validated	yes	KEGG ABCB1, NCBI Orthologs ABCB1
Rattus norvegicus	Rat	ABCB1a		1272	NP_596892.1	170913	Provisional	yes	KEGG ABCB1, (NCBI Orthologs ABCB1 Only lists Rattus rattus)
Rattus norvegicus	Rat	ABCB1b		1275	NP_036755.3	24646	Provisional	yes	KEGG ABCB1, (NCBI Orthologs ABCB1 Only lists Rattus rattus)
Gallus gallus	Chicken	ABCB1		1288	NP_990225.1	395712	Provisional	yes	KEGG ABCB1
Xenopus laevis	African Clawed Frog	ABCB1	L	1287	NP_001081394.1	397812	Provisional	yes	KEGG ABCB1
Xenopus tropicalis	western clawed frog	ABCB1	1	1319	XP_017951387.2	100494753	Model	None	NCBI Search
Xenopus tropicalis	western clawed frog	ABCB1	2	1284	XP_004921510.2	100496268	Model	None	NCBI Search
Danio rerio	Zebrafish	ABCB4	1	1275	NP_001303643.1	100136865	Validated	yes	NCBI Orthologs ABCB4
Danio rerio	Zebrafish	ABCB4	2	650	NP_001108055.2	100136865	Validated	yes	NCBI Orthologs ABCB4, Seems truncated: did not download
Danio rerio	Zebrafish	ABCB5		1338	XP_001922717.3	798527	Model	yes	NCBI Orthologs ABCB5, Listed in KEGG as ABCB1
Oryzias latipes	Japanese Ricefish	MDR1	1	1286	XP_023819737.1	101171435	Model	None	NCBI Orthologs ABCB4, Listed in KEGG as ABCB1
Oryzias latipes	Japanese Ricefish	MDR1	2	1286	XP_023819738.1	101171435	Model	None	NCBI Orthologs ABCB4
Gambusia affinis	Mosquitofish	P-gp		1294	QKW91241.1	None	None	None	NCBI search, direct submit
Fundulus heteroclitus	Atlantic Killifish	ABCB1		1289	XP_035989740.1	105915288	Model	None	NCBI Orthologs ABCB4
Takifugu rubripes	Japanese Pufferfish	MDR1	X1	1280	XP_011603941.1	101067017	Model	None	NCBI Orthologs ABCB4, Listed in KEGG as ABCB1
Takifugu rubripes	Japanese Pufferfish	MDR1	X2	1211	XP_029694127.1	101067017	Model	None	NCBI Orthologs ABCB4
Nothobranchius furzeri	Turquoise Killifish	MDR1		1285	XP_015805983.1	107379650	Model	None	NCBI Orthologs ABCB4, Listed in KEGG as ABCB1
Strongylocentrotus purpuratus	Sea Urchin	ABCB1		1329	NP_001029122.1	591668	Provisional	Yes	NCBI Gene Search, Ref'ed by Amro, (KEGG lists ABCB1-Like, not this one)
Xiphophorus hellerii	green swordtail	ABCB1	Like	1292	XP_032413954.1	116717008	Model	None	NCBI Gene Search, 2 identical seqs on NCBI, gene listing is ABCB4
Stegastes partitus	bicolor damselfish	MDR1		1293	XP_008297781.1	103370486	Model	None	NCBI Gene Search, 5 identical seqs on NCBI, gene listing is ABCB4
Oncorhynchus mykiss	Rainbow Trout	ABCB1	X1	1279	XP_036794808.1	100136278	Model	Yes	NCBI Orthologs ABCB4
Oncorhynchus mykiss	Rainbow Trout	ABCB1	X2	1159	XP_036794815.1	100136278	Model	Yes	NCBI Orthologs ABCB4
Oncorhynchus mykiss	Rainbow Trout	ABCB1	X1	1341	XP_036821525.1	100653442	Model	Yes	NCBI Orthologs ABCB5
Oncorhynchus mykiss	Rainbow Trout	ABCB1	X2	1340	XP_036821532.1	100653442	Model	Yes	NCBI Orthologs ABCB5
Oncorhynchus mykiss	Rainbow Trout	ABCB1	X3	1340	XP_036821542.1	100653442	Model	Yes	NCBI Orthologs ABCB5
Oncorhynchus mykiss	Rainbow Trout	ABCB1	X4	1336	XP_036821552.1	100653442	Model	Yes	NCBI Orthologs ABCB5
Xiphophorus maculatus	southern platyfish	MDR1		1285	XP_014328020.1	102224768	Model	none	NCBI Gene Search, 2 identical seqs on NCBI, gene listing is ABCB4
Poecilia formosa	Amazon molly	MDR1		1283	XP_007556225.1	103140837	Model	None	NCBI Gene Search (ABCB4)
Xiphophorus couchianus	Monterrey platyfish	MDR1	Like	1285	XP_027868031.1	114141597	Model	None	NCBI Gene Search, 2 identical seqs on NCBI, gene listing is ABCB4
Anguilla anguilla	European eel	ABCB4		1279	XP_035243877.1	118211118	Model	None	NCBI Gene Search (ABCB4)
Anguilla anguilla	European eel	ABCB1		1318	XP_035239284.1	118208570	Model	None	NCBI Gene Search, 2 identical seqs on NCBI, gene listing is ABCB5

Table A15: Summary table for bees and pest insects. Listed are the scientific and common names, the protein name and isoform as it appears in NCBI, protein length, accession number, gene ID, refseq status, whether the protein has been published, and the source of the protein (i.e., KEGG, BLAST, NCBI). Proteins in green were used for the pairwise comparisons seen in Ch. 2 Figures 9&10.

	Scientific name	Common Name	Protein	Isoform	Length	Accession	Gene ID	RefSeq Status	Bibliography	Note
Disease	Anopheles albimanus	mosquito	MDR49-Like		1304	XP_035789861.1	118465598	Model	None	NCBI BLASTP
Disease	Anopheles coluzzii	mosquito	MDR49		1304	XP_040221247	120948693	Model	None	NCBI BLASTP
Disease	Anopheles darlingi	mosquito	ABC		1304	ETN61204.1	Direct Submit	---	---	NCBI BLASTP
Disease	Anopheles gambiae	mosquito	AGAP005639-PA		1301	XP_315658	1276325	Provisional	Genome only	KEGG, ABCB1
Disease	Anopheles sinensis	mosquito	AGAP005639-PA-Like		1297	KFB50603.1	Direct Submit	---	---	NCBI BLASTP
Disease	Anopheles stephensi	Asian malaria mosquito	MDR49		1304	XP_035913596.1	118512787	Model	None	NCBI BLASTP
Disease	Aedes aegypti	yellow fever mosquito	MDR49		1307	XP_001654492	5573277	Model	Neurotranscriptome only	KEGG, ABCB1, NCBI Lists 3 identical sequences
Disease	Aedes albopictus	Asian tiger mosquito	MDR49-Like		1307	XP_029735703	109408676	Model	None	NCBI BLASTP
Disease	Culex quinquefasciatus	southern house mosquito	MDR49		1311	XP_038117776.1	6050364	Model	None	KEGG, ABCB1, NCBI Lists 2 identical sequences
Disease	Culex pipiens pallens	northern house mosquito	MDR49-Like	IF1	1311	XP_039451126.1	120430107	Model	None	NCBI BLASTP
Disease	Culex pipiens pallens	northern house mosquito	MDR49-Like	IF2	1311	XP_039451145	120430126	Model	None	NCBI BLASTP
Disease	Pediculus humanus corporis	Body Lice	MDR		1273	XP_002432260	8232191	Provisional	None	KEGG, ABCB1
Disease	Ixodes scapularis	blacklegged tick	ABCB1		1314	XP_029831332	8052808	Model	None	VectorBase
Disease	Ixodes scapularis	blacklegged tick	ABCB1-Like		256	XP_029846821.1	115329389	Model	None	VectorBase
Disease	Ixodes scapularis	blacklegged tick	ABCB1		1002	EEC05534	VB: ISCW004310	Model	None	KEGG, ABCB1
Disease	Ixodes scapularis	blacklegged tick	ABCB1		1070	EEC05109	VB: ISCW017811	Model	None	KEGG, ABCB1
Model	Drosophila melanogaster	fruit fly	MDR65		1302	NP_476831	38726	Reviewed	Yes	KEGG, ABCB1
Model	Drosophila melanogaster	fruit fly	MDR49a		1302	NP_523724	36428	Reviewed	Yes	KEGG, ABCB1
Model	Drosophila melanogaster	fruit fly	MDR49b		1101	NP_001163132.1	36428	Reviewed	Yes	KEGG, ABCB1
Model	Drosophila melanogaster	fruit fly	MDR50		1313	NP_523740	36582	Reviewed	Yes	KEGG, ABCB1
Model	Plutella xylostella	diamondback moth	MDR49		1261	XP_037968795	105383438	Model	Transcriptome only	KEGG, ABCB1
Model	Lucilia cuprina	Australian sheep blowfly	MDR49		1323	XP_023292498	111675874			KEGG, ABCB1
Model	Lucilia cuprina	Australian sheep blowfly	MDR65		1304	XP_023293429	111676690			KEGG, ABCB1
Model	Lucilia cuprina	Australian sheep blowfly	MDR1a		1304	XP_023295699	111678544			KEGG, ABCB1
Model	Bombyx mori	silk moth	MDR49		1307	XP_004929922	101735430			KEGG, ABCB1
Model	Bombyx mori	silk moth	ABCB1	X3	1268	XP_004924686	101735691			KEGG, ABCB1
Model	Bombyx mori	silk moth	MDR49		1309	XP_004929924	101735703			KEGG, ABCB1
Model	Bombyx mori	silk moth	MDR49		1315	XP_012549839	101738993			KEGG, ABCB1
Model	Bombyx mori	silk moth	MDR49		1329	XP_021208843	101741850			KEGG, ABCB1
Pest	Musca domestica	house fly	MDR1A		1303	XP_005177104	101894474	Model	Transcriptome only	KEGG, ABCB1
Pest	Musca domestica	house fly	MDR49	X1	1356	XP_019895136.1	101895168	Model	None	NCBI Search
Pest	Musca domestica	house fly	MDR49	X2	1345	XP_011295840.1	101895168	Model	None	NCBI Search
Pest	Musca domestica	house fly	MDR49	X3	1332	XP_005191448	101895168	Model	None	KEGG, ABCB1
Pest	Musca domestica	house fly	MDR65		1303	XP_005186344	101899244	Model	Transcriptome only	KEGG, ABCB1
Pest	Galleria mellonella	Wax Moth	ABCB1-Like	X1	1274	XP_026762002	113520794	Model	None	NCBI Search
Pest	Galleria mellonella	Wax Moth	ABCB1-Like	X2	1163	XP_031765976.1	113520794	Model	None	NCBI Search
Pest	Galleria mellonella	Wax Moth	ABCB1-Like		1183	XP_026762069.1	113520845	Model	None	NCBI BLASTP
Pest	Galleria mellonella	Wax Moth	ABCB1-Like	X1 2	1254	XP_026765038	113523317	Model	None	NCBI BLASTP
Pest	Galleria mellonella	Wax Moth	ABCB1-Like	X2 2	1254	XP_026765039	113523317	Model	None	NCBI BLASTP
Pest	Varroa destructor	Varroa Mite	MDR1-Like	X1	1607	XP_022661976.1	111250671	Model	None	BLASTP
Pest	Varroa destructor	Varroa Mite	MDR1-Like	X2	1603	XP_022661977.1	111250671	Model	None	BLASTP
Pest	Varroa destructor	Varroa Mite	MDR1-Like	X3	1598	XP_022661978.1	111250671	Model	None	BLASTP
Pest	Varroa destructor	Varroa Mite	MDR1-Like	X4	1585	XP_022661980.1	111250671	Model	None	BLASTP
Pest	Aethina tumida	Small Hive Beetle	MDR1-Like	IF1	1252	XP_019879179.1	109607095	Model	None	BLASTP, LOW QUALITY PROTEIN
Pest	Aethina tumida	Small Hive Beetle	MDR1-Like	IF2	1260	XP_019874216.1	109602313	Model	None	BLASTP, LOW QUALITY PROTEIN
Pest	Aethina tumida	Small Hive Beetle	MDR1-Like	IF3	1253	XP_019878114	109605960	Model	None	BLASTP, LOW QUALITY PROTEIN
Pollinator	Apis mellifera	eastern honeybee	MDR49	X1	1343	XP_006569044	551167	Model	Genome and proteome only	KEGG, ABCB1, NCBI Lists 4 identical sequences
Pollinator	Apis mellifera	eastern honeybee	MDR49	X2	1322	XP_006569046.1	551167	Model	Genome and proteome only	KEGG, ABCB1, NCBI Lists 3 identical sequences
Pollinator	Bombus impatiens	common eastern bumble bee	MDR49	X1	1344	XP_012242648	100745824	Model	Genome only	KEGG, ABCB1, NCBI Lists 4 identical sequences
Pollinator	Bombus impatiens	common eastern bumble bee	MDR49	X2	1323	XP_012242651.1	100745824	Model	Genome only	KEGG, ABCB1, NCBI Lists 2 identical sequences
Pollinator	Bombus terrestris	buff-tailed bumblebee	MDR49		1344	XP_020723751	100650108	Model	None	KEGG, ABCB1
Pollinator	Ceratina calcarata	carpenter bee	MDR49	X1	1346	XP_017884014	108627333	Model	None	KEGG, ABCB1, NCBI Lists 2 identical sequences
Pollinator	Ceratina calcarata	carpenter bee	MDR49	X2	1325	XP_026671324.1	108627333	Model	None	KEGG, ABCB1
Pollinator	Megachile rotundata	alfalfa leafcutting bee	MDR49	X1	1346	XP_003701514.1	100877577	Model	review only	BLASTP, 4 Identical proteins
Pollinator	Megachile rotundata	alfalfa leafcutting bee	MDR49	X2	1325	XP_012136740.1	100877577	Model	review only	BLASTP, 2 Identical Proteins
Pollinator	Osmia bicornis bicornis	Red Mason Bee	MDR49	X1	1346	XP_029036184.1	114872770	Model	None	BLASTP, 5 Identical proteins
Pollinator	Osmia bicornis bicornis	Red Mason Bee	MDR49	X2	1325	XP_029036190.1	114872770	Model	None	BLASTP, 3 Identical Proteins
Pollinator	Osmia lignaria	orchard mason bee	MDR49	X1	1346	XP_034170893.1	117600059	Model	None	BLASTP, 6 Identical proteins
Pollinator	Osmia lignaria	orchard mason bee	MDR49	X2	1325	XP_034170899.1	117600059	Model	None	BLASTP, 3 Identical Proteins

Table A16: Summary table of disease vectors and the disease they carry. Data from https://www.who.int/news-room/fact-sheets/detail/vector-borne-diseases, https://www.cdc.gov/ticks/diseases/index.html, and https://www.cdc.gov/ticks/tickbornediseases/tickID.html.

	Scientific Name	Common Name	Disease
Mosquito	Anopheles gambiae	mosquito	Lymphatic filariasis
Mosquito	Anopheles gambiae	mosquito	Malaria
Mosquito	Aedes aegypti	yellow fever mosquito	Chikungunya
Mosquito	Aedes aegypti	yellow fever mosquito	Dengue
Mosquito	Aedes aegypti	yellow fever mosquito	Lymphatic filariasis
Mosquito	Aedes aegypti	yellow fever mosquito	Rift Valley fever
Mosquito	Aedes aegypti	yellow fever mosquito	Yellow Fever
Mosquito	Aedes aegypti	yellow fever mosquito	Zika
Mosquito	Culex quinquefasciatus	southern house mosquito	Japanese encephalitis
Mosquito	Culex quinquefasciatus	southern house mosquito	Lymphatic filariasis
Mosquito	Culex quinquefasciatus	southern house mosquito	West Nile fever
Ticks	Ixodes scapularis	blacklegged tick	Anaplasmosis
Ticks	Ixodes scapularis	blacklegged tick	Babesiosis
Ticks	Ixodes scapularis	blacklegged tick	Borrelia mayonii
Ticks	Ixodes scapularis	blacklegged tick	Borrelia miyamotoi
Ticks	Ixodes scapularis	blacklegged tick	Lyme disease
Ticks	Ixodes scapularis	blacklegged tick	ehrlichiosis
Ticks	Ixodes scapularis	blacklegged tick	Borrelia burgdorferi
Ticks	Ixodes scapularis	blacklegged tick	Powassan disease
Ticks	Ixodes pacificus	western blacklegged tick	Anaplasmosis
Ticks	Ixodes pacificus	western blacklegged tick	Borrelia miyamotoi
Ticks	Ixodes pacificus	western blacklegged tick	Lyme disease
Ticks	Dermacentor andersoni	Rocky Mountain wood tick	Colorado tick fever
Ticks	Dermacentor andersoni	Rocky Mountain wood tick	tularemia
Ticks	Dermacentor andersoni	Rocky Mountain wood tick	Rocky Mountain spotted fever
Ticks	Ambylomma americanum	lone star tick	Ehrlichiosis
Ticks	Ambylomma americanum	lone star tick	Southern tick-associated rash illness
Ticks	Ambylomma americanum	lone star tick	Bourbon virus
Ticks	Ambylomma americanum	lone star tick	Heartland virus
Ticks	Ambylomma americanum	lone star tick	Tularemia
Ticks	Ixodes cookei	groundhog tick	Powassan disease
Ticks	Amblyomma maculatum	Gulf Coast tick	Rickettsia parkeri rickettsiosis
Ticks	Dermacentor variabilis	American dog tick	Rocky Mountain spotted fever
Ticks	Dermacentor variabilis	American dog tick	Tularemia
Ticks	Rhipicephalus sanguineus	brown dog tick	Rocky Mountain spotted fever
Ticks	Dermacentor occidentalis	Pacific Coast tick	364D rickettsiosis
Ticks	Ornithodoros spp.	Soft Tick	Borrelia hermsii
Ticks	Ornithodoros spp.	Soft Tick	Borrelia turicatae
Ticks	Ornithodoros spp.	Soft Tick	tick-borne relapsing fever

Table A17: Table of honeybee parasites and the disease they cause. See Ch. 2 Table 3 for treatments. Tracheal mites (orange) have yet to be fully cloned and annotated.

	Scientific Name	Common Name	Disease
Parasite	Varroa destructor	Varroa Mite	feed on the hemolymph, vectors for disease
Parasite	Acarapis woodi	Tracheal Mite	Pierce breathing tubes and feed on hemolymph
Parasite	Galleria mellonella	Wax Moth	larvae feed on the wax, pollen, and host's brood
Parasite	Aethina tumida	Small Hive Beetle	eat the wax comb and stored honey and pollen

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