Environmental, Toxicological, and Evolutionary Influences on Membrane Composition in Fish

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Summary

Many factors affect membrane composition in ectotherms, including allometry, temperature, toxins such as PCB-153, and osmotic stress. This thesis seeks to describe the relationship between membrane composition, size, and phylogeny in twelve species of cypriniform fish; to describe interactions between the homeoviscous responses to temperature and to PCB-153 in goldfish and rainbow trout; and to describe the membrane response to hypoosmotic stress in goldfish. Commonalities in these patterns provide insight into shared mechanisms of phospholipid modulation. In particular, such similarities indicate whether the membrane pacemaker theory of metabolism, which connects allometric relationships between body size, membrane phospholipids, and metabolic rate, can serve as a general framework for understanding membrane composition. Chapter 2 investigates how cypriniform membrane unsaturation decreases with mass through different fatty acid substitutions than in endotherms, but these fatty acids are in turn shown to be due to the species' relatedness to one another rather than to purely physiological causes. In Chapter 3, PCB-153 is shown to increase cholesterol in liver and brain, while high temperature primarily reduces phospholipid unsaturation. In Chapter 4, these patterns are further explored in trout. As in goldfish, cholesterol modulation is the primary response to PCB-153, whereas temperature primarily reduces phospholipid unsaturation. Trout show more pervasive fatty acid changes than goldfish in all tissues except the liver, which does not respond to PCB exposure, suggesting that PCB-153 pushes trout's homeoviscous response to a limit that similarly-exposed goldfish do not face. Chapter 5 shows that goldfish intestines decrease membrane saturation; kidneys decrease membrane cholesterol; gills decrease neither; and muscles decrease both in response to long-term exposure to hypoosmotic conditions. The intestine and kidney are both involved in recovering ions from body fluids, but gills suppress ion loss and muscle concentrates ions from the bloodstream. Temperature, osmotic stress, PCB-153, and increasing body size are all addressed via a similar set of membrane responses in fish, which fits with the membrane pacemaker theory's predictions regarding membrane composition, metabolic rate, and size.

Résumé

Plusieurs facteurs affectent la composition membranaire des ectothermes, y compris l'allométrie, la température, les toxines telles que le PCB-153, et le stress osmotique. Cette thèse explore la relation entre la composition des membranes, la taille, et la phylogénie chez douze espèces de poissons cypriniformes; elle décrit les interactions entre les réponses homéovisqueuses à la température et au PCB-153 chez le poisson rouge et la truite; et elle examine la réponse membranaire du poisson rouge au stress hypoosmotique. Des tendances de réponses similaires pourraient suggérer des mécanismes communs utilisés pour la modulation des phospholipides. En particulier, de telles similarités indiquent si la théorie du pacemaker membranaire du métabolisme—qui connecte les relations allométriques entre la taille du corps, les phospholipides de la membrane, et le taux métabolique—peut servir de cadre général pour comprendre la composition membranaire. Le Chapitre 2 démontre que l'insaturation des membranes de cypriniformes diminue avec la masse par l'entremise de substitutions d'autres acides gras que chez les endothermes, cependant, ces changements d'acides gras s'avèrent être des artefacts phylogénétiques. Au Chapitre 3 on voit que le PCB-153 augmente le cholestérol dans le foie et le cerveau, tandis que la hausse de température réduit principalement l'insaturation des phospholipides. Dans le Chapitre 4, ces motifs sont davantage explorés chez la truite. Comme chez le poisson rouge, la modulation du cholestérol est la réponse primordiale au PCB-153, tandis que la température réduit principalement l'insaturation des phospholipides. La truite montre des changements d'acides gras plus généralisés que le poisson rouge dans tous les tissus sauf le foie, qui ne réagit pas à l'exposition au PCB, ce qui suggère que le PCB-153 pousse la réponse homéovisqueuse de la truite vers une limite absente chez le poisson rouge. Dans le Chapitre 5, on voit que l'intestin du poisson rouge réduit la saturation des membranes; le rein réduit le cholestérol membranaire; les branchies ne réduisent ni l'un ni l'autre; et le muscle les réduit tous les deux. L'intestin et le rein sont tous les deux impliqués dans la récupération des ions, mais, les branchies diminuent les pertes d'ions, et le muscle concentre les ions provenant de la circulation. La température, le stress osmotique, le PCB-153, et une augmentation de la taille du corps provoquent tous une suite de

réponses membranaires similaires chez les poissons, en accord avec les prédictions de la théorie du pacemaker membranaire du métabolisme concernant la composition des membranes, le taux métabolique, et la taille.

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16:0	palmitate	Na^+/K^+-ATPase	sodium/potassium adenosine triphosphatase
16:1	palmitoleate	Chol	cholesterol
18:0	stearate	CytB	cytochrome B
18:1	oleate	COI	cytochrome oxidase I
18:2	linoleate	DBI	double bond index
18:3	α-linolenate	DHA	docosahexaenoate
20:0	eicosanoate	IRBP2	interphotoreceptor retinoid-binding protein 2
20:1	gondoate	ND4	nicotinamide adenine dinucleotide hydride dehydrogenase subunit 4
20:2	eicosadienoate		
20:3	eicosatrienoate	PCB	polychlorinated biphenyl
20:4	arachidonate	PDAP	Phenotypic Diversity Analysis Programs
20:5	eicosapentaenoate	PIC	phylogenetically independent contrasts
22:0	behenate	PLFA	phospholipid fatty acid
22:3	docosatrienoate	PUFA	polyunsaturated fatty acid
22:6	docosahexaenoate	s.e.m.	standard error of the mean
24:1	nervonate	SERCA	sarco/endoplasmic reticulum calcium adenosine triphosphatase

CHAPTER 1.
General Introduction

Membrane Physiology

Membranes are essential to life on Earth. By separating inside from outside, membranes enable the creation of concentration gradients, the hoarding of raw materials for growth, the sheltering of genetic material from environmental damage, and every other function of life. Membranes are ubiquitous enough in biology that even some viruses bear them (Salonen et al., 2005). Eukaryotic cells in particular have numerous membranes, thanks to their mitochondria, endoplasmic reticula, Golgi apparata, vesicle systems, and other membrane-bound organelles. This multiplicity of membranes is home to numerous proteins, which serve many vital roles in cell functioning. Membrane proteins include ATP synthase, which produces ATP at the end of the electron transport chain (Zhang et al., 2015); Na^+/K^+-ATPase, an ubiquitous ion pump used in osmoregulation (Grosell, 2006); tyrosine kinases, which are part of numerous molecular pathways (Hanks et al., 1988); and countless others. Membrane-related functions constitute the bulk of the cellular activity contributing to metabolic rate in animals (Hulbert and Else, 2000).

Once regarded as uniform, passive barriers, membranes are now understood as fluid, active participants in metabolism and other cellular activities (Singer and Nicolson, 1972). The principal components of biological membranes are phospholipids, cholesterol, and proteins. Phospholipids provide the primary structure of membranes, forming a bilayer with their hydrophilic head groups facing the inside and outside of the membrane-bound compartment to shelter the hydrophobic tails. This is illustrated in Figure 1.1. Phospholipids with different head groups can have different properties and roles in membranes, and are often preferentially enriched on inner or outer membrane faces, in specific areas, or in specific tissues (Aureli et al., 2016). Cardiolipin is most commonly found in bacterial membranes and in the inner membranes of mitochondria, for example (Garcia Fernandez et al., 2004). Another class, sphingolipids, have sphingomyelin as their head group and are enriched in myelin sheaths (Aureli et al., 2016). Although most membrane molecules are phospholipids, between 20% and 70% of membrane mass is proteins, with mitochondrial inner membranes in particular known for higher values (Warnock et al.,

1993). These proteins can occupy one layer of the bilayer, both layers, or cross between one membrane and another, as is seen in tight junction complexes (Chasiotis et al., 2012a). Membrane proteins often interact strongly with their lipid neighbors, enabling lipids to serve as regulatory elements for protein processes. Some of these interactions take the form of "lipid rafts," in which cholesterol- and sphingolipid-enriched membrane regions thicker than the surrounding membrane transiently coalesce around lipid-anchored proteins and modulate their function (Aliche-Djoudi et al., 2013; Barenholz, 2002; Turk and Chapkin, 2013). In addition to helping maintain lipid rafts, sterols such as cholesterol and ergosterol modulate phase transition temperatures and participate in thermal acclimation (Aureli et al., 2016; Crockett, 1998; Dong et al., 2015). Interestingly, lipoproteins, a series of non-cellular structures used to transport lipids in the blood, are surrounded by lipid monolayers rather than bilayers (Magnoni and Weber, 2007).

Phospholipids are not fixed to one another, and flow around each other and other membrane constituents. Depending on their composition and temperature, phospholipids can therefore assume any of various temperature-dependent phases, including inverted-hexagonal, liquid crystal, and gel (Crockett, 1998). The inverted-hexagonal phase is the natural state of most phospholipids when they are not in membranes and the configuration they assume in very high temperatures, but represents severe damage in a living cell (Vladkova et al., 2010). Living cells maintain a balance of regions in the liquid-crystal and gel phases to preserve membrane integrity and protein function. This balance is often described in terms of membrane fluidity, viscosity, or order. The processes used to maintain membrane fluidity/viscosity/order/phase are united under the terms *homeophasic* or *homeoviscous acclimation*, and involve phospholipid head groups, phospholipid tails, and sterols (Hazel, 1995; Veld et al., 1993). Often, the goal is to keep the membrane's phase transition temperature higher than the ambient temperature, to prevent gel regions from "melting" into liquid crystal and liquid crystal regions from fragmenting entirely—or, conversely, to keep liquid crystal regions from "freezing" into gel (Crockett, 1998; Hazel et al., 1991; Zehmer and Hazel, 2005). Notably, homeoviscous acclimation is rarely perfect, and some

amount of membrane order remains lost or in excess after an animal's homeoviscous response concludes (Raynard and Cossins, 1991; Zehmer and Hazel, 2004). The degree of compensation can only be diagnosed via direct measurements of membrane order, but the presence and relative intensity of homeoviscous responses can be otherwise noted via proxy measurements such as membrane lipid composition.

The best-studied factor affecting membrane fluidity is temperature. Heat makes membranes more fluid, and more fluid membranes give proteins more freedom to change configuration and therefore usually increase their activity, and vice versa (Hazel, 1984), compounding the effects that temperature has on proteins themselves (Hazel, 1972). The first homeoviscous response to temperature stress, beginning within hours, is to modulate the levels of phospholipids with common head groups. Enzymes such as phosphatidylethanolamine N-methyltransferase convert phospholipid head groups into one another directly, while the phospholipids are still within membranes (Vance et al., 1982). Typically, phosphatidylethanolamine, with a very small head group that causes phospholipids to have a conical shape, is replaced with phosphatidylcholine, with a much larger head group, to decrease membrane fluidity in response to heat, and vice versa (Farkas et al., 1994). In some cases, the exchange is more thorough, and sphingomyelin and cardiolipin also decrease (Hazel, 1979). This exchange preserves membrane order in membranes disordered by heat and preserves membrane disorder in membranes ordered by cold, while not affecting other membrane properties, as these head groups are already present in large quantities in most eukaryotic membranes (Dowhan, 1997). Most other head groups, including serine and inositol, have only minimal participation in this process (Dowhan, 1997; Hazel, 1979). Notably, differences in relative amounts of phospholipid head groups between goldfish acclimated to different temperatures are no longer detectable after several weeks, indicating that this response is temporary (Farkas et al., 2001; Hazel and Landrey, 1988; Kemp and Smith, 1970). Phospholipid fatty acids and cholesterol become the primary membrane constituents modified to maintain membrane order in the long term once phospholipid head groups no longer serve this role.

The role of phospholipid fatty acids is the best-known aspect of homeoviscous acclimation. Much as phospholipid head groups have different shapes, the fatty acid tails they bear also take up variable amounts of space in a membrane. Commonly, phospholipids consist of a single polyunsaturated fatty acid and a mono- or unsaturated fatty acid affixed to the sn-2 and sn-1 positions of a glycerol molecule, with the third position occupied by the head group (Cossins et al., 1977; Farkas et al., 2001). The double bonds in a polyunsaturated fatty acid, particularly if they are in a cis configuration, cause the fatty acid to "kink" rather than project directly from the head group, an effect increased by the motion around the single bonds. More saturated phospholipids can therefore pack more closely together than their less saturated counterparts and cause membrane order to increase, for much the same reason that olive oil (less saturated) has a lower melting point than butter (more saturated). To counter this effect, phospholipid fatty acids become less saturated with cold acclimation and more saturated with warmth in numerous species and tissues (Cossins, 1976; de Virville et al., 2002; Hazel, 1995; Overgaard et al., 2008; Raynard and Cossins, 1991; Sinensky, 1974; Wodtke and Cossins, 1991). These changes typically require days to begin and weeks to complete, as opposed to hours for phospholipid head group modulation (Cossins, 1976). Changes in membrane fatty acid composition appear to be mediated by fatty acid desaturases, which increase in activity during cold acclimation in many species (Polley et al., 2003; Schünke and Wodtke, 1983; Snyder and Hennessey, 2003; Trueman et al., 2000; Wodtke and Cossins, 1991). Interestingly, fatty acid changes are not equally prevalent across all tissues. In species whose tissue responses have been investigated separately, brain and muscle tissue in particular are much less responsive than other tissues. The brain maintains a very particular level of polyunsaturated fatty acids that is remarkably consistent across vertebrate species, limiting its ability to modulate fatty acids in response to environmental stressors (Farkas et al., 2000; Roy et al., 1997). At least one researcher asserts that sarcoplasmic reticulum, which comprises the bulk of membrane area in muscle, does not undergo homeoviscous adjustments, and further evidence indicates that enzymes associated with temperature acclimation in crocodile liver do not activate in crocodile muscle (Cossins et al., 1978; Seebacher et al.,

2009). To some degree, muscle tissue may simply endure, rather than resist, temperature-induced disruptions of membrane order.

Sterols, including cholesterol in mammals, ergosterol in yeasts, and several others, have variable effects on membrane order. Cholesterol can increase, decrease, or not respond to increasing temperature in ectotherms (Crockett, 1998). This variability relates to cholesterol's role as a buffer against rapid phase transitions. Where changes in head groups and tails can move phase transition temperatures, cholesterol modulation keeps the transition between gel and liquid crystal phases gentle rather than abrupt, preventing sudden shifts that would destabilize the entire membrane (Zehmer and Hazel, 2003; Zehmer and Hazel, 2004). This potentially gives other membrane constituents time to respond to disturbances. Cholesterol also functions to preserve membrane raft integrity, function, and separation from bulk phospholipids (Zehmer and Hazel, 2005) and helps prevent osmotic ion loss and lipid peroxidation (Barenholz, 2002; Hao et al., 2008; Oliveira et al., 2012). Some evidence suggests that cholesterol also affects permeability to carbon dioxide, suggesting a role in respiration (Itel et al., 2012; Tsiavaliaris et al., 2015). This extensive and heterogeneous set of roles assures that cholesterol is a difficult membrane constituent to predict in stress situations.

Thanks to their generally more consistent body temperatures, endotherms do not exhibit homeoviscous acclimation to the same extent as ectotherms (Meng et al., 1969). Some endotherms face situations in which their body temperatures must shift dramatically, however, and these elicit many of the same processes and effects associated with homeoviscous changes in ectotherms. Hibernating mammals, for example, experience dramatic reductions in both body temperature and metabolic rate, necessitating a membrane response. Syrian hamsters compromise between these two concerns by increasing the proportion of heart phospholipid linoleic acid (18:2n-3) and decreasing saturated fatty acids as body temperature drops during the onset of hibernation, but they also decrease docosahexaenoic acid (22:6n-3), a potent membrane fluidizer (Giroud et al., 2013). Phosphatidylethanolamine decreases, phosphatidylcholine increases, and polyunsaturated fatty acids decrease in preparation for inter-bout

arousal in 13-lined ground squirrels, in keeping with the need to maintain membrane order as body temperature rises for these brief periods of activity (Armstrong et al., 2011). Similar effects are reported for many other mammalian hibernators (Kolomiytseva, 2011), revealing that homeoviscous processes are accessible to endotherms, and invoked when needed.

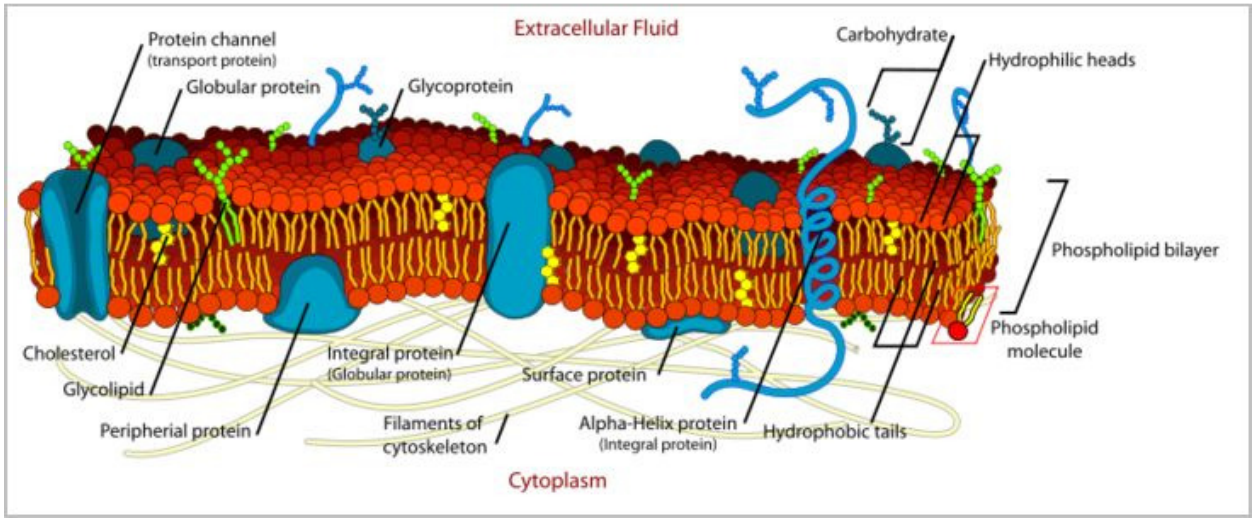

Fig. 1.1. Diagram of plasma membrane according to the fluid mosaic model, showing phospholipids, proteins, cholesterol, and cytoskeleton. Attributed as follows: By Mariana "LadyofHats" Ruiz [Public domain], via http://en.citizendium.org/wiki/Cell_membrane.

Membranes and Osmotic Stress

Given that one of the major reasons for modifying membrane composition in response to temperature is the relationship between membrane lipids and protein activity, it stands to reason that other situations that require changes in membrane protein activity might affect membrane composition. One such situation is osmotic stress. The core of the response to osmotic stress is activating membrane proteins, in particular Na^+/K^+-ATPase, whether to recover ions from the environment, prevent ion losses, or concentrate excess ions in urine and other excretory pathways. These pumps are ubiquitous and their activity is affected by membrane composition (Tang et al., 2010; Wu et al., 2004).

Membrane cholesterol has been shown to increase tolerance for hypoosmotic media and resistance to ion loss in fish cells (Hao et al., 2008; Müller et al., 2008), but the direction of changes in membrane cholesterol in response to membrane-affecting stresses is not universal and depends on whether the model is attempting to solidify the membrane to make it less permeable or fluidize it to increase protein activity (Crockett, 1998). The lipid composition of the tissues of numerous fish species has been shown to change in response to hyperosmotic conditions. *Dicentrarchus labrax* sea bass show decreased phospholipid unsaturation, decreased phosphatidylethanolamine, and increased phosphatidylserine in muscle, liver, and gill during seasonal acclimation to higher salinity, but this is complicated by the association of this same period with increasing temperature and the onset of spawning (Cordier et al., 2002). Total lipids in the whole body and in muscle show decreased mono- and polyunsaturated fatty acids in saline-acclimated *D. labrax* (Hunt et al., 2011). *Poecilia reticulata* guppies show the opposite response, increasing phosphatidylethanolamine, decreasing phosphatidylcholine, and increasing phospholipid unsaturation in their gills and kidneys (Daikoku et al., 1982). Cells isolated from *Salmo salar* Atlantic salmon and *Scophthalmus maximus* turbot also show the opposite of *D. labrax*'s response, increasing the proportion of polyunsaturated fatty acids in their phospholipids with increasing salinity (Tocher et al., 1995). *Acipenser nacarii* Adriatic sturgeon show a much larger proportion of polyunsaturated and lower proportion of saturated fatty acids in gill total lipids when acclimated to 29 ppm salinity than 0 ppm, but show a smaller difference between 0 ppm and 35 ppm (Martínez-Álvarez et al., 2005). Salinity similarly increases the total lipid unsaturation in *Galaxias maculatus* puye (Dantagnan et al., 2007) and increases the proportion of polyunsaturated fatty acids in *Mugil cephalus* mullet total lipids (Khériji et al., 2003). These latter three studies are part of a large body of literature that examines total lipids, rather than the phospholipids examined in the earlier four, and which therefore provide only very limited insight about the potential effects of osmotic stress on membrane composition. Still, in combination with previous studies on membrane lipids in response to osmotic stress in either direction, this body of work strongly suggests that osmotic stress induces the same sorts of changes in membrane composition as temperature, for similar reasons.

The Membrane Pacemaker Theory of Metabolism

The patterns seen in response to temperature and osmotic stress show a curious similarity to those seen on evolutionary scales. Within mammals and birds, phospholipid fatty acid composition varies with body size. Larger species have membranes with more oleate (>%18:1) and less docosahexaenoate (<%22:6), making them consistently less unsaturated (Couture and Hulbert, 1995; Hulbert et al., 2002a). Mass-specific metabolic rate also decreases allometrically in birds, mammals (Schmidt-Nielsen, 1990; White et al., 2006; White and Seymour, 2005), and fish (Clarke and Johnston, 1999; White et al., 2006) and is highly dependent upon membrane processes such as oxidative phosphorylation, cellular fuel intake, and ion transport (Rolfe and Brown, 1997). These are among the processes whose activity is affected by temperature and modulated by homeoviscous acclimation (Guderley et al., 1997; Hazel, 1995; Ibarz et al., 2005; Kraffe et al., 2007; Raynard and Cossins, 1991). Hulbert and Else combined these observations to formulate the *membrane pacemaker theory of metabolism* (Hulbert and Else, 1999). They postulated that the relative abundance of polyunsaturated fatty acids in membrane phospholipids sets metabolic rate by modulating the activity of membrane proteins. The allometric patterns in membrane composition and metabolic rate, effects of homeoviscous acclimation on membrane composition, and membrane response to osmotic stress thereby all point to a common priority: maintaining membrane protein function, and therefore metabolic rate, by maintaining the properties of the proteins' lipid environment. In accordance with the membrane pacemaker proposal, the activity of Na^+/K^+-ATPase from ectotherms is increased by substituting native phospholipids with those from endotherms (Else and Wu, 1999; Wu et al., 2004). Similarly, the activity of succinic dehydrogenase from warm-acclimated goldfish is increased by substituting the native phospholipids with those of cold-acclimated goldfish (Hazel, 1972). Membrane composition can affect intracellular calcium concentration (Yilmaz et al., 2006), which is itself mediated by calcium-ATPase (SERCA), a major protein of the sarcolemma.

However, a substantial body of work suggests that the relationships between membrane composition, metabolic rate, and protein activity proposed by the membrane pacemaker concept may not

hold universally true. Due in part to diet and temperature, fish in general have higher relative levels of polyunsaturated fatty acids in their phospholipids than mammals (Hazel, 1984; Stubbs and Smith, 1984) but lower metabolic rates (Schmidt-Nielsen, 1984). Artificially selecting mice for higher metabolic rate caused an *increase* in membrane saturation (Brzęk et al., 2007), and similar studies show a weak relationship with palmitate only (Haggerty et al., 2008) or a mix of mostly inconsistent shifts in relative fatty acid composition (Wone et al., 2013). Several studies show that increasing trout membrane unsaturation via diet has little to no effect on the activity of major mitochondrial enzymes, despite sometimes affecting oxidative capacity and/or the proportions of rare fatty acids (Guderley et al., 2008; Martin et al., 2013; Martin et al., 2015). The membrane pacemaker concept predicts the opposite in each of these cases.

Most strongly, the original studies indicating a relationship between body size and metabolic rate in mammals and birds did not account for the potential contribution of phylogenetic relatedness to that relationship. Phylogeny gives species shared genetic inheritance that affects their physiology and prevents their trait values from being statistically independent (Díaz-Uriarte and Garland Jr, 1996; Felsenstein, 1985; Garland et al., 1992). This phylogenetic signal can be detected and corrected for when making multispecies comparisons. After reexamining the membrane-metabolic rate relationship using 30 species of mammals and correcting for phylogeny and body mass, Valencak and Ruf (2007) found no link between metabolic rate and any membrane parameter, including % 22:6, % polyunsaturated fatty acids, and overall unsaturation. The original relationships between size, membrane composition, and metabolic rate in mammals are, in this analysis, artifacts of related animals being more similar than unrelated animals in all of these parameters, rather than evidence of a relationship that holds true across taxa.

This multitude of conflicting studies indicates that the membrane pacemaker concept cannot be a complete framework for understanding membrane composition. It almost certainly, however, still provides insight into the factors affecting membrane composition and responses thereto, given the similarity of the responses to factors as disparate as temperature, osmotic stress, and body size. How

closely reality reflects the predictions of the membrane pacemaker concept remains to be more thoroughly investigated, particularly in ectotherms.

Polychlorinated Biphenyls (PCBs)

Polychlorinated biphenyls (PCBs) are a class of organic compounds formerly used for a large variety of applications, including flame retardants, plasticizers, dielectric fluid, electrical insulation, and components of adhesives and caulking. They consist of two 6-carbon benzene rings linked together, with up to 12 hydrogen atoms substituted with chlorine atoms (see Figure 1.2). These many substitution sites mean that the PCB class consists of 209 congeners differing in the number and position of their chlorine substituents, and thus in their chemical properties. Industrial uses of PCBs have much more often involved variable mixtures of congeners rather than pure chemicals, most famously the Arochlor line mixed and sold by weight percentage of chlorine (Arnold et al., 1990). This has meant that the most famous contamination incidents, such as Yusho in Japan and Yu-Cheng in Taiwan, have involved complex mixtures of chemicals with varied toxicological properties (Lung et al., 2005; Mitoma et al., 2015), and that much early research on PCB toxicity has erroneously attributed the effects of some PCB congeners to the whole class (Reich et al., 1981).

PCBs were banned in the United States in the 1970s and Europe in the 1980s due to increasing recognition of their toxicity to humans and to the environment. Because of their environmental persistence, continuing use of pre-existing PCB-containing equipment, and continuing manufacture in other regions, PCBs nevertheless remain prominent pollutants around the world. Physiologically relevant PCB concentrations are reported in *Silurus glanis* wels catfish of the Po watershed in Italy (0.20-1.00 µg/g fresh weight) (Squadrone et al., 2013); *Perca flavescens* yellow perch and *Sander vitreum* walleye of the North American Great Lakes (0.10-1.00 µg/g fresh weight) (Bhavsar et al., 2007); *Salmo salar* Atlantic salmon of the Baltic Sea (0.01-0.09 µg/g fresh weight) (Sørensen et al., 2016); 35 fish species from the Indus watershed (0.00002-0.003 µg/g fresh weight) (Robinson et al., 2016); and five fish species

in the Yangtze River (2.87-3.86 µg/g fresh weight) (Wang et al., 2016). As highly lipophilic toxins, PCBs are typically acquired via the diet and show a strong capacity to biomagnify, becoming more concentrated at higher trophic levels (Bhavsar et al., 2007). They can therefore become a hazard to humans who eat large quantities of high-trophic-level organisms, such as many commonly consumed fish. PCBs are also volatile and can travel long distances on high atmospheric winds, which has caused large concentrations of PCBs to accumulate in Arctic regions far from the kind of industrial development that normally produces PCBs (Armitage et al., 2013; Brown et al., 2013; Jones and de Voogt, 1999). This combination is particularly cruel for the Inuit, Faroese, and other peoples of the far north, who are exposed to this pollution directly and also eat a diet rich in high-trophic-level seafood. As a result, symptoms of chronic PCB poisoning are particularly common in these populations (Kvist et al., 2014; Saint-Amour et al., 2006).

The most famous mode of action for PCB exposure is via the aryl hydrocarbon (ArH or Ah) receptor (Schäfer et al., 2009). This cytosolic receptor is part of a signal transduction pathway that activates cytochrome p450 proteins, which are used to process complex organic compounds. Ligands for this receptor include plant flavonoids and environmental toxins such as benzopyrene, so this response is usually protective (Denison and Nagy, 2003). Some toxins, including 12 PCB congeners, cause this beneficial response to become toxic. These toxins activate the ArH receptor, induce the associated pathway, and are thereby metabolized into free radicals. In addition to generating reactive oxygen species that then generate more free radicals, these free radicals retain the lipophilicity of their source material and thus can easily penetrate intracellular membranes to cause DNA damage, leading to cancer (Nebert et al., 2004). The best-known compound with this mode of action is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), usually called “dioxin,” and the 12 PCBs that behave similarly are characterized by lacking substitutions at any of the four *ortho* positions adjacent to the bond connecting their phenyl rings. Without chlorine atoms to crowd or repel one another at these positions, these congeners assume a coplanar shape, and are often called the coplanar, dioxin-like, or non-*ortho*-substituted PCBs. Other symptoms of ArH-

induced toxicity include characteristic keratinous skin lesions called chloracne and, if encountered in utero or neonatally, developmental disruptions. Because this mode of action is shared between many compounds, it forms the basis of a "toxic equivalency factor" (TEF) that is sometimes used to rank the danger posed by organic toxins, rating them as possessing some fraction of the danger posed by TCDD based on their ability to induce aryl hydrocarbon receptor activity and adding these ratings together to determine the danger posed by mixtures (Safe, 1994). It is worth noting that the nature of the cancer risk posed by dioxin-like compounds is contested, and some researchers maintain that dioxin-like compounds are not genotoxic and induce cancer by some other, unverified means (Dragan and Schrenk, 2000).

The intense scrutiny that has been focused on PCBs has shown that the Toxic Equivalency Factor model is incomplete. Most PCBs—197 of the 209—show minimal or no ability to activate the aryl hydrocarbon receptor or induce the associated cytochrome p450 proteins (Campbell et al., 2008; Gaspar-Ramírez et al., 2015). Some of these have similar oxidative effects via glutathione and other pathways (Zhou and Zhang, 2005). More strikingly, *ortho*-substituted PCBs are much more neurotoxic than their dioxin-like counterparts (Lee and Yang, 2012). These neurotoxic effects are increasingly prevalent among the survivors of the Yusho and Yu-Cheng mass exposure incidents and children born to exposed mothers (Akahane et al., 2015; Chen and Hsu, 1994; Furuya et al., 2005; Mitoma et al., 2015). In-utero and neonatal exposure to *ortho*-substituted PCBs induces behavioral changes and cognitive deficits in rhesus monkeys and in rats, with emphasis on performance on visual and spatial reasoning tests (Arnold et al., 1990; Rice and Hayward, 1997). There is evidence that PCB neurotoxicity is mediated by free radicals, as with their apparent genotoxicity, as antioxidants are sometimes protective against it (Venkataraman et al., 2010). However, non-coplanar PCBs have another mode of action that may explain many of their effects, including neurotoxicity, better than those previously proposed.

Unlike the 12 dioxin-like PCBs, *ortho*-substituted PCBs can intercalate themselves into phospholipid bilayers, either penetrating fully into the membrane core or remaining associated with one of the faces (Bonora et al., 2003; Campbell et al., 2008; Reich et al., 1981). This disrupts membrane order

and increases membrane fluidity (Yilmaz et al., 2006), affecting the activity of numerous membrane proteins of highly heterogeneous function throughout the organism (Lee et al., 1999). Silanols, another chemical class with this mode of action, are potent bactericides because of their ability to disrupt membrane order, leading to cytoskeletal deformations, blebbing, and cell lysis (Kim et al., 2007). It is therefore possible that some of the similar effects of mixed PCB exposure attributed to lipid peroxidation via free radicals instead come partly or fully from this physical effect (Bonora et al., 2003; Katynski et al., 2004; Tan et al., 2004). Toxins capable of affecting membrane order or fluidity are, as well, uniquely poised to interact with homeoviscous acclimation, whereby ectotherms modulate membrane components to maintain consistent membrane order despite the effects of changing temperature on lipid properties.

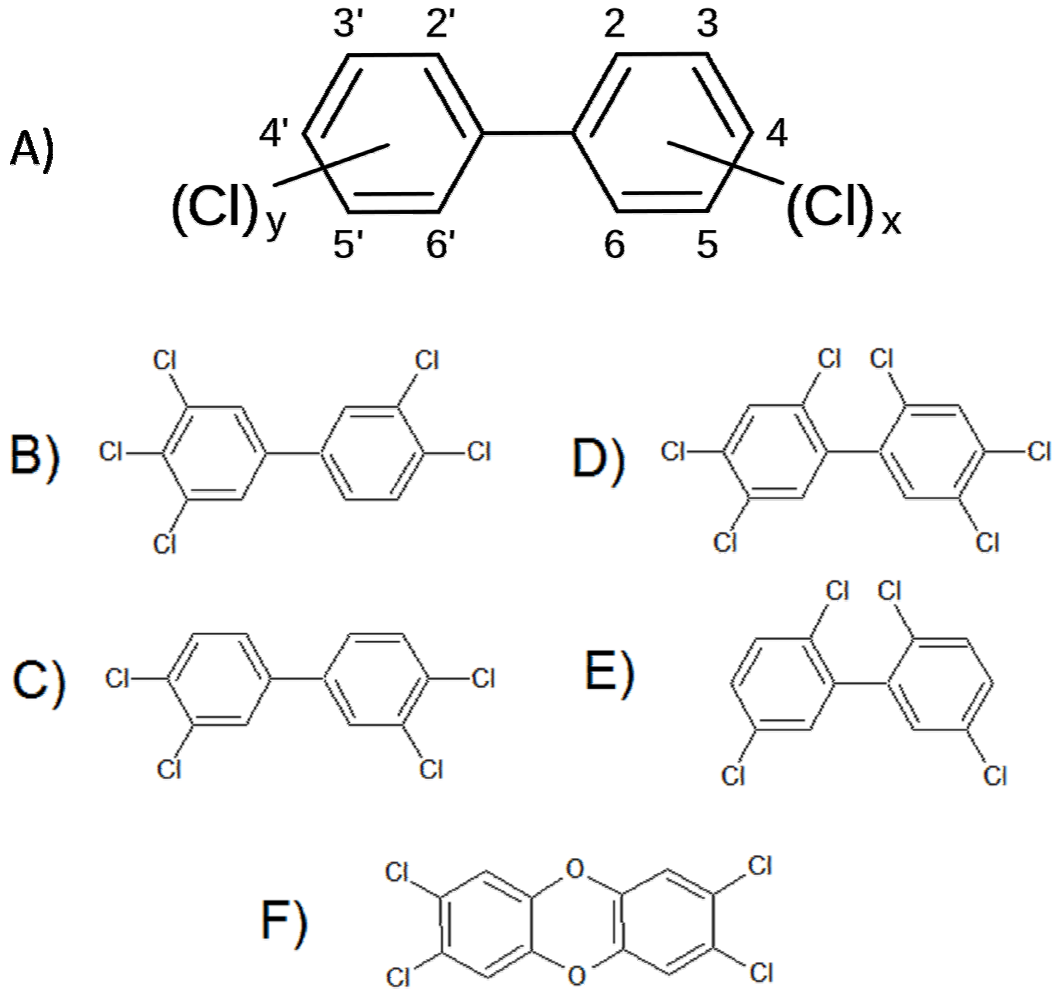

Fig. 1.2. Structure diagrams for common PCB congeners. A) Generic PCB illustrating numbering scheme. B) 3,3′,4,4′,5-pentachlorobiphenyl or PCB-126, coplanar. C) 3,3′,4,4′-tetrachlorobiphenyl or PCB-77, coplanar. D) 2,2′,4,4′,5,5′-hexachlorobiphenyl or PCB-153, non-coplanar (used in Chapters 3 and 4). E) 2,2',5,5'-tetrachlorobiphenyl or PCB-52, non-coplanar. F) 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, a non-PCB. Panel A by Dschanz (own work (drawn with BKchem)) [Public domain], via Wikimedia Commons. Panels B-F generated using ACD/ChemSketch Freeware 2015.

Model Organisms

Twelve wild cypriniform fish species are the subjects of Chapter 2. Goldfish are used as model organisms in Chapter 3 and Chapter 5. Rainbow trout are used as model organisms in Chapter 4. Relationships between the 14 species used in this thesis are summarized as a phylogenetic tree (Figure 1.3). This diagram was generated using PhyML as employed in Phylogeny.fr (Dereeper et al., 2008)..

Approximately half of known living vertebrate species are fish. Fish represent the oldest and most successful lineages of vertebrates and have colonized virtually every aquatic biome. Some have even moved to semi-aquatic lifestyles (Pronko et al., 2013), and one lineage famously gave rise to the tetrapods. Studying fish not only provides insight into the specializations of an ecologically important clade of our distant relatives, but hints at humankind's own history.

The evolutionary relationships among fish have only recently been subjected to the same kind of genetic and cladistic scrutiny that has already massively revised the family trees of mammals (Helgen, 2011), birds (Mirarab et al., 2014), and tetrapods at large (Amemiya et al., 2013). As a result, many seemingly established groups have been shown to be paraphyletic assemblages of creatures united by their primitive characters, rather than shared evolutionary history. The largest changes from once-conventional understanding are that the tetrapods, once held to be a whole series of separate groups, are now known to have a common origin deep within the Sarcopterygii, or lobe-finned fishes; and that the bichirs (Polypteriformes) and sturgeons (Acipenseriformes) are no longer thought to together comprise a monophyletic group (Broughton et al., 2013; Saitoh et al., 2011).

All fourteen of the species used in this thesis are members of the clade Clupeocephala, which includes bony fish other than the bonytongues (Osteoglossiformes), the kin of the true eels (Elopomorpha), gars and bowfins (Holostei), sturgeons, bichirs, and lobe-fins (Saitoh et al., 2011). This gives the fish a common ancestor hypothesized for the mid-Permian period, approximately 275 million years ago. Thirteen of the fourteen are members of Cypriniformes, one of the largest fish orders, which

itself became distinct from its closest relatives in the Mesozoic era approximately 219 million years ago (Saitoh et al., 2011) to 100 million years ago (Broughton et al., 2013). The Cyprinidae may have emerged as a distinct clade within Cypriniformes as early as 155 million years ago (Saitoh et al., 2011), depending on whether Saitoh et al or Broughton et al's molecular clock analyses are closer to the truth. For comparison, the last common ancestor of primates and even-toed ungulates most likely lived approximately 90 million years ago (Nei et al., 2001), making many mammal orders notably younger than Cyprinidae.

The fourteenth, the rainbow trout *Oncorhynchus mykiss*, is a member of the Salmonidae, which likewise diverged from its own closest relatives during the Cretaceous period, approximately 145 million years ago. The salmonids were once held to be members of the "Protacanthopterigii," an assemblage of "intermediate teleosts" with less advanced skeletal structures than the "advanced teleosts" such as Perciformes, but more advanced structures than Cypriniformes, Osteoglossiformes, and other ancient groups. This assemblage is now believed to be paraphyletic, collecting creatures that share a common ancestor not only with each other, but with the "advanced" teleosts (Broughton et al., 2013). Therefore, contrary to what was once proposed, *O. mykiss* is not evolutionarily closer to the other 13 species examined here than most other fish would be.

Cypriniformes has itself seen extensive revision in recent years, and that revision is not yet complete. Some recent studies suggest that cypriniforms other than the Cyprinidae proper are a monophyletic group sister to the cyprinids, termed Cobitoidea (Bufalino and Mayden, 2010; Chen and Mayden, 2012). Another suggests that one family, Catostomidae, is the sister group to Cyprinidae within Cypriniformes, and that the other ostensibly cobitoid families are more distant (Saitoh et al., 2011). Both of these proposals leave the five catostomid species used in this thesis in the same phylogenetic position relative to the eight cyprinids. The monophyly of Cyprinidae, for its part, is widely supported.

Ecologically, the fourteen species in this thesis have widely different diets, habitat preferences, sizes, and reproductive biology. The Cypriniformes are a diverse lineage, containing pelagic predators, benthic scavengers, riffle-dwelling herbivores, and everything in between. They range from a handful of grams to several kilograms in size and are native to every continent except South America, Australia, and Antarctica. Many species around the world are economically important as food, bait, or sport fish. The Cypriniformes also include hundreds of species popular in the aquarium trade and as experimental models, most famously the goldfish *Carassius auratus*, which have been bred as display animals for as long as there has been a trade in pet fish (Froese and Pauly, 2016); and the zebrafish *Danio rerio*, the stock from which the Glo-Fish, the first gene-spliced pet fish, were engineered (Hill et al., 2014). The Salmonidae, similarly, include dozens of Northern-Hemisphere food and sport fish important to the world economy (Froese and Pauly, 2016) and whose semelparous, anadromous life cycles bring crucial oceanic nutrients to terrestrial biomes (Cederholm et al., 1999).

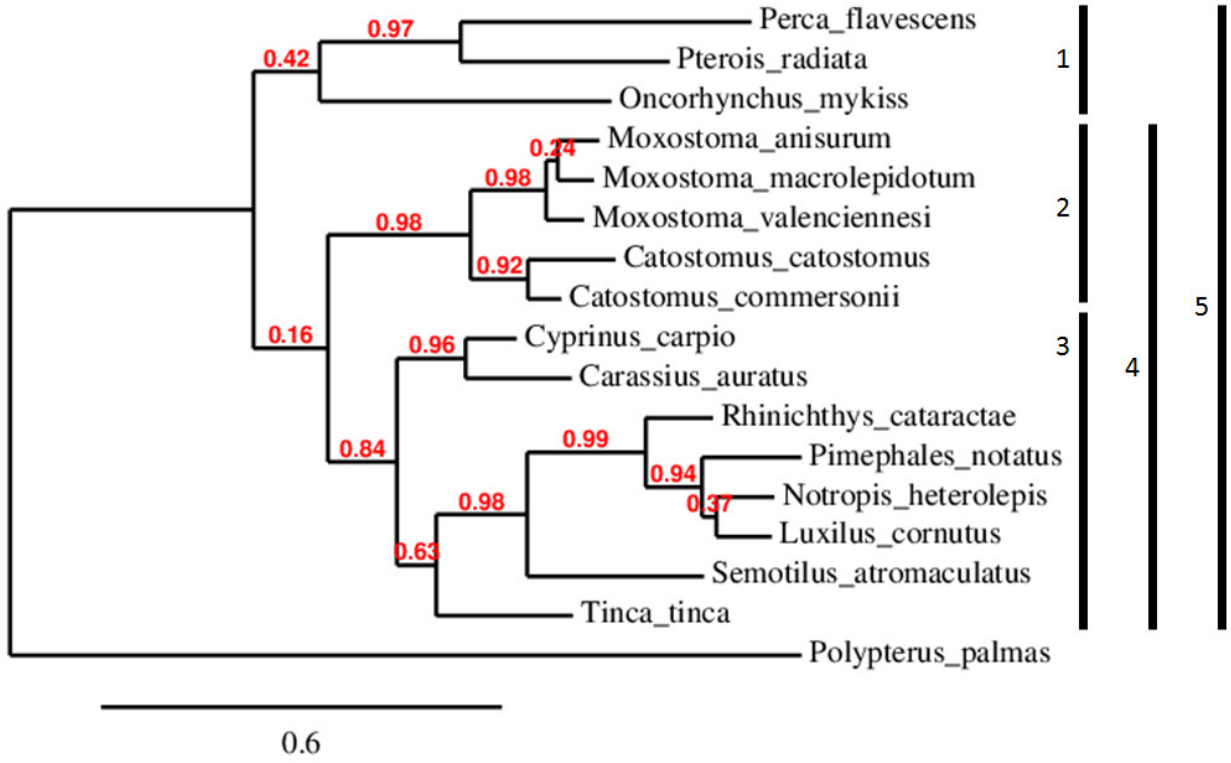

Fig. 1.3. Phylogenetic tree of all 14 species used in this thesis based on cytochrome B (cytB) sequences. Numbers in red are maximum-likelihood estimates. *Perca flavescens*, *Pterois radiata*, and *Polypterus palmas* are used as outgroups to better show the relationships between the 14 and other ray-finned fish. Numbers show clades that include the encompassed species, as proposed in Broughton et al 2013 and Chen and Mayden 2012: 1, Euteleostomorpha; 2, Catostomidae; 3, Cyprinidae; 4, Cypriniformes; 5, Clupeocephala. GenBank accession numbers used are as follows: *C. catostomus* AF454871.1, *C. commersonii* JF799437.1, *C. carpio* DQ868875.1, *L. cornutus* U66597.1, *M. anisurum* JF799452.1, *M. macrolepidotum* JF799476.1, *M. valenciennesi* JF799487.1, *N. heterolepis* AY140696.1, *O. mykiss* D58401.1, *P. flavescens* AF045357.1, *P. notatus* GQ184518.1, *P. palmas* HQ342944.1, *P. radiata* FJ607318.1, *R. cataractae* KF640157.1, *S. atromaculatus* HQ446761.1, *T. tinca* HM167957.1.

Goals of the Investigation

Membrane composition is altered in response to numerous environmental and evolutionary factors, but the degree to which these factors activate the same mechanisms is not clear. The main goal of this thesis was to study the effects of various membrane stressors on membrane composition, and in particular the effects of multiple stressors simultaneously. Additional objectives were to confirm *in vivo* responses previously identified only in cell cultures to determine their physiological relevance. To achieve these objectives, a series of studies were designed to investigate membrane composition and membrane stressors in 14 fish species.

The following questions were addressed:

1. Does the membrane pacemaker hypothesis accurately predict membrane composition in cypriniform fish? (Chapter 2)
2. Do allometric differences in membrane composition across species affect the activity of membrane proteins? (Chapter 2)
3. Does PCB-153 affect membrane composition in living fish? (Chapters 3 and 4)
4. Does PCB-153 interact with the homeoviscous response to temperature? (Chapters 3 and 4)
5. Does sensitivity to temperature change alter the response to PCB-153? (Chapter 4)
6. Does hypoosmotic water induce a change in membrane composition in goldfish? (Chapter 5)

In order for the membrane pacemaker concept to serve as a unifying framework for responses to membrane stressors in fish, it must first accurately describe membrane composition across fish species of various sizes, per its original formulation. In Chapter 2, the goal was to collect membrane composition and muscle calcium-ATPase (SERCA) data from 12 cypriniform fish and correct it for the effect of phylogenetic relatedness. In this way, the study verifies whether the patterns are truly allometric, or whether they have more to do with how the fish are related to one another, as well as whether they relate to the activity of an important membrane protein. I predicted that larger cypriniforms would have more

saturated membranes and lower muscle SERCA activities than smaller ones, in keeping with their lower mass-specific metabolic rates.

The goal of Chapter 3 is to quantify the effect of PCB-153 exposure on membrane composition in goldfish acclimated to two temperatures. This 2×2 factorial design enables the detection of the independent effects of temperature and PCB exposure as well as any synergistic effects that appear specifically when both membrane stressors are in place. I hypothesized that PCB-153 would induce a homeoviscous response in goldfish tissues comparable to the one induced by increasing temperature, using the same mechanisms, and that the responses in brain and muscle would be weaker than those in other tissues.

After confirming that PCB-153 does induce a homeoviscous response in Chapter 3, the goal of Chapter 4 was to extend the experiment of Chapter 3 to a new species, the rainbow trout. This permits a comparison between the response of a more sensitive fish and the goldfish's response. Within each tissue, I hypothesized that PCB-153 would induce a homeoviscous change in cholesterol content and temperature would induce a corresponding homeoviscous change in phospholipid fatty acid saturation, as was shown in Chapter 2. Further, I hypothesized that the combination of high temperature and PCB exposure would elicit strong responses in most or all tissues. The two stimuli impose similar stresses and are more likely to synergize to place higher demands on membrane physiology in trout than in goldfish. In particular, I predicted that muscle and brain would respond more strongly than in previous experiments, because of the intensity of the combined stress. By pushing the homeoviscous response to its limits, this experiment provides further insight into the heterogeneity of the homeoviscous response across an animal's tissues, especially how some organs exhibit larger fatty acid changes in response to temperature than others.

In order to extend this thesis to an additional membrane stressor, the goal of Chapter 5 is to determine whether hypoosmotic stress induces membrane changes in goldfish. This experiment will

determine whether the effect of osmotic stress, if any, is comparable to the differences induced by homeoviscous acclimation to temperature. I hypothesized that phospholipid fatty acid saturation will decrease in response to long-term exposure to ion-poor conditions, but that cholesterol might not behave similarly because of its more complicated role. I also anticipated that the gill, kidney, and intestine would show much stronger responses than muscle, because of their role in osmoregulation.

Finally, general conclusions are presented in Chapter 6, where I discuss the physiological significance of the similarity of response to these various factors, and the value of the membrane pacemaker theory of metabolism as a unifying framework for understanding membrane composition.

CHAPTER 2.
Membranes as possible pacemaker of metabolism in cypriniform fish: does phylogeny matter?

Chapter 2

Membranes as possible pacemaker of metabolism in cypriniform fish: does phylogeny matter?

Based on

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Author Contributions: AG and JMW designed this study, analyzed the data and wrote the manuscript. AG executed the experiments. BP developed and verified the cypriniform phylogeny.

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Introduction

Membranes are the boundaries of every cell compartment. They are dynamic structures that include proteins serving numerous transport, catalytic, and sensory functions (Hulbert and Else, 1999). Their phospholipid composition impacts the activity of many enzymes of energy metabolism, ATPases, hormone receptors, and ion channels by determining local molecular environment (Yilmaz et al., 2006). On an evolutionary scale, it has been shown that phospholipid composition varies with body size in mammals and birds: larger species having membranes with more oleate (>%18:1) and less docosahexaenoate (<%22:6) (Couture and Hulbert, 1995; Hulbert et al., 2002a). Mass-specific metabolic rate also decreases allometrically (Schmidt-Nielsen, 1990), and is highly dependent upon membrane processes like oxidative phosphorylation, cellular fuel intake, and ion transport (Rolfe and Brown, 1997). For fish, the size–metabolic rate relationship was established by surveying 69 species from 12 orders (Clarke and Johnston, 1999) and was later confirmed in a smaller number of species (White et al., 2006). Temperature acclimation of ectotherms involves altering membrane composition to maintain membrane function, such as changing membrane unsaturation to compensate for loss of protein activity with changing temperature (Guderley et al., 1997; Hazel, 1995; Ibarz et al., 2005; Kraffe et al., 2007; Raynard and Cossins, 1991). Hulbert and Else combined all these observations to formulate the membrane pacemaker theory of metabolism (Hulbert and Else, 1999). They postulated that the relative abundance of polyunsaturated fatty acids in membrane phospholipids sets metabolic rate by modulating the activity of membrane proteins. Support for this comes not only from multispecies correlations between membrane composition and metabolism, but also from experimental manipulations of phospholipids. In accordance with the theory, the activity of Na^+/K^+-ATPase from ectotherms is increased by replacing native phospholipids with those from endotherms (Else and Wu, 1999; Wu et al., 2004) and the activity of succinic dehydrogenase from warm-acclimated goldfish is increased by substituting the native phospholipids with those of cold-acclimated goldfish (Hazel, 1972). Similarly, membrane composition can affect intracellular calcium concentration (Yilmaz et al., 2006), but it is unclear whether this response

is mediated by changes in the activity of sarco/endoplasmic reticulum calcium ATPase (SERCA), a major protein and predominant ATPase of the sarcolemma.

By contrast, several recent studies using intraspecific selection or interspecific correlations corrected for phylogeny fail to support the theory. Artificially selecting mice for higher metabolic rate caused an increase in membrane saturation, the opposite of the theory's predictions (Brzęk et al., 2007), and similar studies show a weak relationship with palmitate only (Haggerty et al., 2008) or a mix of mostly inconsistent shifts in relative fatty acid composition (Wone et al., 2013). Several studies show that increasing trout membrane unsaturation via diet has little to no effect on the activity of major mitochondrial enzymes, despite sometimes affecting oxidative capacity and/or the proportions of rare fatty acids (Guderley et al., 2008; Martin et al., 2013; Martin et al., 2015). Phylogeny gives species shared genetic inheritance that affects their physiology and prevents their trait values from being statistically independent (Díaz-Uriarte and Garland Jr, 1996; Felsenstein, 1985; Garland et al., 1992). This phylogenetic signal can be detected and corrected for when making multispecies comparisons. With that in mind, Valencak and Ruf (2007) reexamined the membrane-metabolic rate relationship using 30 species of mammals. After correcting for phylogeny and body mass, they found no link between metabolic rate and any membrane parameter, including percentage 22:6, percentage polyunsaturated fatty acids, and overall unsaturation. With or without correction for phylogeny, the relationship between metabolism and membrane composition has never been characterized for ectotherms, possibly because homeoviscous adjustments would obscure potential correlations (Cossins, 1976). Also, seemingly contradicting the theory, fish have higher relative levels of polyunsaturated fatty acids in their phospholipids than mammals (in part because of diet) (Hazel, 1984; Stubbs and Smith, 1984), and a higher rate of proton leak (Brookes et al., 1998), but lower metabolic rates (Schmidt-Nielsen, 1984).

The goal of this study was to determine whether the membrane pacemaker theory of metabolism applies to ectotherms, using fish as a model. Cypriniforms were selected for this purpose because: (1) The allometric relationship between fish size and metabolic rate is well established (Clarke and Johnston,

1999; White et al., 2006) and cypriniforms may show the same pattern; (2) locally available species range from 1 gram to 10 kilograms in mass, a 10,000-fold range; (3) detailed information about genetic relatedness is readily available for this order, making phylogenetic correction possible; and (4) by including closely related species (and cypriniforms are particularly so), this model enables a test of the theory on a much narrower genetic scale than previously done across all mammals or birds. More specifically, my aim was (1) to test whether the fatty acid composition of muscle and liver membranes changes with body mass in cypriniforms; (2) to determine whether phylogeny affects membrane composition; and (3) to quantify whether the activity of an abundant ATPase important for muscle function, SERCA, varies with body mass and membrane composition in a manner consistent with the theory. I hypothesized that larger cypriniforms would have more saturated membranes and lower muscle Ca^{2+}-ATPase activities than smaller ones, in keeping with their lower mass-specific metabolic rates.

Materials and methods

Animals and experimental design

Adult wild cypriniform fish of 12 species were collected from eastern Ontario and southwestern Quebec by electrofishing, nets, or traps, and they were euthanized via a blow to the head. Carp were acquired from a fish market. Juvenile fish were avoided because of potential ontogenetic differences in fatty acid composition. Details about the species, size, provenance, diet, and water temperature are presented in Table 2.1. The species selected are mostly omnivorous, eating variable mixes of insects, detritus, algae, crustaceans, and plant matter, with a handful of more carnivorous species dispersed across the cladogram, and they include a mix of fast-water and slow-water species. Diet and water speed preference are not associated with each other or with size among these species (Froese and Pauly, 2016; Roberts et al., 2006), nor do they exhibit a phylogenetic pattern, in keeping with previous researchers' work with mammals and birds (Hulbert and Else, 2005; Hulbert et al., 2002a; Hulbert et al., 2002b; Valencak and Ruf, 2007). Temperature was not significantly correlated with size, membrane composition,

or calcium-ATPase activity ($p > 0.05$). White muscle and liver samples were taken and stored at -80°C until analyses. Liver samples were not collected for the carp (*Cyprinus carpio*) and tench (*Tinca tinca*). All procedures were approved by the Animal Care Committee of the University of Ottawa and adhered to the guidelines established by the Canadian Council on Animal Care for the use of animals in research.

Phospholipid composition

Total lipids were extracted from ~30 mg of tissue using chloroform:methanol (2:1 v/v). Samples were homogenized (Polytron, Kinematica, Littau, Switzerland) and centrifuged (10 min at 2000 *g*). Supernatants were filtered and 0.25% KCl added to separate aqueous and organic phases. The organic phase was evaporated and the lipids resuspended in chloroform before loading on solid-phase extraction columns (Supelclean 3 mL 500 mg LC-NH2; Sigma-Aldrich; St. Louis, MO, USA) to separate the phospholipids. Separation was achieved by sequential elution of lipid classes using solvents of increasing polarity: chloroform:isopropanol (3:2 v/v), isopropyl ether:acetic acid (98:2 v/v), and methanol, respectively (Maillet and Weber, 2006). The fatty acid composition of phospholipids was measured after acid transesterification. Fatty acid methyl esters were analyzed on an Agilent Technologies 6890N gas chromatograph (Mississauga, Ontario, Canada) equipped with a fused silica capillary column (Supelco DB-23, 60m, 0.25 mm i.d., 0.25µm film thickness; Sigma-Aldrich) using hydrogen as carrier gas as previously (Magnoni and Weber, 2007). Only the fatty acids accounting for >1% of total fatty acids in phospholipids are reported in this study, although traces of myristoleate (14:1), gondoate (20:1), eicosatrienoate (20:3), and docosatrienoate (22:3) were also detected. Phospholipid recovery could not be verified.

SERCA activity

Calcium-ATPase (SERCA) activity was assayed using a method modified from Tupling et al (Duhamel et al., 2007; Tupling et al., 2011). Muscle samples (~20 mg) were homogenized (Polytron, Kinematica, Littau, Switzerland) in a buffer solution containing 250 mM sucrose, 5 mM HEPES, 0.2 mM PMSF, and 2 g/L NaN_3 at pH 7.5. A 20 µL sample of homogenate was added to 1 mL of assay cocktail

containing 200 mM KCl, 20 mM HEPES, 10 mM NaN_3, 1 mM EGTA, 15 mM $MgCl_2$, 10 mM PEP, 5 mM ATP, 5.73 nM calcium ionophore A23187, 11.47 U/mL lactate dehydrogenase, 3.95 U/mL pyruvate kinase, and 0.93 mM $CaCl_2$ at pH 7.0. This master mix was aliquoted into two Eppendorf tubes, one of which additionally received 1 µL of 40 mM cyclopiazonic acid (CPA) to block SERCA activity and provide a measurement of basal ATPase activity. Ca^{2+}-ATPase activity was initiated with 2 µL of 19 mg/mL NADH and measured as a 340 nm kinetic assay for 30 minutes at room temperature on a spectrophotometer (SpectraMax Gemini XS, Molecular Devices, Sunnyvale, CA, USA) to determine Ca^{2+}-ATPase activity.

Phylogenetic reconstruction

A phylogenetic tree of all 12 species (Fig. 2.1) was obtained from cytochrome oxidase I, cytochrome B, interphotoreceptor retinoid-binding protein 2, and NADH dehydrogenase subunit 4 gene sequences from GenBank as shown in Table 2.2. The analysis was based on earlier phylogenetic reconstructions of the Catostomidae and Cyprinidae (Bufalino and Mayden, 2010; Chen and Mayden, 2012). Two out-group species were included in the analysis (*Hiodon alosoides* and *Scaphirhynchus platorhynchus*). The four sets of sequences were independently aligned using MUSCLE software (Edgar, 2004). The best fit model was determined using MrModel software (Nylander et al., 2004). The aligned sequences were concatenated into a single long sequence with the help of a Perl script, substituting a "?" for any missing data. MrBayes 3.1.2 served to reconstruct the phylogeny with an averaged gamma-distributed Generalized Time Reversible (GTR) model with invariant sites (Ronquist and Huelsenbeck, 2003). Through an MCMC method, 4.5 million trees were generated, keeping every thousandth generation, giving a total of 4500 trees. PhyML was used to confirm the results of MrBayes via 10,000 bootstraps, using the same model as for the Bayesian analysis (Guindon et al., 2010). Note that, although Bayesian methods were used to confirm the probabilities of each monophyletic group in Figure 2.1 and maximum-likelihood methods in Figure 1.3 and the two trees were constructed with different gene

sequences, the two trees are in perfect agreement on the relationships between the 12 species used in this study, indicating that these relationships are robustly supported.

Calculations and statistics

All statistical analyses were performed using SigmaPlot 12 (Systat, San Jose, CA, USA). The relative abundance of each fatty acid (expressed in %) in the phospholipid fraction was plotted against species body mass. All values were log-transformed to achieve linearity (Hulbert, 2007). All values presented are means ± s.e.m. Double bond index (DBI) was calculated as the average number of double bonds divided by the fraction of saturated fatty acids. This index was chosen because it is more sensitive to changes in membrane composition than degree of unsaturation (Maillet and Weber, 2006). Dependent and independent variables were first tested for normality using the Shapiro-Wilk test. Linear regressions were used to test for relationships between relative fatty acid abundance and body mass.

To assess and correct for the degree of relatedness between species, I analyzed the data using phylogenetic comparative methods in two steps. I used the phylosig function (v0.2) included in the phytools package in the R environment (Team, 2016) to evaluate the presence of a phylogenetic signal in the data, using 10 randomly selected trees from the set of 4500 (Revell, 2012). Results did not differ for any of the 10 trees. I then conducted an analysis of PIC using the PDAP module in Mesquite (Garland et al., 1999; Maddison and Maddison, 2011) for each parameter where the phylosig function indicated the presence of a signal ($P<0.05$). To compute phylogenetically independent contrasts for a given parameter, each node or common ancestor on the cladogram is assigned a value equal to the average of the values of the species or nodes immediately descendant from it. The value for each species is then subtracted from that of the other species or node descendant from the same common ancestor and divided by the standard deviation of the their branch lengths, generating a number of contrasts equal to the number of species minus one. This removes from the data any inter-species similarity due to phylogeny while retaining the other relationships between the parameters from which the contrasts were generated (Felsenstein, 1985; Garland et al., 1992). I obtained standardized independent contrasts from the log-transformed character

data. These contrasts thereby show the relationship between the data points when the effect of the fishes' phylogenetic relatedness is removed.

Results

Double bond index

Fig. 2.2 shows the level of unsaturation of membrane phospholipids in muscle and liver expressed as the double bond index. In both tissues, double bond index decreased significantly with body mass ($P<0.05$).

Fatty acid composition of membranes

The relative abundance of individual fatty acids in membrane phospholipids is shown in Table 2.3 for muscle and Table 2.4 for liver. Figs 2.3 and 2.4 show the relationship between six specific phospholipid fatty acids and body mass for both tissues. These six fatty acids were selected because they showed significant changes with body mass in at least one of the tissues examined. They include 18:1 and 22:6, the two fatty acids that were identified in previous membrane pacemaker studies. In muscle, palmitate (16:0) increased and docosahexaenoate (22:6) decreased significantly with mass ($P<0.05$, Fig. 2.3). In liver, palmitoleate (16:1), oleate (18:1), and linoleate (18:2) decreased and stearate (18:0) increased significantly with mass ($P<0.05$, Fig. 2.4). All other membrane fatty acids, as well as average chain length in both tissues, showed no significant relationship with mass ($P>0.05$).

Ca^{2+}-ATPase (SERCA) activity

Ca^{2+}-ATPase (SERCA) activity is related to body mass in Fig. 2.5A, to phospholipid double bond index in Fig. 2.5B, to percent docosahexaenoate in Fig. 2.5C, and to percent linoleate in Fig. 2.5D. None of these relationships were significant ($P>0.05$).

Phylogenetic analysis

The phylogenetic tree derived for all the fish species of this experiment is shown in Fig. 2.1, based on the sequences of cytochrome oxidase I (*COI*), cytochrome B (*CytB*), interphotoreceptor retinoid-binding protein 2 (*IRBP2*), and NADH dehydrogenase subunit 4 (*ND4*) (see Table 2.2 for accession numbers). I used this tree to check for the presence of a phylogenetic signal in the relationships between tissue parameters and body mass using Pagel's lambda (Garland et al., 1992). These parameters were the relative abundance of all the fatty acids reported in Figs. 2.2 and 2.3 (selected because they showed significant changes with size in at least one of the tissues), double bond index in both tissues, and muscle Ca^{2+}-ATPase activity. A significant phylogenetic signal was only identified for muscle palmitate, oleate, and docosahexaenoate, as well as for liver stearate, oleate, and linoleate ($P < 0.05$). All other membrane fatty acids, double bond indices, chain lengths, and Ca^{2+}-ATPase activity showed no significant relationship with phylogeny ($P>0.05$). These fatty acids showing a phylogenetic signal and body mass were used to generate phylogenetically independent contrasts (PIC). These PIC values for % fatty acid and body mass were plotted against each other in Fig. 2.6 to reveal corrected relationships between the two quantities when the contribution of phylogeny is removed. Therefore, a loss of significance between uncorrected and corrected correlations indicates that the relationship is only based on phylogenetic relatedness. None of the fatty acid contrasts showed a significant relationship with body mass contrasts (Fig. 2.6). Therefore, the significant relationships identified in Figs. 2.2 and 2.3 (except for 16:1 in Fig. 2.4) were based on phylogeny.

Discussion

This study demonstrates that key parameters of membrane composition are correlated with body mass in multiple species of ectotherms. I show that the membranes of cypriniform fish decrease unsaturation with body mass, as seen in endotherms, but through different mechanisms in muscle and liver. For muscle, docosahexaenoate (a polyunsaturated fatty acid) is replaced with palmitate (a saturated

fatty acid). By contrast, several different unsaturates (palmitoleate, oleate, and linoleate) are replaced with stearate (saturated) in liver membranes. These patterns of allometric changes are consistent with the membrane pacemaker theory of metabolism, despite involving different fatty acids from those previously characterized in birds and mammals (Hulbert and Else, 2005) and in rainbow trout (Martin et al., 2013). After correcting for phylogeny, however, all these correlations lose significance except for overall unsaturation in both tissues, and for liver palmitoleate. In addition, no relationship between muscle calcium-ATPase activity and body mass could be demonstrated, even though the theory posits that membrane composition should set metabolic rate by modulating the activity of membrane proteins. In this fish model, therefore, the membrane pacemaker concept provides useful predictions for broad-scale membrane parameters like overall unsaturation, but fails to account for finer membrane properties.

Allometric changes in cypriniform membranes

This test of the allometry of membrane composition in fish shows that cypriniforms decrease phospholipid unsaturation with increasing body size. In both muscle and liver, double bond index decreases with mass (Fig. 2.2), following the change observed in endotherm muscles across similar ranges in size (Couture and Hulbert, 1995; Hulbert et al., 2002a; Hulbert et al., 2002b). This observation is consistent with the assumption that the metabolic rate of cypriniforms scales with body size as it does in other fish orders (Clarke and Johnston, 1999; White et al., 2006). However, cypriniforms exhibit tissue-specific patterns of allometric changes in membrane fatty acids that are different from those of endotherms (Figs. 2.3 and 2.4). Currently known changes in membrane composition with increasing body size are summarized in Table 2.5. In nearly all vertebrate muscles measured to date, docosahexaenoate (22:6) is replaced with a more saturated fatty acid as size increases. Rainbow trout of 200-800 g show a much less pronounced pattern, replacing 24:1n11, 24:1n9, and 22:5n3 with 22:5n6 (Martin et al., 2013). Cypriniforms are unusual in using palmitate (16:0) rather than oleate (18:1) like endotherms. It is worth noting, however, that this difference may be driven by the unusual fatty acid profile of the market-purchased common carp; when the carp's data are excluded, the relationship between 18:1 and size

becomes highly significant ($p < 0.001$). This may be related to diet, as farmed fish often have far more saturated diets than wild fish. The more limited information available for liver shows that 22:6 decreases with body mass in endotherms, but does not change significantly with size in cypriniforms. Instead, these fish decrease membrane unsaturation of liver membranes by reducing levels of 16:1, 18:1 and 18:2, and replacing them with 18:0 (Fig 2.3). The specific fatty acids replacing 22:6 in hepatic membranes of large endotherms have not been identified (Table 2.5). It is also worth noting that none of these correlation studies take into account the possibility that different membrane types within a single tissue may show different allometric changes. Two separate studies of avian livers suggest that this might be the case because different fatty acids were identified in whole tissue samples in one study and in isolated mitochondria in the other (Brand et al., 2003; Szabó et al., 2010) (see Table 2.5). Therefore, future examinations of the membrane pacemaker hypothesis will have to consider that allometric patterns can vary between endo- and ectotherms, between tissues, and even possibly between membrane types of the same tissue.

Correcting allometric relationships for phylogeny

A novel phylogenetic tree of the cypriniform species of this study was created (Fig. 2.1) and it was used to assess whether phylogeny plays a role in determining allometric patterns of membrane composition. No phylogenetic signal was detected for the double bond index of either tissue, revealing that the general pattern for membrane unsaturation holds across taxa despite differences in genetic heritage, activity level, diet, and habitat. This robust pattern and its pervasiveness are consistent with the predictions of the membrane pacemaker theory. By contrast, the other apparent allometric changes in the abundance of specific fatty acids nearly all disappear when the data is corrected for cypriniform phylogeny (Fig. 2.6). This corroborates earlier studies of phylogenetic signals in fatty acid composition of muscle membranes (Ruf et al., 2006; Turner et al., 2006; Valencak and Ruf, 2007), but support is now provided on a much narrower genetic scale and in an additional tissue: the liver. Interestingly, 22:6 in muscle remains significant when corrected for phylogeny if only the Cyprinidae species are considered,

but this relationship vanishes when the additional five, more distantly related Catostomidae species are included. Put together, my results reveal that the observed relationships between relative fatty acid abundance and body mass are mostly based on kinship rather than size. These patterns are adaptations to ecological factors or inherited legacies of a species's evolutionary history. I could find no evidence for an association with metabolic challenges that are similar across taxa, despite a weak but non-phylogenetic relationship between liver palmitoleate and size. Genetic constraints prevent the membrane pacemaker concept from being useful to predict the finer properties of membrane phospholipids (i.e. variation in the relative abundance of individual fatty acids). Interestingly, the fact that a phylogenetic signal explains part of the natural variation in phospholipids suggests that membrane composition could be used in future evaluations of phylogenies (e.g. see Moser et al., 2011).

Calcium-ATPase activity shows no allometric pattern

Calcium-ATPase is a highly abundant protein pump that is vital for muscle function and is responsible for most muscle ATPase activity. In addition to being itself a transmembrane protein of the sarcoplasmic reticulum, it is regulated by several membrane-bound proteins that include sarcolipin and phospholamban (Cerra and Imbrogno, 2012; Gorski et al., 2013). If the membrane pacemaker concept accurately predicts cypriniform biochemistry, calcium-ATPase activity should vary with body mass and/or membrane composition. For example, a relationship with % linoleate or % docosahexaenoate should be detectable because these two fatty acids have been shown to affect the enzyme in mammals (Giroud et al., 2013). The cypriniform calcium-ATPase exhibits no such variation because its activity is not correlated with body mass, double bond index, or the abundance of any specific fatty acid (Fig. 2.5). In addition, the activity of the enzyme fails to show a phylogenetic signal. In this multispecies comparison, neither body size nor membrane composition exerted detectable effects on calcium-ATPase activity, contrary to the theory's prediction that these parameters should be tightly correlated and in keeping with earlier findings on mitochondrial enzymes in trout (Martin et al., 2013; Martin et al., 2015). The method used here cannot distinguish between a change in enzyme number and a change in the

activity of each enzyme molecule, so I cannot exclude the possibility that calcium-ATPase enzyme number changes with size or with the relative amount of particular fatty acids. Therefore, the network of regulatory proteins that controls cypriniform calcium-ATPase appears to do so without being affected by membrane composition in any consistent way.

Conclusions

This study shows that the membrane composition of ectotherms can vary with body mass, but also greatly with phylogeny. Cypriniform fish decrease membrane unsaturation with increasing body mass, as previously observed in endotherms, but through different mechanisms that also vary among tissues. The specific fatty acids showing an allometric change are different between muscle and liver, and do not match those previously identified in endotherm membranes (Hulbert and Else, 2005) or in rainbow trout (Martin et al., 2013). When ignoring the effects of phylogeny in cypriniforms, the mass-related patterns of changes in unsaturation and in relative abundance of specific fatty acids are all consistent with the predictions from the membrane pacemaker theory of metabolism. These results are comparable to those from the intraspecific study of Martin et al. (2013) wherein a phylogenetic contribution to the pattern was impossible to detect. However, accounting for the contribution of phylogeny in cypriniforms renders almost all these relationships non-significant except for overall unsaturation. Specific membrane components are set by genetic attributes that vary over evolutionary time rather than by size-based signals. Previous studies on endotherm muscle had come to similar conclusions (Ruf et al., 2006; Turner et al., 2006; Valencak and Ruf, 2007), but this effect of phylogeny is demonstrated here on a much narrower genetic scale, and in ectotherm liver as well as muscle. In addition, no relationship between calcium-ATPase activity and body mass or phospholipid composition could be demonstrated, even though the theory proposes that membranes set metabolic rate by modulating the activity of their proteins. I conclude that the membrane pacemaker concept accurately predicts general membrane properties like unsaturation, but does not explain allometric patterns of fatty acid composition.

Acknowledgements

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Figures

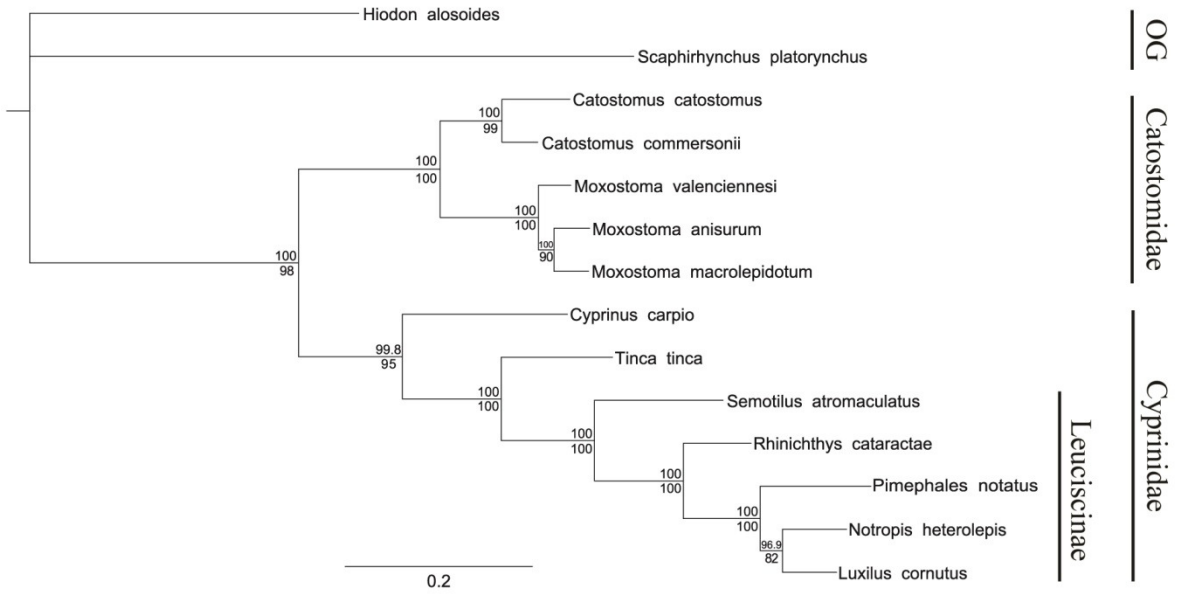

Fig. 2.1: Phylogenetic tree hypothesized for 12 cypriniform species based on *COI*, *CytB*, *IRBP2*, and *ND4* sequences and inferred using a Bayesian method. Bayesian posterior probabilities and PhyML bootstrap values as percentages are reported above and below each node, respectively. Accession numbers for these genes are listed in Table 2.2. Bars on the right indicate classification following Bufalino and Mayden (2010) and Chen and Mayden (2012) for the families Catostomidae and Cyprinidae and the cyprinid subfamily Leuciscinae. OG = outgroups.

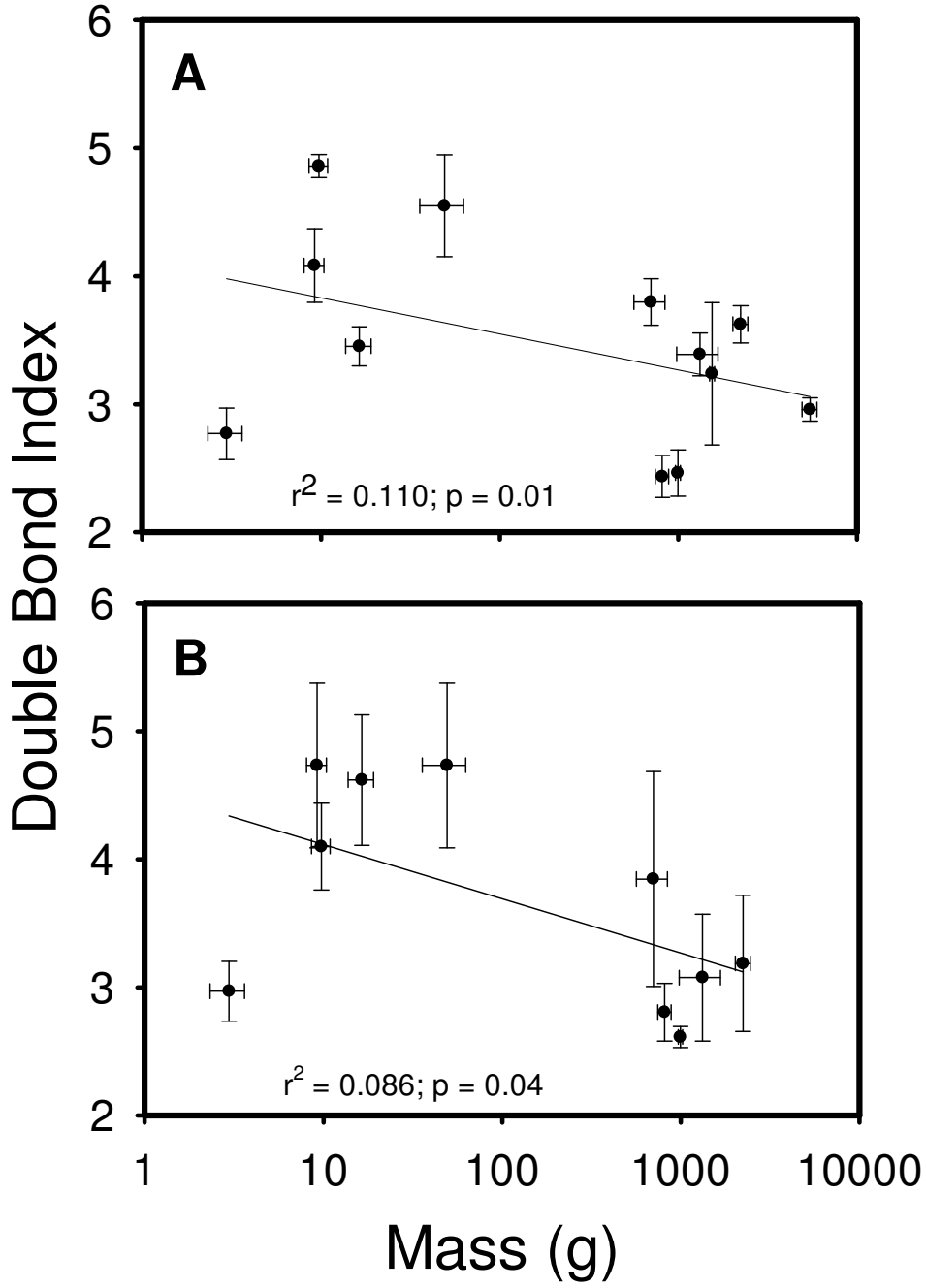

Fig. 2.2. Relationship between the double bond index of membrane phospholipids and body mass of cypriniform species. Data are for (A) white muscle and (B) liver. Lines were fitted by linear regression and values are means ± s.e.m (N = 5).

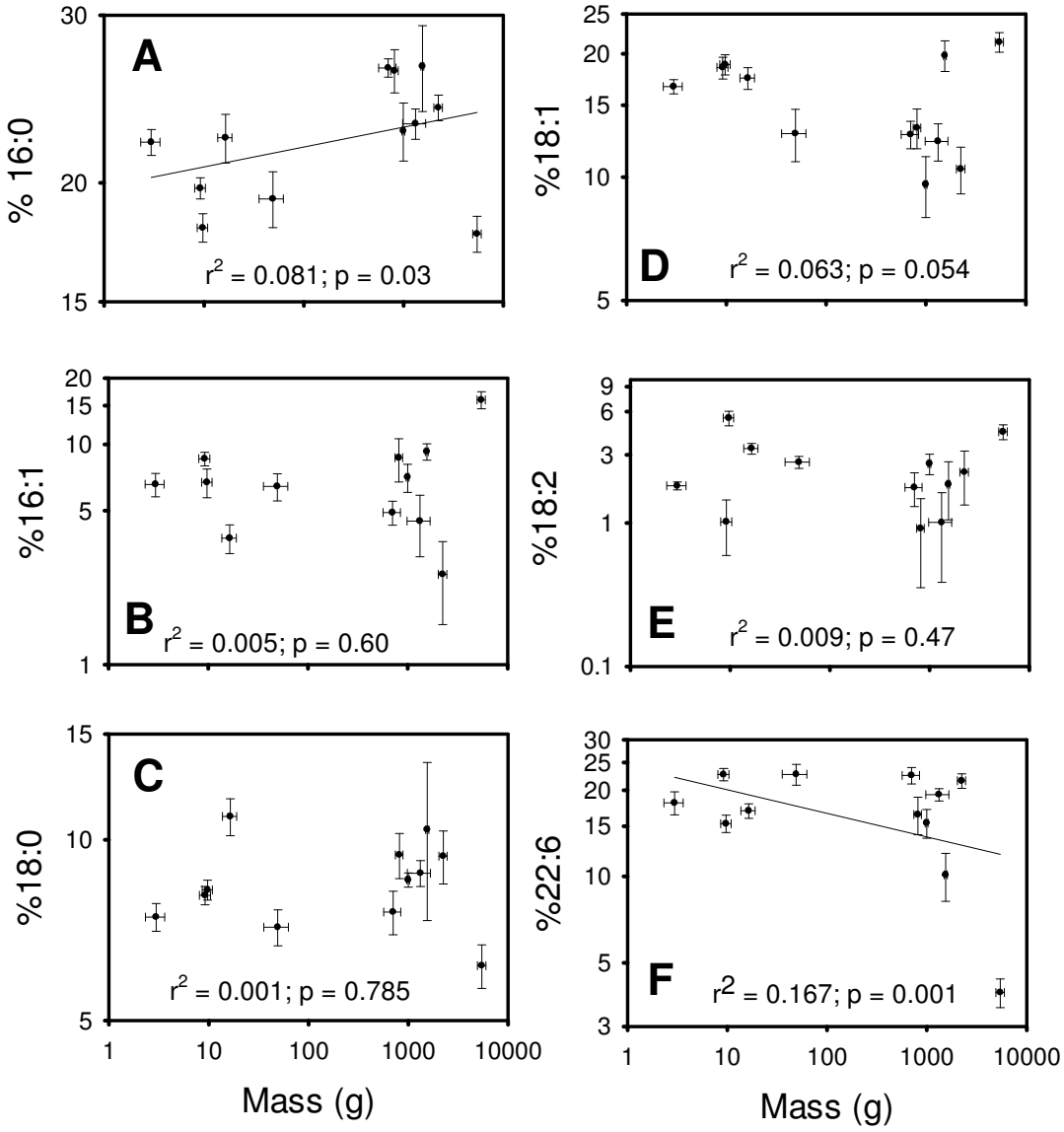

Fig. 2.3. Relationships between body mass and the relative abundance of selected fatty acids in muscle membrane ephospholipids. Fatty acid abundance is given as a percentage of total fatty acids for 12 cypriniform species on log-log plots: (A) palmitate or 16:0; (B) palmitoleate or 16:1; (C) stearate or 18:0; (D) oleate or 18:1; (E) linoleate or 18:2; (F) docosahexaenoate or 22:6. Fatty acids were selected because they showed a significant relationship with body mass in either liver or muscle. Lines fitted by linear regression are indicated when the slope is different from 0 ($P<0.05$) in muscle. Values are means ± s.e.m ($N = 5$).

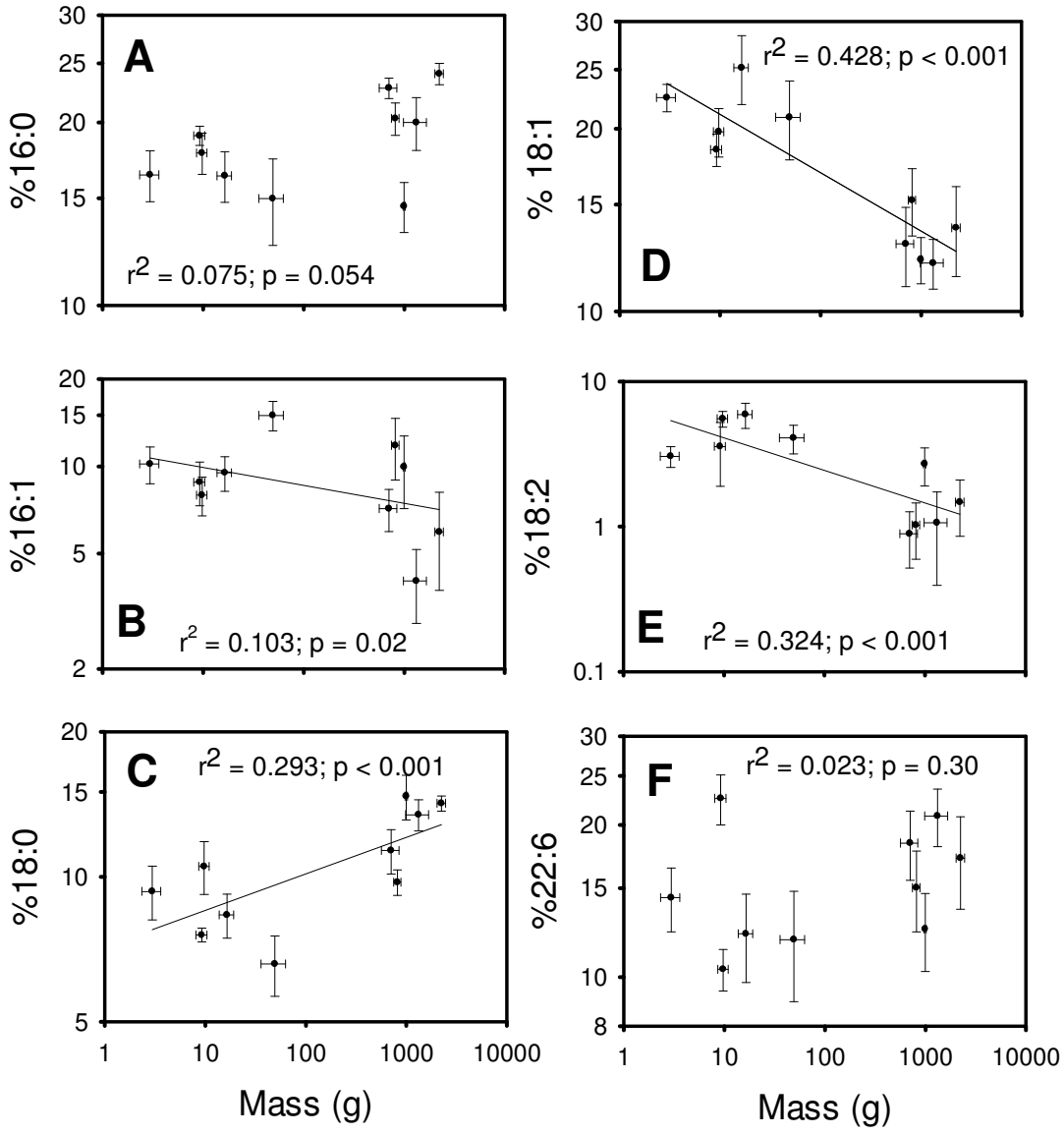

Fig. 2.4. Relationships between body mass and the relative abundance of selected fatty acids in liver membrane phospholipids. Fatty acid abundance is given as a percentage of total fatty acids for 12 cypriniform species on log-log plots: (A) palmitate or 16:0; (B) palmitoleate or 16:1; (C) stearate or 18:0; (D) oleate or 18:1; (E) linoleate or 18:2; (F) docosahexaenoate or 22:6. Fatty acids were selected because they showed a significant relationship with body mass in either liver or muscle. Lines fitted by linear regression are indicated when the slope is different from 0 ($P<0.05$) in muscle. Values are means ± s.e.m ($N = 5$).

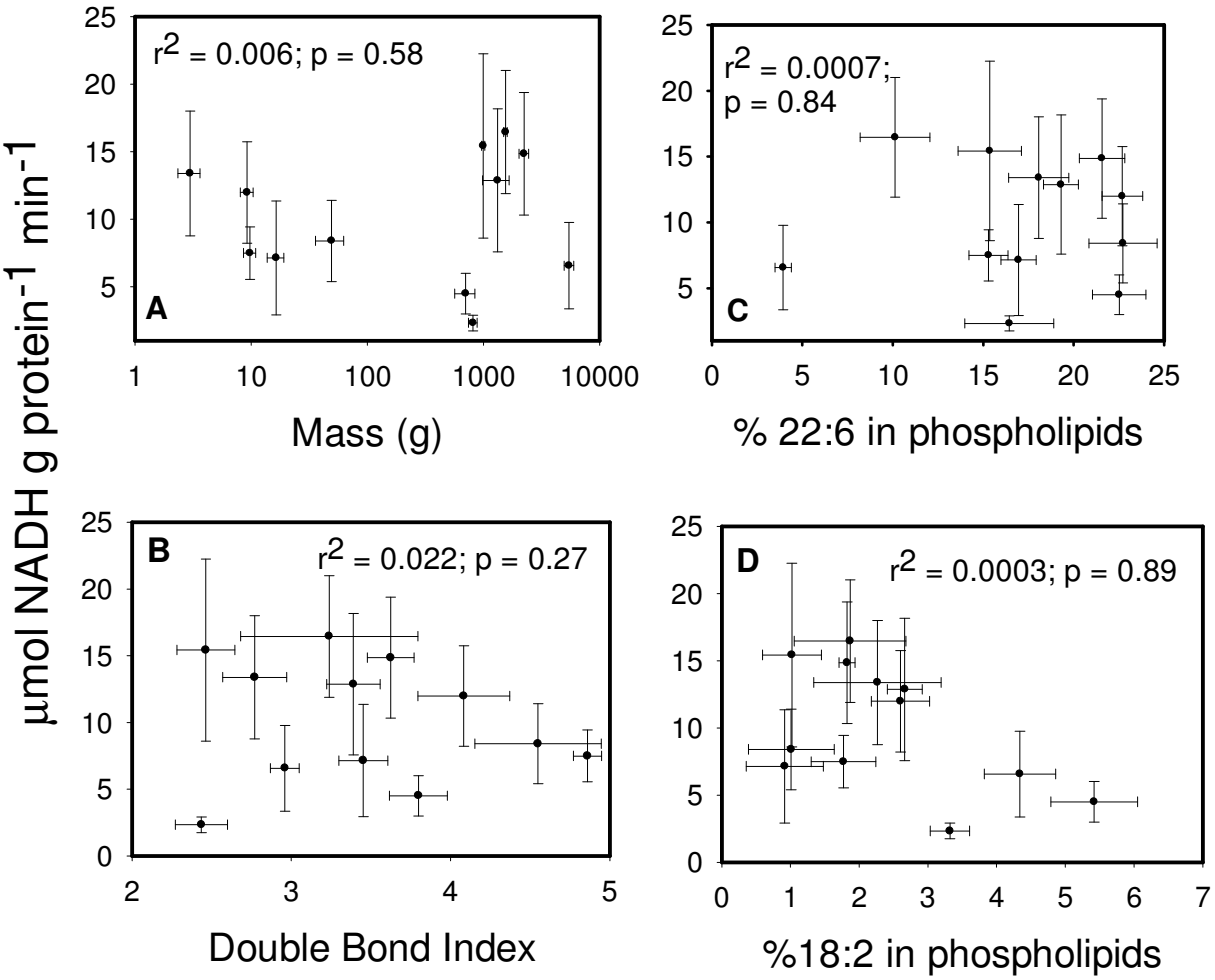

Fig. 2.5. Calcium-ATPase (SERCA) activity in 12 cypriniform species. Activity is shown in relation to (A) body mass; (B) double bond index; (C) percentage docosahexaenoate (22:6); and (D) percentage linoleate of muscle membranes. Values are means ± s.e.m (N = 5).

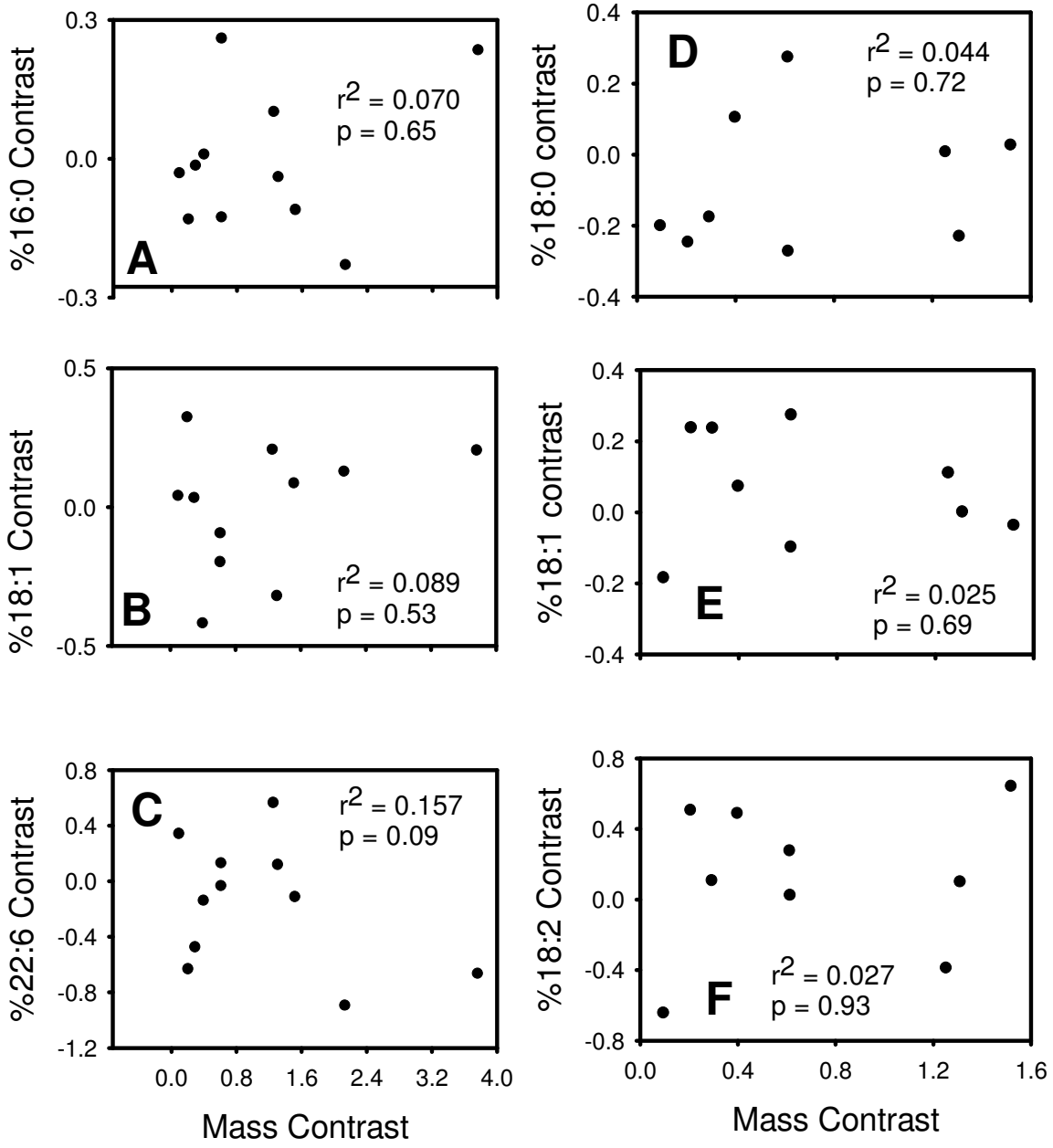

Fig. 2.6. Relationship between body mass and % phospholipid fatty acid independent contrasts obtained for white muscle and liver. Data are from *COI*, *CytB*, *IRBP2*, and *ND4* phylogeny (Fig. 2.1) for white muscle [(A) palmitate; (B) oleate; (C) docosahexaenoate] and liver [(D) stearate; (E) oleate; (F) linoleate], showing the shape of each relationship with the contribution of phylogeny removed. No significant correlation was found.

Tables

Table 2.1. Cypriniform species used in this study, mean body mass ± s.e.m. (n=5), sources, water temperature, and diet. Diet from (Froese and Pauly, 2016).

Species	Common Name	Mass (g)	Source	Temp (°C)	Diet
Catostomidae					
Catostomus commersonii	White sucker	703.2±138.7	Rideau Canal, Ottawa, ON	20	Omnivorous
Catostomus catostomus	Longnose sucker	813.2±70.1	Pont Champlain, St. Lawrence River, QC	16	Omnivorous
Moxostoma anisurum	Silver redhorse	2233±215.3	Rideau Canal, Ottawa, ON	20	Omnivorous
Moxostoma macrolepidotum	Shorthead redhorse	995.8±32.7	Pont Champlain, St. Lawrence River, QC	16	Carnivorous
Moxostoma valenciennesi	Greater redhorse	1322.6±342.3	Rideau Canal, Ottawa, ON	20	Omnivorous
Cyprinidae					
Cyprinus carpio	Common carp	5453.5±424.4	Lapointe Fish Market, Orleans, ON	15	Omnivorous
Luxilus cornutus	Common shiner	16.40±2.67	Hoople Creek, Ottawa, ON	28	Omnivorous
Notropis heterolepis	Blacknose shiner	9.67±1.16	Lac Lannigan, Déléage, QC	21	Carnivorous
Pimephales notatus	Bluntnose minnow	4.24±0.93	Watts Creek, Ottawa, ON	15	Omnivorous
Rhinichthys cataractae	Longnose dace	9.21±1.17	Watts Creek, Ottawa, ON	15	Carnivorous
Semotilus atromaculatus	Creek chub	49.14±13.47	Richelieu River, St-Paul-de-l'île-aux-Noix, QC	17	Omnivorous
Tinca tinca	Tench	1545.6±41.90	Richelieu River, St-Paul-de-l'île-aux-Noix, QC	17	Omnivorous

Table 2.2. List of the GenBank accession numbers used in the phylogenetic reconstruction. *COI*, cytochrome oxidase I. *CytB*, cytochrome B. *IRBP2*, interphotoreceptor retinoid-binding protein 2. *ND4*, NADH dehydrogenase subunit 4.

Species	**COI**	**CytB**	**IRBP2**	**ND4**
Catostomus catostomus	186884210	28201351	325112634	260766555
Catostomus commersonii	324023510	347949515	172050603	N/A
Cyprinus carpio	460418872	115490837	217069416	N/A
Luxilus cornutus	324024172	1519414	N/A	N/A
Moxostoma anisurum	186884978	347949545	325112680	260766624
Moxostoma macrolepidotum	186885074	347949591	325112698	260766657
Moxostoma valenciennesi	186885092	347949615	325112712	260766678
Notropis heterolepis	339771397	186885264	N/A	N/A
Pimephales notatus	407232072	336317785	N/A	N/A
Rhinichthys cataractae	324023472	442559611	384369807	N/A
Semotilus atromaculatus	324024188	299893141	172050643	N/A
Tinca tinca	460421432	307090444	217069456	N/A
Outgroups				
Hiodon alosoides	186884570	46391328	N/A	N/A
Scaphirhynchus platorhynchus	339773117	4204893	N/A	N/A

Table 2.3. Fatty acid composition of membrane phospholipids in white muscle of 12 cyprinoforms expressed as % of total membrane fatty acids. -: trace amounts (indicated when species average <2% of total fatty acids). CL: chain length. DBI: double bond index. Values are means ± s.e.m. (n=5).

	Pimephales notatus	*Rhinichthys cataractae*	*Notropis heterolepis*	*Luxilus cornutus*	*Semotilus atromaculatus*	*Catostomus commersonii*	*Catostomus catostomus*	*Moxostoma macrolepidotum*	*Moxostoma anisurum*	*Tinca tinca*	*Moxostoma valenciennesi*	*Cyprinus carpio*
16:0	22.1±0.7	19.7±0.5	17.9±0.6	22.3±1.3	19.2±1.3	26.4±0.6	26.2±1.4	22.7±1.6	24.0±0.7	26.5±2.7	23.1±0.9	17.7±0.8
16:1	6.6±0.8	8.6±0.6	6.7±1.0	3.6±0.6	6.5±0.9	4.9±0.6	8.7±1.9	7.1±1.0	2.6±1.1	9.3±0.8	4.5±1.4	15.9±1.4
18:0	7.4±0.4	8.1±0.3	8.3±0.3	10.9±0.8	7.2±0.5	7.6±0.6	9.4±0.8	8.6±0.2	9.4±1.0	10.4±2.1	8.8±0.4	6.2±0.5
18:1	16.6±0.7	18.5±1.1	18.8±1.1	17.5±1.1	12.8±1.9	12.7±1.0	13.2±1.5	9.6±1.6	10.5±1.4	19.8±1.7	12.2±1.3	21.4±1.2
18:2n-6	-	-	5.4±0.6	3.3±0.3	2.7±0.3	-	-	2.6±0.4	2.3±0.9	-	-	4.3±0.5
18:3n-6	-	-	2.5±0.8	-	-	-	-	2.8±0.9	-	-	-	4.3±0.5
20:0	-	-	-	-	-	-	-	-	-	-	-	2.3±0.7
20:4n-6	3.2±2.9	4.2±0.4	6.5±2.2	7.7±0.3	7.9±1.7	7.6±0.7	3.3±0.5	3.9±0.6	10.6±0.7	7.7±2.1	9.7±0.7	4.7±0.6
20:5n-3	-	-	3.9±2.2	-	-	-	-	-	-	-	-	-
22:0	18.9±1.0	12.9±1.4	8.0±0.9	11.6±1.5	12.9±1.9	11.6±0.6	15.0±0.6	16.9±0.6	13.7±1.8	6.5±1.9	16.1±1.1	8.5±0.4
22:6n-3	18.1±1.7	22.7±1.1	15.3±1.1	17.0±0.9	22.7±1.9	22.5±1.5	16.4±2.5	15.4±1.8	21.6±1.3	10.1±1.9	19.3±1.0	3.9±0.5
24:0	4.9±0.4	3.9±0.4	4.1±0.2	2.8±0.4	5.5±0.3	4.2±0.2	4.8±0.4	7.2±0.5	5.5±1.6	2.6±1.1	4.4±1.1	2.8±0.2
CL	19.3±0.1	19.2±0.1	18.9±0.1	18.9±0.1	19.2±0.2	19.2±0.1	18.8±0.2	18.9±0.2	19.4±0.2	18.4±0.3	19.4±0.2	17.9±0.1
DBI	2.8±0.2	4.1±0.3	4.9±0.1	3.5±0.2	4.6±0.4	3.8±0.1	2.4±0.2	2.5±0.2	3.6±0.2	3.2±0. 6	3.4±0.2	3.0±0.1

Table 2.4. Fatty acid composition of membrane phospholipids in liver of 10 cyprinoforms expressed as % of total membrane fatty acids.
-: trace amounts (indicated when species average <2% of total fatty acids). CL: chain length. DBI: double bond index. Values are means ± s.e.m. (n=5).

	Pimephales notatus	*Rhinichthys cataractae*	*Notropis heterolepis*	*Luxilus cornutus*	*Semotilus atromaculatus*	*Catostomus commersonii*	*Catostomus catostomus*	*Moxostoma macrolepidotum*	*Moxostoma anisurum*	*Moxostoma valenciennesi*
16:0	9.3±4.5	19.0±0.7	17.7±1.4	16.3±1.6	15.0±2.4	22.8±0.9	20.3±1.2	14.6±1.4	24.0±1.0	20.0±2.0
16:1	5.0 ±2.2	8.8±1.5	8.0±1.2	9.5±1.3	15.0±1.8	7.2±1.2	11.8±2.8	9.9±2.8	5.9±2.2	4.0±1.2
18:0	5.3±2.5	7.6±0.3	10.5±1.3	8.3±0.9	6.6±0.9	11.3±1.2	9.8±0.6	14.7±1.6	14.2±0.5	13.5±1.0
18:1	12.5±5.9	18.5±1.1	19.8±1.8	25.2±3.3	20.9±3.1	12.9±1.9	15.3±1.9	12.2±1.1	13.7±2.3	12.0±1.1
18:2n-6	3.3±1.4	3.6±1.7	5.6±0.7	5.9±1.2	4.1±0.9	-	-	2.7±0.8	-	-
18:3n-6	-	-	2.2±1.1	3.1±0.7	-	-	-	2.9±1.1	-	-
22:0	4.1±2.0	10.9±1.5	7.1±0.7	7.9±0.7	11.1±1.3	12.8±4.6	13.1±1.7	14.1±1.2	10.5±3.2	19.4±8.1
20:4n-6	5.1±2.4	3.6±0.7	11.3±1.6	6.2±1.1	8.2±1.1	6.3±2.3	2.8±0.5	5.2±1.2	7.9±2.9	4.4±1.1
20:5n-3	-	-	-	-	-	2.8±2.3	-	-	-	-
22:6n-3	6.5±2.8	22.6±2.6	10.4±1.0	12.2±2.4	11.9±2.9	18.4±2.9	15.0±2.7	12.5±2.2	17.2±3.6	20.8±2.7
24:0	-	2.5±0.4	4.1±0.4	-	3.7±0.9	2.1±0.7	4.3±0.5	5.6±0.3	3.4±0.9	3.7±0.9
CL	18.9±0.2	19.0±0.1	18.7±0.1	18.2±0.3	18.5±0.2	18.7±0.3	18.4±0.4	19.0±0.2	18.9±0.2	19.5±0.3
DBI	2.4±1.1	4.7±0.6	4.1±0.3	4.6±0.5	4.6±0.8	3.8±0.8	2.8±0.2	2.6±0.1	3.2±0.5	3.1±0.5

Table 2.5. Summary of changes in membrane composition associated with size in bird, mammal, and cypriniform muscle and liver.

Muscle		**As size increases**		**Reference**
Cypriniforms	22:6	→	16:0	This study
Rainbow trout	24:1n-11, 24:1n-9, and 22:5n-3		22:5n-6	(Martin et al., 2013)
Mammals	22:6	→	18:1	(Hulbert et al., 2002b)
Birds	22:6	→	18:1	(Hulbert et al., 2002a)
Liver				
Cypriniforms	16:1, 18:1, and 18:2	→	18:0	This study
Mammals	22:6	→	Not identified	(Couture and Hulbert, 1995)
Birds	22:6	→	Not identified	(Szabó et al., 2010)
Bird mitochondria	18:0 and 22:3	→	18:1	(Brand et al., 2003)

CHAPTER 3.
PCB-153 and temperature cause restructuring of goldfish membranes: homeoviscous response to a chemical fluidizer

Chapter 3

PCB-153 and temperature cause restructuring of goldfish membranes: homeoviscous response to a chemical fluidizer

Based on

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Author Contributions: AG and JMW designed this study, analyzed the data and wrote the manuscript. AG executed the experiments. AO performed most of the phospholipid extractions.

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Introduction

Polychlorinated biphenyls (PCBs) are an exceptionally well-studied group of environmental toxicants formerly used for applications as diverse as flame retardants and dielectric fluid. Though banned in much of the developed world, PCBs remain a toxicological concern in many places (Drouillard et al., 2007). Earlier research about PCB exposure focused on the carcinogenic effects of the 12 coplanar, dioxin-like PCBs, acting via the aryl hydrocarbon receptor (Safe, 1994). The other 197 PCB congeners are non-coplanar and do not activate this receptor. However, these toxic compounds intercalate between phospholipids in a manner similar to cholesterol (Campbell et al., 2008; Yilmaz et al., 2006) and have been shown to increase membrane fluidity in rodent and chicken cells (Bonora et al., 2003; Katynski et al., 2004; Reich et al., 1981; Tan et al., 2004). Such fluidity perturbations affect the activity of membrane proteins, including enzymes from oxidative pathways and hormone receptors, with far-reaching physiological consequences (Corcoran et al., 2007; Guderley et al., 2008). Therefore, membrane fluidizers are a toxicological concern, alongside the known estrogenic and neurotoxic effects also attributed to non-coplanar PCBs (Arnold et al., 1990; Saint-Amour et al., 2006; Venkataraman et al., 2010).

Membrane fluidity also varies with temperature, but ectotherms have a well-developed homeoviscous response to counter the effects of potentially harmful thermal fluctuations (Hazel, 1995; Seebacher et al., 2009). The primary mechanism of homeoviscous acclimation is to modulate the saturation state of phospholipids and the concentration of cholesterol. Desaturases mediate phospholipid restructuring by adding double bonds on fatty acid chains and cholesterol buffers membranes against abrupt phase transitions (Cossins et al., 2002; Crockett, 1998; Zehmer and Hazel, 2005). Ectotherms show a strong homeoviscous response in the liver, but not in other tissues such as brain and muscle (Cossins, 1977; Farkas et al., 2001; Hazel et al., 1991). Because most of the detailed information on this response comes from studies on trout liver (Hazel et al., 1991; McKinley and Hazel, 2000; Vagner and Santigosa, 2011), it may be instructive to examine other tissues and different ectotherms to assess the variability in

membrane restructuring. Similarly, the effects of fluidizing chemicals have mainly been investigated in the membranes of isolated mammalian cells and chicken embryos (Katynski et al., 2004; Lopez-Aparicio et al., 1994; Suwalsky et al., 1997; Tan et al., 2004). Unfortunately, mammalian cells and embryos do not naturally undergo homeoviscous acclimation and the membrane effects of non-coplanar PCBs have never been examined in ectotherms (Cossins and Wilkinson, 1982; Vaish and Sanyal, 2011). It remains unknown whether chemical fluidization can activate a homeoviscous response or interfere with normal acclimation to temperature. Polar species are especially threatened by the combined effects of thermal stress from climate change and chemical stressors from pollution (Armitage et al., 2013; Brown et al., 2013; Saint-Amour et al., 2006). Therefore, understanding PCB-membrane interactions may prove important for the conservation of fragile polar biomes.

The goal of this study was to examine whether PCB-153 can cause membrane restructuring or interfere with the normal homeoviscous response of an ectotherm during temperature acclimation. More specifically, my aim was to measure potential changes in membrane composition (phospholipids and cholesterol) and in the expression of the main desaturases (Δ6 and Δ9) in goldfish organs (gill, brain, muscle, and liver) in response to PCB-153 and to temperature (5 or 20°C). A 2 x 2 factorial design was chosen to detect possible synergistic effects between PCB exposure and temperature. PCB-153 was the *ortho*-substituted congener selected here because of its environmental pervasiveness and particularly long half-life in animal tissues (Armitage et al., 2013; Drouillard et al., 2007). Within each tissue, I hypothesized that PCB-153 would induce a homeoviscous change in fatty acid unsaturation, cholesterol content, and desaturase expression similar to that elicited by temperature acclimation.

Materials and methods

Animals and experimental design

All procedures were approved by the Animal Care Committee of the University of Ottawa and adhered to the guidelines established by the Canadian Council on Animal Care for the use of animals in

research and the code of ethics of the World Medical Association (Declaration of Helsinki) for animal experiments. Adult goldfish *Carassius auratus auratus* (Linnaeus) (21.13 ± 4.99 g, N = 81) were purchased from Aleongs International (Mississauga, Ontario, Canada) and held in four 70-L flow-through tanks in dechlorinated, well-oxygenated water at 18°C under a 12h:12h light-dark photoperiod. Fish were from the same batch and therefore the same age. They were fed floating fish pellets (Profishent; Martin Mills; Elmira, Ontario, Canada) daily until satiation. Fish were habituated to these conditions for at least 14 days before temperature acclimation. The experiments were designed as 2 x 2 matrices testing simultaneously the effects of temperature (5°C vs 20°C) and PCB exposure (sham injection vs PCB injection). For each experiment, fish were randomly assigned to one of four groups: cold sham-injected, warm sham-injected, cold PCB-injected, and warm PCB-injected. Acclimation temperature was reached gradually (2°C/day). In the first experiment, 61 fish were used to measure changes in membrane composition. They were maintained at their acclimation temperatures for 21 days before starting sham/PCB injections and maintained at these temperatures for an additional 30 days. In the second experiment, a separate group of 20 fish was used to assess desaturase expression. They were maintained at their acclimation temperatures for one week before starting sham/PCB injections and were maintained at these temperatures for an additional 15 days.

Sham or PCB injection and tissue sampling

After temperature acclimation, the fish from experiment 1 (membrane composition) received three sham/PCB injections under benzocaine anaesthesia (0.33 g/L) at 10-day intervals. The fish from experiment 2 (desaturase expression) received a sham/PCB injection immediately after temperature acclimation and another one 10 days later. In both experiments, one group each of warm and cold goldfish were injected with 3 µg/g body mass of PCB-153 (2,2',4,4',5,5'-hexachlorobiphenyl) (Ultra Scientific; North Kingstown, RI, USA) in sunflower oil (1.25 mg PCB / mL). This dose is comparable to concentrations used in previous studies (Andersson et al., 2001; Duffy-Whritenour et al., 2010) and measured in wels catfish from Italy (Squadrone et al., 2013), but approximately ten times those reported

in wild fish from the Great Lakes region (Abdolahpur Monikh et al., 2013; Scheider et al., 1998). The remaining two groups received an equivalent volume of pure sunflower oil as a sham injection. Fish were injected on the left side, behind the dorsal fin and into the body cavity at a 45° angle. For each temperature, the sham-injected and PCB-injected groups had identical food intake. The goldfish were then euthanized by cervical dislocation 10 days (membrane composition experiment) or five days (desaturase experiment) after the last sham/PCB injection. The brain, gill, white muscle, and liver were sampled from the right side and freeze-clamped in liquid N_2. Tissue samples were stored at -80°C until analyses.

Membrane lipids

Total lipids were extracted from ~40 mg of tissue using chloroform:methanol (2:1 v/v). Samples were homogenized (Polytron, Kinematica, Littau, Switzerland) and centrifuged (10 min at 2000 *g*). Supernatants were filtered and 0.25% KCl added to separate aqueous and organic phases. The organic phase was evaporated and the lipids resuspended in chloroform before loading on solid-phase extraction columns (Supelclean 3 mL 500 mg LC-NH2; Sigma-Aldrich; St. Louis, MO, USA) to separate the phospholipids. Separation was achieved by sequential elution of lipid classes—neutral lipids, non-esterified fatty acids, and phospholipids (PL)—using solvents of increasing polarity: chloroform:isopropanol (3:2 v/v), isopropyl ether:acetic acid (98:2 v/v), and methanol, respectively (Maillet and Weber, 2006). The fatty acid composition of PL was measured after acid transesterification. Fatty acid methyl esters were analyzed on an Agilent Technologies 6890N gas chromatograph (Mississauga, Ontario, Canada) equipped with a fused silica capillary column (Supelco DB-23, 60m, 0.25 mm i.d., 0.25µm film thickness; Sigma-Aldrich) using hydrogen as carrier gas as previously (Magnoni and Weber, 2007). Only the fatty acids accounting for >1% of total fatty acids in membrane phospholipids are reported in this study, although traces of myristate (14:0), myristoleate (14:1), heptadecaenoate (17:1), α-linolenate (18:3), arachidate (20:0), gondoate (20:1), erucate (22:1), brassate

(22:2), tricosanoate (23:0), eicosapentaenoate (20:5), lignocerate (24:0), nervonate (24:1), and docosapentaenoate (22:5) were also detected. Phospholipid recovery could not be verified.

Membrane cholesterol was measured as non-esterified (free) cholesterol in ~50 mg of tissue. Tissues were homogenized in chloroform:methanol (2:1 v/v). KCl/EDTA (2 M / 5 mM) was added to separate aqueous and organic phases prior to centrifugation (10 min at 2000 *g*). The organic phase was dried, resuspended in 2-methoxyethanol, and stored at -80°C. Cholesterol was measured by fluorometry (SpectraMax Gemini XS, Molecular Devices, Sunnyvale, California, USA) using a commercial assay kit (Cayman Chemical, Ann Arbor, Michigan, USA). This kit was selected because it allows the separate measurement of membrane (free, non-esterified) cholesterol and cholesterol esters that are found only outside membranes. Cholesterol recovery could not be verified.

Tissue PCB concentration

Tissue samples were finely diced before adding a PCB 205 standard to correct for recovery. They were placed in an accelerated solvent extractor (Dionex ASE 200; Thermo Fisher Scientific) with petroleum-ether-rinsed silica hydromatrix (Agilent) occupying the remaining dead space. The samples were extracted overnight using dichloromethane:hexane (1:1 v/v) and filtered through sodium sulfate cartridges (Chromafix, Macherey-Nagel, Düren, Germany). The PCBs were eluted from solid-phase extraction columns (6 mL 1 g LC-Si; Supelco; Sigma-Aldrich) with hexane:dichloromethane (19:1 v/v). They were resuspended in 500 µL isooctane with10 µL octachloronapthalene as internal standard before gas chromatography (Agilent 6890N with G2350A micro-electron capture detector). The average recovery of the PCB-205 standard was 69%.

To assess the level of PCB exposure in goldfish tissues, I have measured the PCB-153 content of gill and muscle in six sham-injected and six PCB-injected fish (no brain tissue was left after analyzing membrane composition and the high lipid content of liver prevented accurate measurement of PCB-153 levels). Gill and muscle values were pooled because the two tissues had the same PCB content within each group (P=0.87 for sham-injected fish and P=0.81 for PCB-injected fish). The tissues of PCB-injected

fish had significantly higher amounts of PCB-153 than their sham-injected counterparts ($P<0.001$). PCB-153 levels averaged 1112±367 ng/g (N=12) in PCB-injected fish, but only 18.4±10.3 ng/g (N=12) in sham-injected fish.

Desaturase expression

Total RNA was extracted from ~200 mg of tissue using TRIzol (Invitrogen). Samples were taken only of gill and liver, since the brain and muscle showed no fatty acid response to PCB exposure. Samples were homogenized and left to incubate at room temperature. Samples were extracted and centrifuged with chloroform (15 min at 2000 *g*) to separate phases. The aqueous supernatants were removed and treated with isopropanol to precipitate RNA, dried, and reconstituted in DEPC-treated water. Complementary DNA was produced using the QuantiTect reverse transcription kit (Qiagen; Hilden, Germany). Negative controls were performed without reverse transcriptase.

Primers were designed using sequences from GenBank and provided by Integrated DNA Technologies (Coralville, Iowa, USA). Primer sequences are shown in Table 3.1. Sequence identity was confirmed using a PureLink Plasmid MiniPrep kit (Invitrogen) and agarose gels. The primers and cDNA were used with QuantiFast SYBR-Green for real-time PCR (Qiagen) on a BioRad CFX96^{TM} Real-Time System (Berkeley, California, USA). Cycling parameters were as follows: 95°C for five minutes, 41 cycles of 95°C for 10 seconds and 60°C for 30 seconds, 95°C for 10 seconds, and five seconds of melt-curve analysis from 60°C to 95°C in 0.5°C increments.

Calculations and statistics

Membrane saturation was expressed by the double bond index, calculated as the average number of double bonds divided by the fraction of saturated fatty acids. Data were analyzed using two-way ANOVA with the Holm-Sidak post-hoc test to determine which treatments differed from one another. Statistical analyses were performed using SigmaPlot 12 (Systat, San Jose, CA, USA). All values presented are means ± s.e.m. and a level of significance of $p<0.05$ was used in all tests. Normality of the data was always tested prior to analyses using the Shapiro-Wilk test. This test revealed that the PCB-153

concentrations measured were not normally distributed. Normality was achieved by natural logarithmic transformation and the log-transformed PCB concentrations were used for statistical analysis. All percentages were transformed to the arcsine of their square root before analyses. Gene expression results are presented as relative expression normalized to elongation factor 1α (EF1A), calculated using the ΔΔCt method (Rao et al., 2013), and were also log-transformed to achieve normality.

Results

Fatty acid composition of membranes

The relative abundance of individual fatty acids in membrane phospholipids is shown in Table 3.2 for brain, Table 3.3 for gill, Table 3.4 for liver, and Table 3.5 for muscle. Figs. 3.1 and 3.2 show the changes in phospholipid composition caused by acclimation temperature and PCB exposure. In gill, high temperature and PCB exposure caused an increase in percent palmitate (16:0), but only temperature decreased percent eicosadienoate (20:2) ($P<0.05$, Fig. 3.1B). In brain, high temperature caused an increase in percent oleate (18:0) and a decrease in percent eicosenoate (20:1) and arachidonate (20:4) ($P<0.01$, Fig. 3.1A). In muscle, high temperature caused an increase in percent palmitate ($P<0.05$) and a decrease in percent arachidonate ($P<0.01$, Fig. 3.1C). In liver, high temperature caused an increase in percent palmitate and docosahexanoate (22:6) ($P<0.05$) and a decrease in percent palmitoleate (16:1), stearate (18:0), oleate (18:1), linoleate (18:2), eicosenoate, and eicosadienoate (Fig. 3.2A, 3.2B). PCB-exposed liver also had higher arachidonate and lower palmitoleate than sham-injected liver ($P<0.05$). No significant interaction between temperature and PCB exposure was detected in any fatty acid in any tissue ($P>0.05$), except for liver oleate, in which the difference between cold and warm-acclimated fish was higher when the fish were exposed to PCB-153 ($P<0.05$). All the other membrane fatty acids were not affected by any treatment ($P>0.05$).

Double bond index and chain length

Figure 3.3 shows the level of unsaturation of membrane phospholipids in brain, gill, liver, and muscle expressed as the double bond index. In gill (Fig. 3.3B), both high temperature and PCB exposure caused a decrease in the double bond index ($P<0.05$). In muscle (Fig. 3.3D) and brain (Fig. 3.3A), neither treatment had an effect on double bond index ($P>0.05$). In liver (Fig. 3.3C), high temperature caused an increase in the double bond index ($P<0.01$), a difference magnified via an interaction with PCB exposure ($P<0.01$). No significant interaction was detected between temperature and PCB exposure in other tissues ($P>0.05$).

Figure 3.4 shows the chain length of membrane phospholipid fatty acids in brain, gill, liver, and muscle. In liver (Fig. 3.4C), high temperature caused an increase in chain length ($P<0.05$). No other responses to temperature or PCB exposure were detected ($P>0.05$). No significant interaction was detected between temperature and PCB exposure in any tissue ($P>0.05$).

Cholesterol

The effects of temperature and PCB exposure on membrane cholesterol concentration in µmol/g tissue are shown in Fig. 3.5. In gill, high temperature ($P<0.05$), but not PCB exposure ($P>0.05$) caused a decrease in cholesterol concentration (Fig. 3.4B). Neither treatment had an effect on the membrane cholesterol content of muscle ($P>0.05$) (Fig. 3.4D). In brain ($P<0.001$) and liver ($P<0.05$), PCB exposure caused an increase in membrane cholesterol, but temperature had no effect ($P>0.05$) (Fig. 3.4A and 3.4C). No significant interaction was detected between temperature and PCB exposure in any tissue ($P>0.05$).

Desaturase expression

The effects of temperature and PCB exposure on Δ9 and Δ6 desaturase gene expression are shown in Fig. 3.6 for gill and liver. In gill, neither high temperature nor PCB exposure had a significant effect on the expression of either desaturase ($P>0.05$) (Fig. 3.6A). In liver, high temperature ($P<0.05$), but not PCB exposure ($P>0.05$), caused a decrease in Δ9 desaturase expression relative to EF1α (Fig. 3.6B).

Neither treatment had a significant effect on Δ6 expression (P>0.05). No significant interaction was detected between temperature and PCB exposure for either mRNA (P>0.05).

Discussion

Novelty of the study

This study is the first to show that *in vivo* exposure to a membrane fluidizer can cause a homeoviscous response in an ectothermic animal. Nakanishi et al exposed *Gnathopogon caerulescens* to sodium dodecylbenzenesulfonate and sodium stearate (Nakanishi et al., 1987a; Nakanishi et al., 1987b), which also destabilize phospholipid membranes, but did not examine membrane lipids in any detail or in multiple organs, nor did they examine the relationship between chemical fluidization and thermal acclimation. Modulating membrane cholesterol is mostly used to cope with PCB-153, whereas changes in unsaturation dominate temperature acclimation. Results also reveal that the relative importance of these two mechanisms does not only vary between chemical and thermal stressors, but also varies greatly between tissues.

Homeoviscous response to PCB exposure

Membranes are known to respond to fluctuations in ambient temperature by changing phospholipid unsaturation and cholesterol content (Hazel, 1995). These changes are usually homeoviscous, maintaining stable fluidity and preserving the environment of membrane proteins. Phospholipid unsaturation decreases as temperature rises and vice versa. Cholesterol content increases as the temperature deviates from the gel/liquid crystal phase transition temperature (Crockett, 1998). In some cases, a phospholipid or cholesterol change can be anti-homeoviscous, decreasing order at the same time that heat is decreasing order or vice versa, but it is compensated for by a large homeoviscous change in the other parameter (Crockett and Hazel, 1995). Changes in phospholipid head groups can also participate in the homeoviscous response, but they are reversed after long-term acclimation and were therefore a poor target for 30-day experiments (Farkas et al., 2001).

The injection procedure used here increased the tissue concentration of PCB-153 by 60-fold to reach levels similar to what has been reported in previous studies (Andersson et al., 2001; Duffy-Whritenour et al., 2010). These values are approximately ten times higher than measured in wild fish from the Great Lakes region, but comparable to concentrations found in deer mice from highly contaminated soil in northern Canada and in apex-predator wels catfish from Italy (Abdolahpur Monikh et al., 2013; Ficko et al., 2013; Scheider et al., 1998; Squadrone et al., 2013).

In this study, I show that exposure to PCB-153 induces changes in membrane composition in goldfish gill, brain, and liver: responses that are consistent with homeoviscous acclimation. Many changes in membrane composition, particularly changes in cholesterol concentration, serve other roles, including modulating membrane permeability (Hao et al., 2008) and maintaining the activity of specific proteins that require specific fatty acids in their vicinity (Barenholz, 2002), and more accurately determining which changes serve which roles in this experiment would require additional data, such as measurements of membrane order. However, the tissue-wide patterns observed here are consistent with the predicted effects of homeoviscous acclimation to membrane fluidization. Even though the adjustments of gill and brain to PCB-153 are both apparently homeoviscous, these two tissues use completely different mechanisms to restructure their membranes. Fluidity is controlled by decreasing phospholipid unsaturation in gill (Fig. 3.3B), but by increasing cholesterol in brain (Figs. 3.1A, 3.3A, 3.5A). These results show that some ectotherms are capable of protecting their membranes from chemical fluidization, potentially mitigating the disruption of numerous membrane processes.

Mechanism of homeoviscosity varies between tissues

This study shows that goldfish are able to adjust their membranes to cope with chemical and thermal stressors, but the mechanism and magnitude of the response vary greatly among tissues. Overall, the primary mechanism of the temperature response is a change in fatty acid unsaturation, whereas it is mainly cholesterol modulation for the PCB response. With higher temperature, gill, muscle, and brain replace polyunsaturated fatty acids such as arachidonate [20:4] and eicosadienoate [20:2] with saturated

fatty acids such as palmitate [16:0] and stearate [18:0]. The exact fatty acids used to respond to temperature or PCBs seem to be unconnected to their individual abundance (common fatty acids are not modulated more often than rare ones; see Tables 3.2-3.6). Instead, particular fatty acids may be selected to meet the specific requirements of local proteins that vary between tissues.

Cholesterol does not change with temperature, except in gill. In this tissue, the large homeoviscous adjustment in unsaturation (Fig. 3.3B) may compensate for the anti-homeoviscous change in cholesterol (Fig. 3.5B). The cholesterol response may relate to the osmoregulatory function of the gill because this steroid is known to interact directly with Na^+/K^+-ATPase in addition to influencing membrane fluidity. Reducing phospholipid unsaturation and cholesterol simultaneously may preserve fluidity as well as Na^+/K^+-ATPase activity in a way that a purely homeoviscous response would not (Crockett, 1998; Zehmer and Hazel, 2004). By contrast, PCB exposure increases cholesterol in brain and liver, but decreases fatty acid unsaturation in gill where it does not affect cholesterol. It is unclear whether such diversity in mechanisms between tissues and between stimuli offers advantages over a single, universal response.

It is possible that differences in membrane cholesterol between tissues are related to different quantities of total membrane or particular intracellular membranes between tissues. Membranes were not separated from one another prior to analysis and phospholipid and cholesterol recovery could not be verified, preventing the accurate determination of a cholesterol:PL molar ratio. There is no evidence that PCB exposure or temperature induces changes in the relative contribution of different organelles to total membranes or to the amount of total membranes present in a cell (Kraffe et al., 2007; Tan et al., 2004). It is likewise worth acknowledging that the degree of compensation for loss of membrane order cannot be conclusively determined with the experiments presented here. The extent to which the membrane responses demonstrated here restore normal membrane order can be determined with direct measurements of membrane order using 1,3,5-diphenylhexatriene (DPH), Fourier-transform infrared (FTIR) spectroscopy, or another method (Katynski et al., 2004). As membrane order is rarely perfectly

compensated (Zehmer and Hazel, 2004), this would be a useful future experiment and a more conclusive demonstration of homeoviscous responses to chemical fluidization. It remains true, however, that the responses observed here are consistent with large-scale and multi-organ efforts to restore and maintain membrane order in the face of stressors that would otherwise disrupt it, even if the degree of membrane order compensation cannot be precisely determined.

Desaturase expression

Desaturases are membrane-bound enzymes that add double bonds to saturated fatty acids prior to incorporation into phospholipids. Δ9 and Δ6 desaturases add double bonds to the C9-C10 and C6-C7 positions on fatty acids and thereby modulate phospholipid composition in response to temperature (Trueman et al., 2000). Here, desaturase expression levels were not affected by PCB-153 but Δ9 expression did change with temperature in liver (Fig. 3.6). The fact that PCB-153 and temperature did not stimulate the expression of the desaturase genes in most tissues is insufficient evidence to eliminate the involvement of desaturases in this experiment. This is possibly because the recruitment of desaturases may only occur with early activation of gene expression, prior to the 15-day tissue collection point. For example, changes in desaturase activity have been measured within 24 h of cold exposure (Cossins et al., 2002). Alternately, if mRNA turnover is high, expression changes may not be detectable. More experiments are needed to establish whether desaturase-related mechanisms are activated by PCB-153 in goldfish tissues. Determining potential changes in protein amounts or whether desaturase inhibitors can affect the homeoviscous response are promising avenues for future work.

Goldfish liver

Liver membranes respond very differently in goldfish than in rainbow trout, the model species and the tissue on which much of our understanding of homeoviscous acclimation is based (Hazel, 1995; McKinley and Hazel, 2000; Zehmer and Hazel, 2005). In goldfish liver, increases in docosahexaenoate (22:6) and arachidonate (20:4) create an anti-homeoviscous fatty acid response to both temperature and PCB-153 (Figs. 3.2, 3.3C, 3.4C). The two stimuli act synergistically, with one exacerbating the effect of

the other. The underlying causes of these responses are unknown, but the different liver response of goldfish compared to trout may be related to the highly different thermal tolerance of the two species. In addition, goldfish liver membranes may vary for other reasons than homeoviscosity. For example, the responses may also be associated with lipid raft maintenance or with the supply of docosahexaenoate and arachidonate as transduction signals (Rovito et al., 2013; Turk and Chapkin, 2013). The observed increase in membrane cholesterol (Fig. 3.5C) supports this notion because it is known to preserve lipid raft and ion pump function (Oliveira et al., 2012; Zehmer and Hazel, 2005). Recent evidence also shows that PCB-153 causes degradation of β-catenin and other cell adhesion proteins in rodent hepatocytes (Šimečková et al., 2009). The downstream effects of PCB-induced protein damage are likely multiplied when high temperature increases cellular activity. As more fluid membranes are associated with greater membrane protein activity, synergistic effects on unsaturation may be a means to increase activity and mitigate protein damage. The observed responses and their variability between tissues suggest that focusing on a single species or a single tissue in future examinations of homeoviscous acclimation could be misleading.

Conclusions

This study shows that goldfish membranes are not only able to cope with changes in temperature, but can also protect themselves from chemical fluidization. Contrary to expectation, the mechanisms used to deal with thermal and chemical stressors are different. Thermal acclimation mostly causes changes in phospholipid unsaturation, whereas PCB exposure induces changes in cholesterol. If also present in other species, these protective responses may prove particularly important for polar fish that face the combined effects of thermal stress from climate change and chemical fluidization from organochlorine deposition (Armitage et al., 2013; Saint-Amour et al., 2006). Results also show that each tissue has a distinct pattern of changes, suggesting that different local factors contribute to the stress response. The different behaviour of liver membranes observed in goldfish compared to trout may be related to the widely different thermal tolerance of the two species. Therefore, the homeostatic mechanisms that preserve normal membrane function vary: (1) with the nature of the stressor that perturbs fluidity, (2) with local

conditions within each tissue, and (3) possibly with the thermal tolerance of individual species. These complicating factors should be considered in future studies of homeoviscous adjustments.

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Figures

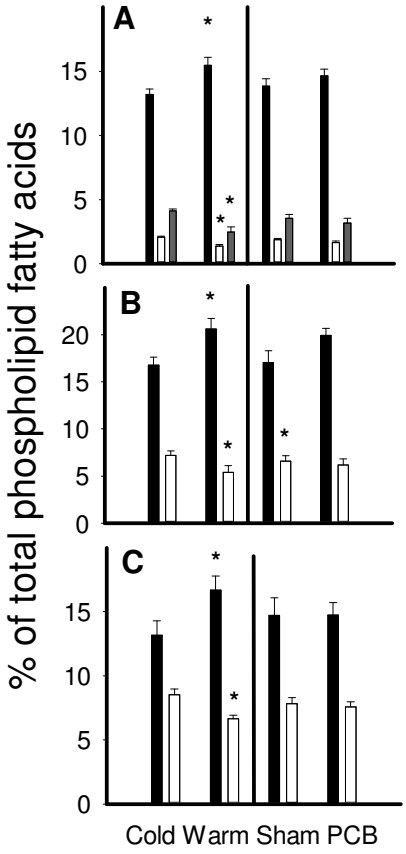

Fig. 3.1. Individual fatty acids from goldfish membrane phospholipids significantly affected by temperature and PCB-153 exposure. (A) Brain stearate (■), eicosenoate (□), and arachidonate (■). (B) Gill palmitate (■) and eicosadienoate (□). (C) Muscle palmitate (■) and arachidonate (□). Left panels show the effects of temperature only (fish exposed and not exposed to PCB-153 were pooled). Right panels show the effects of PCB-153 exposure only (warm and cold fish were pooled). Values are means ± s.e.m (Gill: n = 29 for cold and 24 for warm; n = 26 for sham-injected and 27 for PCB. Brain: n = 26 for cold and 22 for warm; n = 24 for sham-injected and 24 for PCB. Muscle: n = 29 for cold and 23 for warm; n = 25 for sham-injected and 27 for PCB.). Significant effects of temperature or PCB exposure are indicated by * ($P<0.05$). The percentages of all the other phospholipid fatty acids did not change and can be found in Tables 3.1, 3.2, and 3.4.

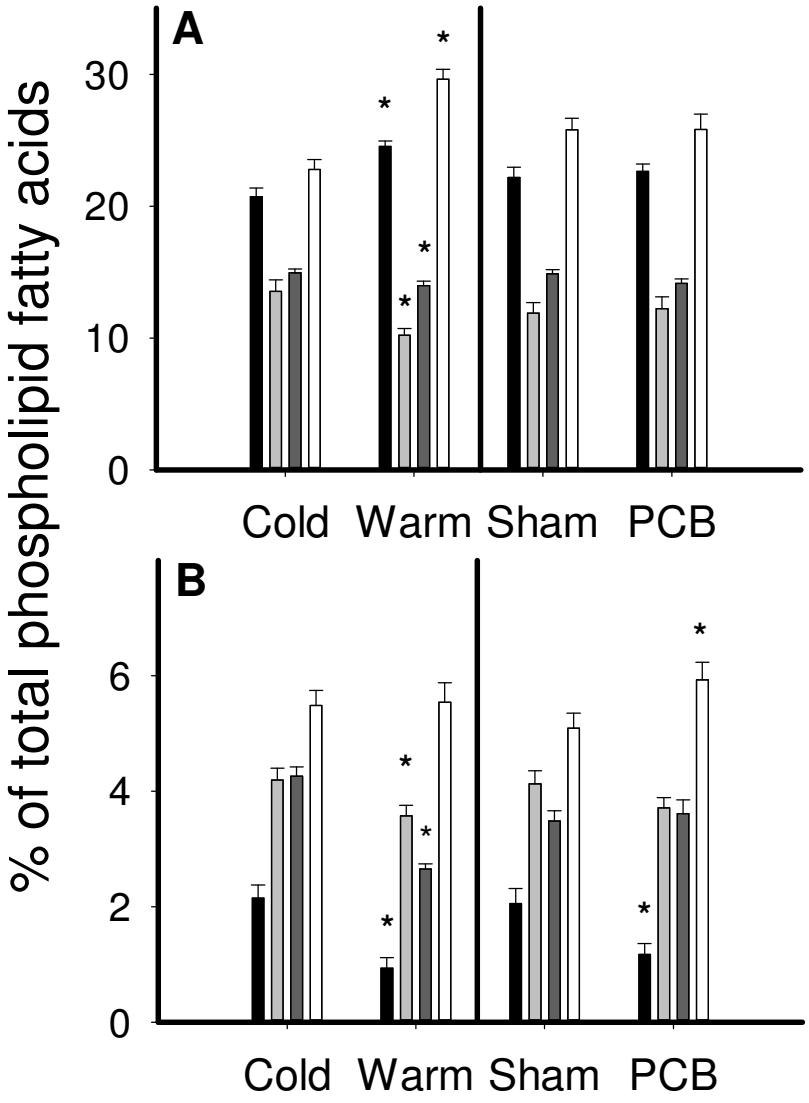

Fig. 3.2. Individual fatty acids from membrane phospholipids significantly affected by temperature and PCB-153 exposure in goldfish liver. (A) Liver palmitate (■), stearate (■), oleate (■), and docosahexaenoate (□). (B) Liver palmitoleate (■), linoleate (■), eicosenoate (■), and arachidonate (□). Left panels show the effects of temperature only (fish exposed and not exposed to PCB-153 were pooled). Right panels show the effects of PCB-153 exposure only (warm and cold fish were pooled). Values are means ± s.e.m (n = 29 for cold and 24 for warm; n = 26 for sham-injected and 27 for PCB). Significant effects of temperature or PCB exposure are indicated by * ($P<0.05$). The percentages of all the other phospholipid fatty acids did not change and can be found in Table 3.3.

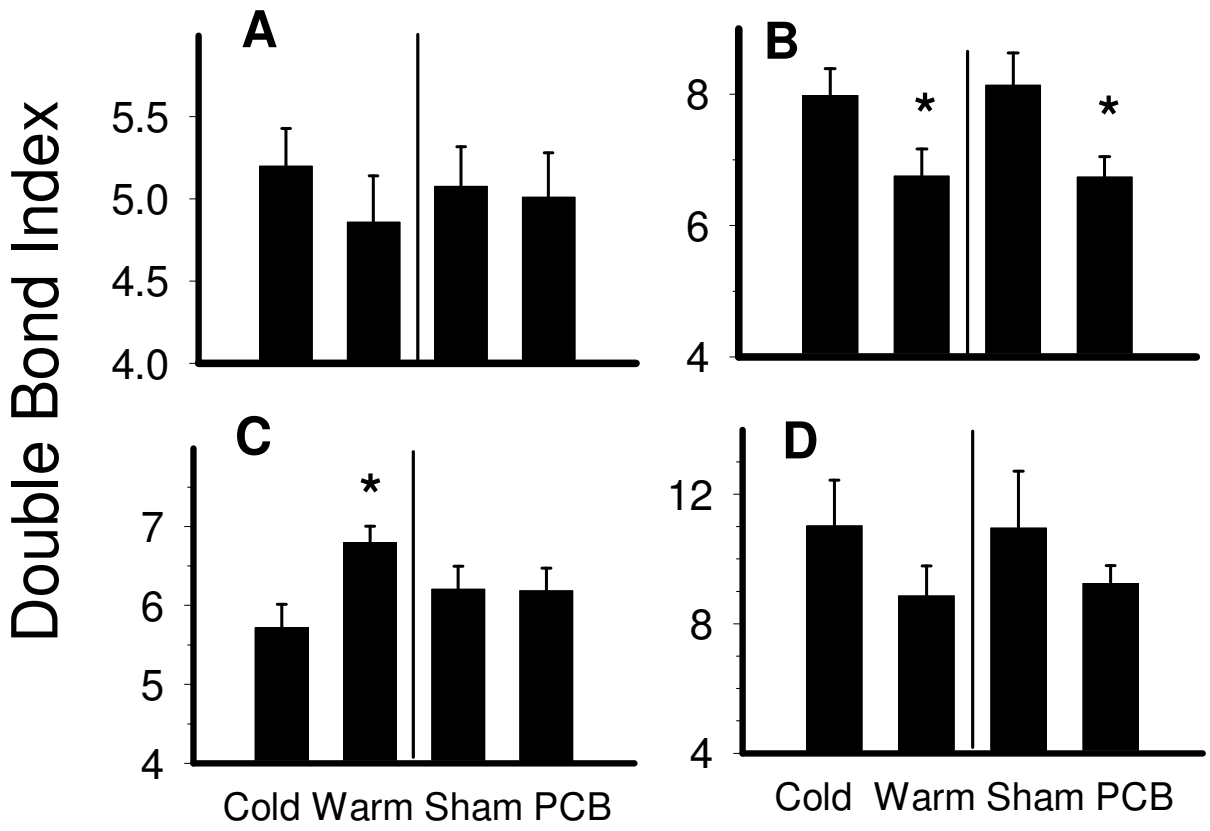

Fig. 3.3. Effects of temperature and PCB-153 exposure on the double bond index of membranes in goldfish brain (A), gill (B), liver (C), and muscle (D). Left panels show the effects of temperature only (fish exposed and not exposed to PCB-153 were pooled). Right panels show the effects of PCB-153 exposure only (warm and cold fish were pooled). Values are means ± s.e.m (brain, gill, and liver: n = 29 for cold and 24 for warm; n = 26 for control and 27 for PCB. Muscle: n = 29 for cold and 23 for warm; n = 25 for sham-injected and 27 for PCB.). Significant effects of temperature or PCB exposure are indicated by * ($P<0.05$).

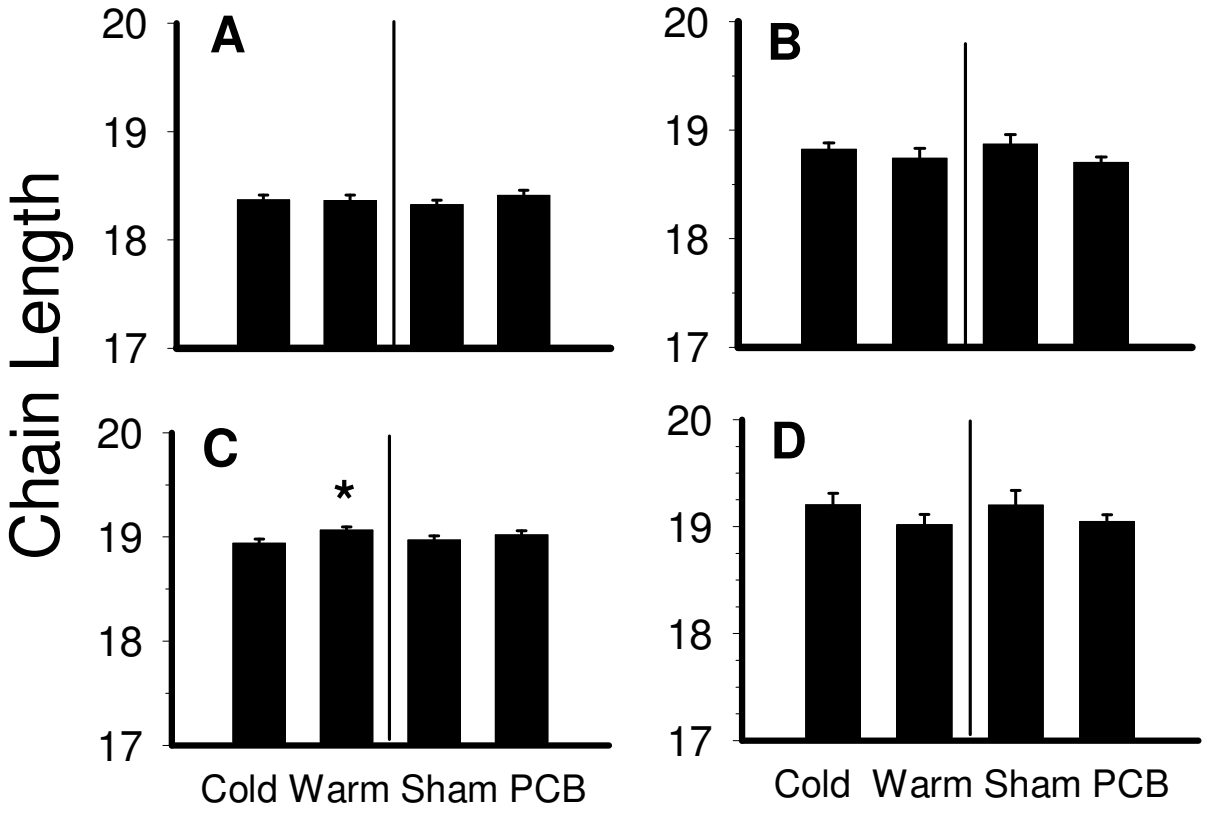

Fig. 3.4. Effects of temperature and PCB-153 exposure on the chain length of membrane phospholipid fatty acids in goldfish brain (A), gill (B), liver (C), and muscle (D). Left panels show the effects of temperature only (fish exposed and not exposed to PCB-153 were pooled). Right panels show the effects of PCB-153 exposure only (warm and cold fish were pooled). Values are means ± s.e.m (brain, gill, and liver: n = 29 for cold and 24 for warm; n = 26 for control and 27 for PCB. Muscle: n = 29 for cold and 23 for warm; n = 25 for sham-injected and 27 for PCB.). Significant effects of temperature or PCB exposure are indicated by * ($P<0.05$).

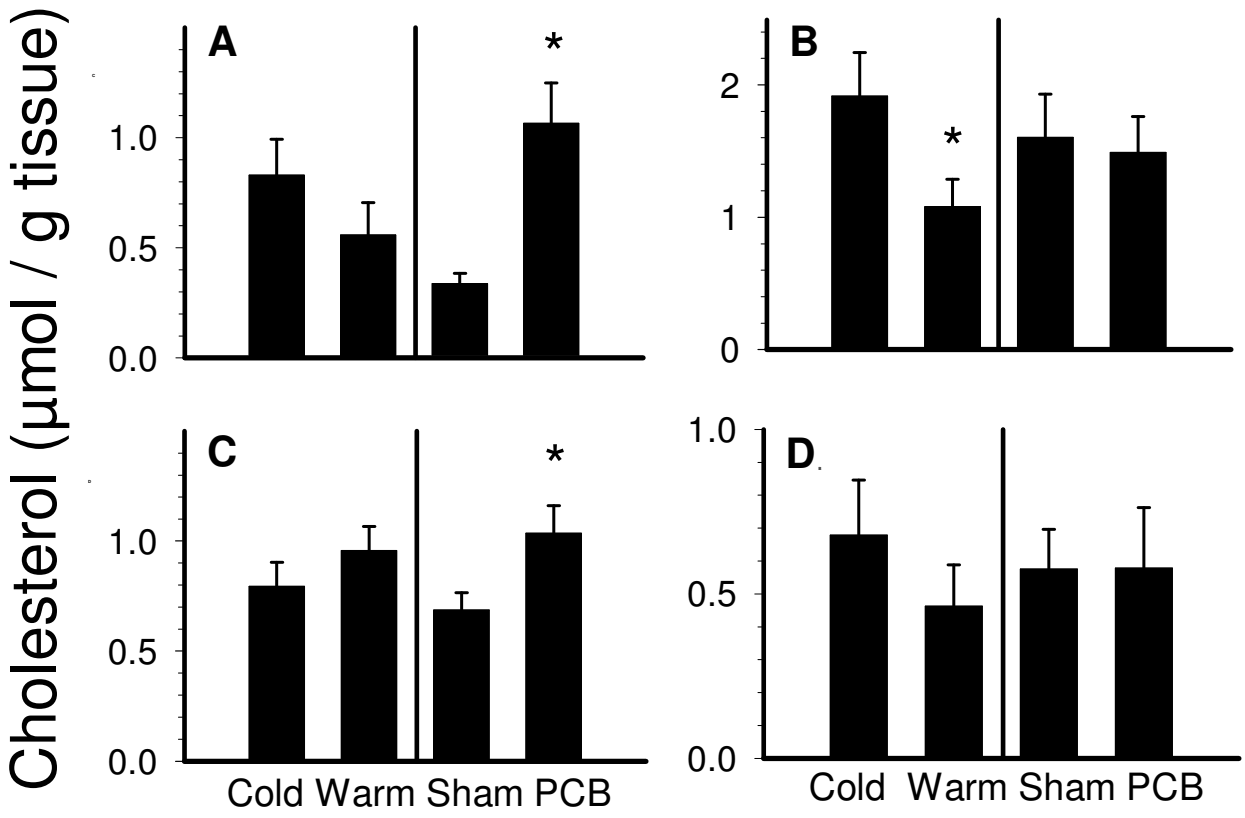

Fig. 3.5. Effects of temperature and PCB-153 exposure on membrane cholesterol concentration in goldfish brain (A), gill (B), liver (C), and muscle (D). Left panels show the effects of temperature only (fish exposed and not exposed to PCB-153 were pooled). Right panels show the effects of PCB-153 exposure only (warm and cold fish were pooled). Values are means ± s.e.m (Brain: n = 18 for cold and 20 for warm; n = 19 for sham-injected and 18 for PCB. Gill: n = 28 for cold and 23 for warm; n = 25 for sham-injected and 26 for PCB. Liver: n = 28 for cold and 21 for warm; n = 24 for sham-injected and 25 for PCB. Muscle: n = 28 for cold and 20 for warm; n = 24 for sham-injected and 24 for PCB.). Significant effects of temperature or PCB exposure are indicated by * ($P<0.05$).

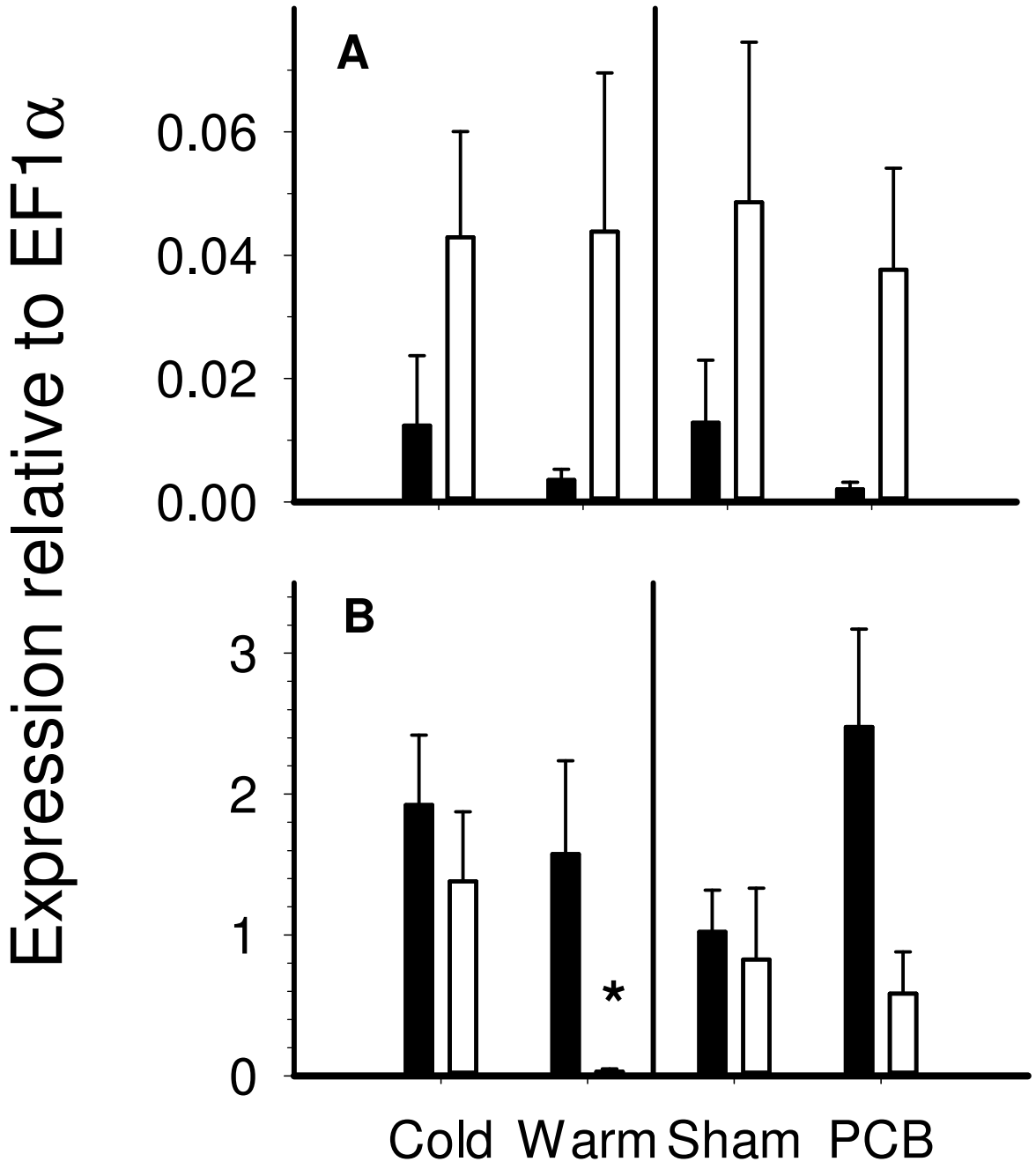

Fig. 3.6. Effects of temperature and PCB-153 exposure on Δ6 desaturase (■) and Δ9 desaturase (□) expression relative to elongation factor 1α (EF1α) in gill (A) and liver (B) of goldfish calculated using ΔCT. Values are means ± s.e.m (n = 10). Significant differences are indicated by * ($P<0.05$).

Tables

Table 3.1. Primer sequences for goldfish elongation factor 1α, Δ6 desaturase, and Δ9 desaturase.

Primer	Sequence
Elongation factor 1α forward	5'- GAT TGT TGC TGG TGG TGT TG -3'
Elongation factor 1α reverse	5'- GCA GGG TTG TAG CCG ATT T -3'
Δ6 desaturase forward	5'- ACA CGG CCG TCG TTG CTG TT -3'
Δ6 desaturase reverse	5'- ACG CTC CCT TCA GGT GTC CGA -3'
Δ9 desaturase forward	5'- GGC CAG AGA CCA TCG TGT CCA -3'
Δ9 desaturase reverse	5'- GCG AGCTCC AGT TTG CGT CCT -3'

Table 3.2. Fatty acid composition of membrane phospholipids in the brains of goldfish from four treatment groups expressed in % of total membrane fatty acids.

	Cold Sham	Warm Sham	Cold PCB	Warm PCB
n	15	11	14	13
Fatty acids				
16:0	21.3 ± 0.7	21.0 ± 1.0	20.2 ± 0.7	21.5 ± 1.1
16:1	7.1 ± 0.5	6.3 ± 0.5	6.6 ± 0.6	5.2 ± 0.5
18:0	13.1 ± 0.6	14.7 ± 1.1	13.2 ± 0.6	16.1 ± 0.7
18:1	26.2 ± 0.8	26.6 ± 0.7	25.6 ± 0.7	26.0 ± 0.6
18:2	1.8 ± 0.1	1.5 ± 0.1	1.8 ± 0.1	1.3 ± 0.3
20:1	2.1 ± 0.1	1.6 ± 0.1	2.0 ± 0.1	1.2 ± 0.2
20:3	-	1.7 ± 0.6	-	1.7 ± 0.6
22:0	1.4 ± 0.1	1.1 ± 0.1	1.5 ± 0.1	-
20:4	4.0 ± 0.2	2.9 ± 0.7	4.3 ± 0.2	2.1 ± 0.5
24:0	-	1.2 ± 0.8	1.9 ± 1.1	1.4 ± 0.6
22:6	21.2 ± 1.5	21.5 ± 1.6	21.9 ± 1.7	22.2 ± 1.7
Cholesterol (ng/g tissue)	1.1 ± 0.3	0.7 ± 0.1	3.0 ± 0.6	2.4 ± 0.8

Values are means ± s.e.m. -, trace component (average < 1% of total). Cold, 5°C. Warm, 20°C. Sham, injected with sunflower oil. PCB, injected with 3 µg/g body mass PCB-153 in sunflower oil.

Table 3.3. Fatty acid composition of membrane phospholipids in the gills of goldfish from four treatment groups expressed in % of total membrane fatty acids.

	Cold Sham	Warm Sham	Cold PCB	Warm PCB
n	15	11	14	13
Fatty acids				
16:0	15.3 ± 1.4	19.4 ± 2.1	18.3 ± 0.8	21.6 ± 1.2
16:1	3.0 ± 0.5	1.4 ±0.4	3.6 ± 0.4	1.8 ±0.4
18:0	7.7 ± 1.0	8.3 ± 0.4	7.7 ± 0.5	10.0 ± 1.2
18:1	20.5 ± 1.6	19.4 ± 0.6	17.2 ± 0.6	19.7 ± 1.0
18:2n-6	7.5 ± 1.1	5.6 ± 0.2	6.6 ± 0.6	5.5 ± 0.6
20:2n-6	7.5 ± 0.9	5.4 ± 0.4	6.9 ± 0.2	5.4 ± 1.3
21:0	-	1.5 ± 0.5	-	-
20:3n-6	1.4 ± 0.4	1.5 ± 0.3	1.9 ± 0.3	1.6 ± 0.4
22:0	3.0 ± 0.5	3.7 ± 2.1	3.4 ± 0.2	2.5 ± 0.4
20:4n-6	7.0 ± 1.0	6.8 ± 0.5	7.2 ± 0.5	8.2 ± 1.0
22:3	-	1.1 ± 0.2	1.4 ± 0.2	1.4 ± 0.3
24:1	-	-	1.7 ± 1.6	-
22:6n-3	22.4 ± 2.1	24.1 ± 1.8	20.5 ± 1.8	20.9 ± 2.0
Cholesterol (ng/g tissue)	5.6 ± 1.3	2.0 ± 0.4	4.2 ± 1.1	3.5 ± 0.9

Values are means ± s.e.m. -, trace component (average < 1% of total). Cold, 5°C. Warm, 20°C. Sham, injected with sunflower oil. PCB, injected with 3 µg/g body mass PCB-153 in sunflower oil.

Table 3.4. Fatty acid composition of membrane phospholipids in the livers of goldfish from four treatment groups expressed in % of total membrane fatty acids.

	Cold Sham	Warm Sham	Cold PCB	Warm PCB
n	15	11	14	13
Fatty acids				
16:0	21.6 ± 0.8	19.9 ± 1.0	25.3 ± 0.3	23.8 ± 0.7
16:1	1.6 ± 0.2	2.7 ± 0.3	1.2 ± 0.2	-
18:0	14.8 ± 1.2	12.4 ± 1.2	11.2 ± 0.8	9.3 ± 0.5
18:1	14.3 ± 0.4	15.5 ± 0.4	14.0 ± 0.4	14.0 ±0.5
18:2	4.0 ± 0.2	4.4 ± 0.3	3.8 ± 0.2	3.4 ± 0.3
18:3	2.7 ± 0.8	2.5 ± 0.6	1.7 ± 0.4	1.3 ± 0.5
20:1	4.4 ± 0.3	4.1 ± 0.2	2.6 ± 0.1	2.7 ± 0.1
20:3	2.6 ± 0.2	2.7 ± 0.2	3.0 ± 0.2	2.5 ± 0.3
22:0	2.4 ± 0.2	2.8 ± 0.3	1.8 ± 0.1	1.8 ± 0.1
20:4	5.6 ± 0.4	5.4 ± 0.4	4.6 ± 0.3	6.4 ± 0.5
24:0	1.2 ± 0.3	1.2 ± 0.2	-	-
22:3	1.2 ± 0.2	1.0 ± 0.2	1.6 ± 0.2	2.3 ± 0.3
22:6	21.7 ± 1.1	23.8 ± 1.0	28.5 ± 1.1	30.6 ± 0.9
Cholesterol (ng/g tissue)	1.5 ± 0.3	2.3 ± 0.3	2.7 ± 0.5	2.6 ± 0.5

Values are means ± s.e.m. -, trace component (average < 1% of total). Cold, 5°C. Warm, 20°C. Sham, injected with sunflower oil. PCB, injected with 3 µg/g body mass PCB-153 in sunflower oil.

Table 3.5. Fatty acid composition of membrane phospholipids in the muscles of goldfish from four treatment groups expressed in % of total membrane fatty acids.

	Cold Sham	Warm Sham	Cold PCB	Warm PCB
n	15	10	14	13
Fatty acids				
16:0	13.6 ± 1.8	16.4 ± 2.2	12.7 ± 1.4	16.9 ± 1.0
16:1	-	-	1.1 ± 0.3	-
18:0	7.7 ± 0.6	6.8 ± 0.2	7.9 ± 0.8	7.8 ± 0.5
18:1	16.5 ± 0.2	16.7 ± 1.6	16.7 ± 0.8	20.2 ± 0.6
18:2n-6	8.7 ± 1.2	9.7 ± 0.8	11.6 ± 0.6	9.4 ± 0.4
20:1	2.3 ± 0.9	-	-	-
20:2n-6	7.6 ± 1.0	6.9 ± 1.0	7.9 ± 0.6	7.3 ± 0.5
20:3n-6	-	1.3 ± 0.4	2.1 ± 0.4	1.6 ± 0.3
22:0	5.3 ± 1.1	3.6 ± 0.4	3.7 ± 0.3	3.0 ± 0.3
20:4n-6	8.5 ± 0.7	6.8 ± 0.5	8.5 ± 0.6	6.5 ± 0.2
22:3	2.2 ± 0.4	2.5 ± 0.8	2.1 ± 0.4	1.9 ± 0.3
22:5n-3	1.7 ± 0.3	-	1.1 ± 0.3	-
22:6n-3	23.3 ± 3.3	25.3 ± 2.7	21.9 ± 1.3	20.8 ± 0.7
Cholesterol (ng/g tissue)	1.6 ± 0.5	1.4 ± 0.4	1.9 ± 0.7	1.0 ± 0.5

Values are means ± s.e.m. -, trace component (average < 1% of total). Cold, 5°C. Warm, 20°C. Sham, injected with sunflower oil. PCB, injected with 3 µg/g body mass PCB-153 in sunflower oil.

CHAPTER 4.
Homeoviscous response to PCB-153 and temperature use different mechanisms in rainbow trout

Chapter 4

Homeoviscous response to PCB-153 and temperature use different mechanisms in rainbow trout

Based on

Alex Gonzalez[1] and Jean-Michel Weber[1]

Toxicology and Applied Pharmacology (in preparation)

Author Contributions: AG designed this study, performed the experiments, and analyzed the data. AG and JMW wrote the manuscript.

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Introduction

Polychlorinated biphenyls (PCBs) are an exceptionally well-studied group of environmental contaminants once commonly used for applications as diverse as paint additives and dielectric fluid. Though banned in much of the developed world, PCBs remain a toxicological concern, particularly in the Arctic (Drouillard et al., 2007). Earlier research about PCB exposure focused on the carcinogenic effects of the 12 coplanar, dioxin-like PCBs, acting via the aryl hydrocarbon receptor (Safe, 1994). The other 197 PCB congeners are non-coplanar and do not strongly activate this receptor. However, these PCB congeners intercalate between phospholipids in a manner similar to cholesterol (Campbell et al., 2008; Yilmaz et al., 2006) and have been shown to increase membrane fluidity in rodent and chicken cells (Bonora et al., 2003; Katynski et al., 2004; Reich et al., 1981; Tan et al., 2004). Such fluidity perturbations affect the activity of membrane proteins, including enzymes from oxidative pathways and hormone receptors, with far-reaching physiological consequences (Corcoran et al., 2007; Guderley et al., 2008). Therefore, membrane fluidizers are a toxicological concern, alongside the additional estrogenic and neurotoxic effects also attributed to non-coplanar PCBs (Arnold et al., 1990; Berg et al., 2011; Saint-Amour et al., 2006; Venkataraman et al., 2010). Given that polar species face the combined effects of thermal stress from climate change and chemical fluidization from pollution (Armitage et al., 2013; Brown et al., 2013; Saint-Amour et al., 2006), a broader understanding of PCB-membrane interactions is important for the conservation of fragile polar biomes.

Membrane fluidity also varies with temperature, but ectotherms have a well-developed homeoviscous response to counter the effects of thermal fluctuations on membrane properties (Hazel, 1995; Seebacher et al., 2009). The primary mechanisms of homeoviscous acclimation are to modulate the saturation state of phospholipids and the concentration of membrane cholesterol. More saturated phospholipids make for less fluid, more ordered membranes and vice versa. Membrane cholesterol can likewise be increased or decreased to buffer membranes against abrupt phase transitions (Cossins et al., 2002; Crockett, 1998; Zehmer and Hazel, 2005). Ectotherms usually show a strong homeoviscous

response in the liver and much more limited responses in other tissues, such as brain and muscle (Cossins, 1977; Farkas et al., 2001; Hazel et al., 1991). Data on the interaction between temperature-induced homeoviscous acclimation and membrane-fluidizing chemicals are scant, as most studies of membrane fluidizers have been conducted in the membranes of isolated mammalian cells and chicken embryos (Katynski et al., 2004; Lopez-Aparicio et al., 1994; Shen et al., 2011; Suwalsky et al., 1997; Tan et al., 2004; Vaish and Sanyal, 2011). While mammals do undergo homeoviscous acclimation under certain circumstances (Armstrong et al., 2011; Giroud et al., 2013; Kolomiytseva, 2011; Kolomiytseva et al., 2008), the models used for previous studies of PCB-induced membrane fluidization do not. Nakanishi et al exposed *Gnathopogon caerulescens* to sodium dodecylbenzenesulfonate and sodium stearate (Nakanishi et al., 1987a; Nakanishi et al., 1987b), which also destabilize phospholipid membranes, but did not examine membrane lipids in any detail or in multiple organs, nor did they examine the relationship between chemical fluidization and thermal acclimation. One study showed that the effects of PCB-153 on goldfish vary between tissues and that the primary mechanism of response to PCB-153 is cholesterol modulation, rather than the phospholipid adjustments used to acclimate to temperature in this species (Chapter 3). The goldfish is a highly eurythermic model organism whose liver response in particular differs from established patterns, however. How the intensity of its homeoviscous response compares to that of other, less eurythermal species is not yet fully elucidated. A species more vulnerable to increasing temperature may exhibit a more extensive membrane response to prevent damage to its more sensitive proteins or heart-failure-induced mortality (Somero, 2004; Somero, 2010), or its vulnerability to high temperature may be mediated by an inability to restore membrane order under these conditions. Membrane-fluidizing chemicals thus provide an opportunity to specifically push membrane responses to their limits, and to learn whether this combination of stressors poses a multiplied threat to animals otherwise vulnerable to high temperature.

The goal of this study was to examine whether PCB-153 can cause membrane restructuring or interfere with the normal homeoviscous response of a more sensitive ectotherm than goldfish during

temperature acclimation. More specifically, my aim was to measure potential changes in membrane composition (phospholipids and cholesterol) in rainbow trout organs (gill, brain, heart, muscle, and liver) in response to PCB-153 and to temperature (5 or 18°C) . A 2 x 2 factorial design was chosen to detect possible synergistic effects between PCB exposure and temperature. PCB-153 was the *ortho*-substituted congener selected here because of its environmental pervasiveness and particularly long half-life in animal tissues (Armitage et al., 2013; Drouillard et al., 2007). Within each tissue, I hypothesized that PCB-153 would induce a homeoviscous change in cholesterol content and temperature would induce a corresponding homeoviscous change in phospholipid fatty acid saturation. Further, I hypothesized that the combination of high temperature and PCB exposure would elicit strong responses in most or all tissues, because the two stimuli impose similar stresses and are likely to synergize to place higher demands on membrane physiology than either would alone. In particular, I hypothesized that trout would show pronounced responses in muscle and brain, whose responses are reduced or absent in goldfish.

Materials and methods

Animals and experimental design

All procedures were approved by the Animal Care Committee of the University of Ottawa and adhered to the guidelines established by the Canadian Council on Animal Care for the use of animals in research and the code of ethics of the World Medical Association (Declaration of Helsinki) for animal experiments. Juvenile rainbow trout *Oncorhynchus mykiss* (Walbaum) (40.03 ± 1.58 g, n = 74) were purchased from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada) and held in four 70-L flow-through tanks in dechlorinated, well-oxygenated water at 13°C under a 16h:8h light-dark photoperiod. Fish were from the same batch and therefore the same age. They were fed floating fish pellets (Profishent; Martin Mills; Elmira, Ontario, Canada) daily until satiation. Fish were habituated to these conditions for at least 14 days before temperature acclimation. The experiments were designed as 2 x 2 matrices simultaneously testing the effects of temperature (5°C vs 18°C) and PCB exposure (sham injection vs

PCB injection). For each experiment, fish were randomly assigned to one of four groups: cold sham-injected, warm sham-injected, cold PCB-injected, and warm PCB-injected. Acclimation temperature was reached gradually (2°C/day). The fish were maintained at their acclimation temperatures for 21 days before starting sham/PCB injections and maintained at these temperatures for an additional 30 days.

Sham or PCB injection and tissue sampling

After temperature acclimation, the fish received three sham or PCB injections under MS-222 anaesthesia (20 mg/L) at 10-day intervals. One group each of warm and cold trout were injected with 6 µg/g body mass of PCB-153 (2,2',4,4',5,5'-hexachlorobiphenyl) (Ultra Scientific; North Kingstown, RI, USA) in sunflower oil (1.25 mg PCB / mL). This dose is comparable to concentrations used in previous studies (Andersson et al., 2001; Duffy-Whritenour et al., 2010) as well as in Chapter 3. The remaining two groups received an equivalent volume of pure sunflower oil as a sham injection. Fish were injected on the left side, behind the dorsal fin and into the body cavity at a 45° angle. This injection protocol was previously verified as increasing tissue PCB concentration at least 60-fold (Chapter 3) to levels comparable to what is measured in wels catfish from Italy (Squadrone et al., 2013) and approximately twenty times those reported in wild fish from the Great Lakes region (Bhavsar et al., 2007). For each temperature, the sham-injected and PCB-injected groups had identical food intake. The trout were then euthanized by MS-222 overdose (80 mg/L) 10 days after the last sham/PCB injection. The brain, gill, white muscle, heart, and liver were sampled from the right side and freeze-clamped in liquid N_2. Tissue samples were stored at -80°C until analyses.

Membrane lipids

Total lipids were extracted from ~40 mg of tissue using chloroform:methanol (2:1 v/v). Samples were homogenized (Polytron, Kinematica, Littau, Switzerland) and centrifuged (10 min at 2000*g*). Supernatants were filtered and 0.25% KCl added to separate aqueous and organic phases. The organic phase was evaporated and the lipids resuspended in chloroform before loading on solid-phase extraction columns (Supelclean 3 mL 500 mg LC-NH2; Sigma-Aldrich; St. Louis, MO, USA) to separate the

phospholipids. Separation was achieved by sequential elution of lipid classes—neutral lipids, non-esterified fatty acids, and phospholipids (PL)—using solvents of increasing polarity: chloroform:isopropanol (3:2 v/v), isopropyl ether:acetic acid (98:2 v/v), and methanol, respectively (Maillet and Weber, 2006). The fatty acid composition of PL was measured after acid transesterification. Fatty acid methyl esters were analyzed on an Agilent Technologies 6890N gas chromatograph (Mississauga, Ontario, Canada) equipped with a fused silica capillary column (Supelco DB-23, 60m, 0.25 mm i.d., 0.25μm film thickness; Sigma-Aldrich) using hydrogen as carrier gas as previously (Magnoni and Weber, 2007). Only the fatty acids accounting for >1% of total fatty acids in membrane phospholipids are reported in this study. Phospholipid recovery could not be verified.

Membrane cholesterol was measured as non-esterified (free) cholesterol in ~50 mg of tissue. Tissues were homogenized in chloroform:methanol (2:1 v/v). KCl/EDTA (2 M / 5 mM) was added to separate aqueous and organic phases prior to centrifugation (10 min at 2000 *g*). The organic phase was dried, resuspended in 2-methoxyethanol, and stored at -80°C. Cholesterol was measured by fluorometry (SpectraMax Gemini XS, Molecular Devices, Sunnyvale, California, USA) using a commercial assay kit (Cayman Chemical, Ann Arbor, Michigan, USA). This kit was selected because it allows the separate measurement of membrane (free, non-esterified) cholesterol and cholesterol esters that are found only outside membranes. Cholesterol recovery could not be verified.

Calculations and statistics

Membrane saturation was expressed by the double bond index, calculated as the average number of double bonds divided by the fraction of saturated fatty acids. Because absolute phospholipid values were highly variable and dependent on recovery calculations, cholesterol data are presented as μmol cholesterol per gram tissue. Data were analyzed using two-way ANOVA with the Holm-Sidak post-hoc test to determine which treatments differed from one another. Statistical analyses were performed using SigmaPlot 12.5 (Systat, San Jose, CA, USA). All values presented are means ± s.e.m. and a level of

significance of $P < 0.05$ was used in all tests. Normality of the data was always tested prior to analyses using the Shapiro-Wilk test.

Results

Cholesterol

The effects of temperature and PCB exposure on trout membrane cholesterol concentration in µmol/g tissue are shown in Table 4.1 and Fig. 4.1. In gill, high temperature caused an increase in membrane cholesterol ($p < 0.05$) and PCB exposure caused a decrease ($p < 0.05$). Neither treatment had an effect on the membrane cholesterol of brain or liver ($p > 0.05$). PCB exposure caused a decrease in heart cholesterol ($p < 0.05$), but temperature had no effect ($p > 0.05$). There was a significant interaction between temperature and PCB exposure on brain and muscle membrane cholesterol ($p < 0.05$).

At the level of pairwise comparisons, the brain membrane cholesterol of the cold sham-injected fish was significantly higher than that of the warm sham-injected and cold PCB-injected fish (Fig. 4.1A, $p < 0.05$). The brain membrane cholesterol of the warm PCB-injected fish did not differ significantly from that of the warm sham-injected or cold PCB-injected fish ($p > 0.05$). In gills, only the cold sham-injected and cold PCB-injected fish had significantly different membrane cholesterol content (Fig. 4.1B, $p < 0.05$). The hearts of cold PCB-injected fish had significantly less membrane cholesterol than those of cold sham-injected and warm PCB-injected fish (Fig. 4.1C, $p < 0.05$), but warm sham-injected hearts did not differ significantly from cold sham-injected or warm PCB-injected hearts ($p > 0.05$). No pairwise comparisons showed significant differences in liver (Fig. 4.1D). In muscle, warm sham-injected trout muscles had significantly less membrane cholesterol than their warm PCB-injected fish counterparts (Fig. 4.1E, $p < 0.05$).

Double bond index

The effects of temperature and PCB exposure on trout membrane double bond index are shown in Table 4.2 and Fig. 4.2. In all tissues, double bond index was higher in cold-acclimated fish ($p < 0.05$) and did not differ significantly between the sham-injected and PCB-injected fish ($p > 0.05$). There was a significant interaction between temperature and PCB exposure in trout gill ($p < 0.05$).

At the level of pairwise comparisons, the brains (Fig. 4.2A) and livers (Fig. 4.2D) of the two warm-acclimated groups had significantly higher double bond indices than those of the corresponding cold-acclimated groups ($p < 0.05$), but the PCB-injected groups did not differ significantly from their sham-injected counterparts in these tissues ($p > 0.05$). The gill membranes (Fig 4.2B) of warm-acclimated fish are likewise significantly more saturated than those of the corresponding cold-acclimated fish ($p < 0.05$). Additionally, warm sham-injected gill membranes are significantly less unsaturated than warm PCB-injected gill membranes ($p < 0.05$), but gills from cold sham-injected and cold PCB-injected fish do not have significantly different double bond indices ($p > 0.05$). In the hearts (Fig. 4.2C), warm sham-injected fish have lower membrane unsaturation than cold sham-injected fish ($p < 0.05$), but sham-injected fish do not differ from their PCB-injected counterparts, nor do cold and warm PCB-injected fish differ from each other ($p > 0.05$). Similarly, in muscle (Fig. 4.2E), warm PCB-injected membranes have lower double bond indices than cold PCB-injected membranes ($p < 0.05$), but PCB exposure does not lead to differences in double bond index in fish exposed to the same temperature, nor do cold and warm sham-injected fish differ from one another ($p > 0.05$).

Chain length

The effects of temperature and PCB exposure on trout fatty acid chain length are shown in Table 4.3 and Fig. 4.3. In gill, high temperature caused a decrease in chain length ($p < 0.05$) and PCB exposure caused an increase ($p < 0.05$). Neither treatment had an effect on the chain length of heart or liver ($p > 0.05$). PCB exposure increased chain length in brain ($p < 0.05$), and there was a significant interaction between temperature and PCB exposure on brain chain length ($p < 0.05$).

At the level of pairwise comparisons, the brain chain length of the warm sham-injected fish was significantly lower than that of the cold sham-injected and warm PCB-injected fish (Fig. 4.3A, $p < 0.05$). The brain chain length of the warm PCB-injected fish did not differ significantly from that of the warm sham-injected or cold PCB-injected fish ($p > 0.05$). In gill and muscle, warm fish had lower chain lengths than cold fish in both the sham-injected and PCB-injected groups ($p > 0.05$), and the gills warm PCB-

injected fish had higher chain lengths than the gills of cold PCB-injected fish ($p > 0.05$). No pairwise comparisons showed significant differences in heart or liver (Fig. 4.1D).

Phospholipid fatty acids

The effects of temperature and PCB exposure on the molar fractions of individual fatty acids are shown for brain, gill, heart, liver, and muscle in Tables 4.5-4.9. Statistical analysis of these data is presented in Table 4.10. Pairwise comparisons are not shown. In brain, nearly all fatty acids detected above trace levels differ significantly in percentage in response to temperature, PCB exposure, or both. In response to higher temperature, brain palmitate (16:0), brain stearate (18:0), and docosatrienoate (22:3) increase while linoleate (18:2), arachidonate (20:4), behenate (22:0), nervonate (24:1), and docosahexaenoate (22:6) decrease. In response to PCB exposure ($p< 0.05$), palmitate, palmitoleate, and eicosatrienoate (20:3) increase while stearate, oleate (18:1), linoleate, arachidonate, docosatrienoate, nervonate, and docosahexaenoate decrease. Brain stearate, oleate, linoleate, eicosatrienoate, docosatrienoate, nervonate, and docosahexaenoate have significant interaction terms as well, indicating that the effect of one treatment varies based on which level of the other is present ($p<0.05$). In gill, palmitate and oleate increase and eicosatrienoate, behenate, and docosahexaenoate decrease in response to warmth, no fatty acids change in response to PCB exposure, and palmitoleate, stearate, linoleate, eicosatrienoate, behenate, and docosahexaenoate have significant interaction terms ($p<0.05$). In heart, palmitate and docosatrienoate increase and oleate, linoleate, and linolenate (18:3) decrease in response to temperature ($p<0.05$) and no fatty acids change in response to PCB exposure or have significant interaction terms. In liver, palmitate, palmitoleate, stearate, and oleate increase and arachidonate and docosahexaenoate decrease in response to warmth, no fatty acids respond to PCB exposure, and nervonate shows a significant interaction term ($p<0.05$). In muscle, only docosahexaonate responds to either treatment, decreasing in response to both high temperature and PCB exposure, and no fatty acid shows a significant interaction between temperature and PCB-153.

Discussion

PCB-153 provides a similar stress to high temperature—disruption of membrane order—but is compensated for by different membrane changes than temperature. Cholesterol is a major mode of response to PCB exposure, whereas temperature is handled principally via changes in phospholipid fatty acid unsaturation. Each tissue has its own response pattern, presumably related to pre-existing differences in membrane composition as well as functional needs. The brain's response is particularly intense, involving pervasive fatty acid alterations as well as cholesterol modulation, despite the brain not being known for large homeoviscous changes (Farkas et al., 2001). Muscle and heart show much more limited responses, indicating that these tissues have relatively little capacity for addressing chemically-induced losses of membrane order. Gill shows homeoviscous responses as well, which differ in important ways from the responses reported for this combination of stressors in goldfish (Chapter 3). The trout's response to combined PCB exposure and high temperature show that the homeoviscous response is in keeping with the trout's much greater sensitivity to temperature than the goldfish and revealing that the trout's sensitivity to heat is at least partially related to a reduced ability to modulate membrane composition in response to temperature.

Response to PCB exposure

Membranes are known to respond to fluctuations in ambient temperature by changing phospholipid unsaturation and cholesterol content (Hazel, 1995). These changes are usually homeoviscous, maintaining membrane order and preserving the environment of membrane proteins. Phospholipid unsaturation decreases as temperature rises and vice versa. Cholesterol content increases as the temperature deviates from the gel/liquid crystal phase transition temperature (Crockett, 1998). Many changes in membrane composition, particularly changes in cholesterol concentration, serve other roles, including modulating membrane permeability (Hao et al., 2008) and maintaining the activity of specific proteins that require specific fatty acids in their vicinity (Barenholz, 2002), and more accurately determining which changes serve which roles in this experiment would require additional data, such as

measurements of membrane order. However, the tissue-wide patterns observed here are consistent with the predicted effects of homeoviscous acclimation to membrane fluidization. In some cases, a phospholipid or cholesterol change can be anti-homeoviscous and would exacerbate the stress, but it is compensated for by a large homeoviscous change in the other parameter (Crockett and Hazel, 1995). Changes in phospholipid head groups can also participate in the homeoviscous response, but they are reversed after long-term acclimation and were therefore a poor target for 30-day experiments (Farkas et al., 2001).

The rainbow trout's response to PCB exposure is primarily through modulation of membrane cholesterol and fatty acid chain length rather than fatty acid unsaturation. No tissue shows a change in double bond index in response to PCB exposure, but two (brain and heart) show a PCB-induced reduction in membrane cholesterol; a third (muscle) shows an increase in membrane cholesterol in warm-acclimated fish only, resulting in a significant interaction between the two treatments; and two show PCB-induced reductions in chain length. Conversely, only the gills show a significant effect of temperature on membrane cholesterol and chain length, while all five tissues decrease their double bond index in response to warmth. Interestingly, the direction of cholesterol and chain length changes with PCB exposure and temperature is different in gills. Gill cholesterol increases and chain length decreases with warmth, but PCB exposure elicits changes in the opposite direction in both parameters. Increasing cholesterol with warmth and increasing chain length with PCB exposure are anti-homeoviscous effects offset by changes in double bond index and cholesterol, respectively, which are presumably related to gill-specific functional concerns such as CO_2 permeability and osmoregulation (Itel et al., 2012; Müller et al., 2008; Tsiavaliaris et al., 2015). Notably, however, both brain and muscle feature individual phospholipid fatty acids that increase or decrease in response to PCB exposure, and all tissues but muscle show one or more significant interactions between temperature and PCB exposure affecting the levels of individual phospholipid fatty acids. This shows that, while cholesterol is much more involved in the response to PCB exposure than in the response to temperature, both membrane components are invoked

to respond to PCB-153 in trout. These results show that some ectotherms are capable of protecting their membranes from chemical fluidization, potentially mitigating the disruption of numerous membrane processes. They also show that some membrane changes in response to fluidizing stressors address effects other than fluidization, to the point of being anti-homeoviscous on their own.

Mechanism of homeoviscosity varies between tissues

This study shows that trout are able to adjust their membranes to cope with chemical and thermal stressors, but the mechanism and magnitude of the response vary greatly among tissues. Overall, the primary mechanism of the temperature response is a change in fatty acid unsaturation, whereas it is mainly cholesterol and chain length modulation for the PCB response, with a lesser contribution from phospholipid double bonds. All tissues primarily replace highly unsaturated fatty acids with saturated fatty acids in response to temperature, and some also do so in response to PCB exposure. The exact fatty acids used to respond to temperature or PCBs seem to be unconnected to their individual abundance (common fatty acids are not modulated more often than rare ones; see Tables 4.5-4.10). Instead, particular fatty acids may be selected to meet the specific requirements of local proteins that vary between tissues.

Brain tissue shows a pervasive fatty acid response to both treatments, despite not exhibiting a change in double bond index with PCB exposure, and additionally its membrane cholesterol and chain length show an interactive response to PCB-153. Membrane cholesterol decreases with warmth in sham-injected fish, but shows a trend toward an increase in PCB-injected fish, and similarly, PCB exposure decreases membrane cholesterol in cold-acclimated fish but shows a trend toward an increase in warmth. Although the pairwise comparisons do not reveal significant differences in response in all pairs, the interaction terms demonstrate that combined exposure to PCB-153 and high temperature elicits a different response than either stimulus does on its own. In particular, the role of cholesterol as a means to mitigate the effect of PCB-153 appears to be different in warm brains versus cold ones, in keeping with established patterns (Crockett, 1998). Overall, trout brain membrane lipids are thoroughly restructured in response to 18°C acclimation and PCB-153 injection, despite being shown as relatively unresponsive in other studies

(Farkas et al., 2001). This is most likely related to the critical need to preserve brain membrane function regardless of environmental conditions. Notably, the chain length increase observed in response to PCB exposure is anti-homeoviscous, indicating that membrane thickness is needed to address PCB-related toxicity even as other membrane components are modified to instead maintain membrane order.

Gills are the only tissue to show a clear cholesterol response to temperature as well as PCB exposure, and these responses are opposed to one another. PCB exposure decreases cholesterol, as predicted, but high temperature increases it. This increase is an anti-homeoviscous response that is offset by the gill's fatty acid response, whereas the fatty acids that change with PCB exposure act in concert with membrane cholesterol. Goldfish show the same pattern (Chapter 3), perhaps because of the osmoregulatory function of the gill or the fact that membrane cholesterol increases CO_2 permeability (Itel et al., 2012; Tsiavaliaris et al., 2015). Cholesterol is known to interact directly with Na^+/K^+-ATPase, so reducing phospholipid unsaturation and cholesterol simultaneously may preserve fluidity as well as Na^+/K^+-ATPase activity in a way that a purely homeoviscous response would not (Crockett, 1998; Zehmer and Hazel, 2004). Interestingly, PCB-153 and temperature have an interactive effect on double bond index, with the difference between warm and cold gill membranes being much smaller in PCB-injected groups. This suggests that the anti-homeoviscous effect of increasing chain length with PCB exposure cannot be sufficiently mitigated via changes in unsaturation and that cholesterol, which would otherwise increase, must be reduced to compensate.

Heart cholesterol decreases with PCB exposure and double bond index decreases with high temperature, in keeping with predictions. The phospholipid composition of the heart is, uniquely, totally unaffected by PCB exposure, meaning that membrane cholesterol bears the entire burden of compensating for PCB-induced fluidization in trout hearts. Such a reduced d response is unlikely to be sufficient to maintain membrane order against both heat and PCB-153, even if the heart is unusually resistant to membrane disturbance. This means that the combination of PCB-153 and high temperature is particularly dangerous for the heart, which is the first organ to fail during severe heat stress (Somero, 2004; Somero,

2010) and whose homeoviscous response is critical for maintaining its function during temperature acclimation (Giroud et al., 2013; Iftikar and Hickey, 2013).

Similarly, livers show no response to PCB other than a significant interaction between PCB exposure and high temperature in nervonate, a rare fatty acid, but show a homeoviscous response to temperature. Liver is one of the most strongly responsive tissues in other investigations of homeoviscous responses in trout and other species (Zehmer and Hazel, 2003), but does not respond to PCB-153's fluidizing effect at all. Even at high temperatures, trout liver offers no resistance to or accommodation for PCB-induced membrane order loss, and is vulnerable to disruptions and damage to its membrane proteins (Šimečková et al., 2009).

Refining established patterns, muscle is largely unresponsive to either stimulus. One fatty acid responding to both temperature and PCB exposure appears to be responsible for the muscle's significant, homeoviscous change in double bond index and chain length with temperature. This effect was not sufficient to reveal a similar change in double bond index or chain length with PCB exposure, and muscle cholesterol was likewise unresponsive. Notably, warm PCB-injected fish have significantly higher cholesterol than cold PCB-injected fish (Fig. 4.1E) and the difference in chain length between cold and warm PCB-injected fish is smaller than that between their sham-injected counterparts (Fig. 4.3E), suggesting that other physiological priorities may become important, or the role of cholesterol may reverse, when these stressors combine.

It is possible that differences in membrane cholesterol between tissues are related to different quantities of total membrane or particular intracellular membranes between tissues. Membranes were not separated from one another prior to analysis and phospholipid and cholesterol recovery could not be verified, preventing the accurate determination of a cholesterol:PL molar ratio. There is no evidence that PCB exposure or temperature induces changes in the relative contribution of different organelles to total membranes or to the amount of total membranes present in a cell (Kraffe et al., 2007; Tan et al., 2004).

It is unclear whether such diversity in mechanisms between tissues and between stimuli offers advantages over a single, universal response. Most likely, each response is tailored to the available fatty acids, changing those that provide the fastest path to the needed effect on membrane order, and to the functional needs and limitations of each tissue.

Conclusions

PCB-153 provides a similar stress to high temperature—disruption of membrane order—but is addressed by different membrane changes than temperature. Cholesterol is a major mode of response to PCB exposure, whereas temperature is handled principally via changes in phospholipid fatty acid unsaturation. Both membrane constituents are modulated in response to both stressors in at least some tissues. The response to PCB exposure is, in most cases, indicative of a system pushed to or beyond its limits. Trout show few of the peculiarities of the goldfish studied under similar conditions in Chapter 3 while introducing some of their own. Instead of a reversed cholesterol response or anti-homeoviscous change in liver fatty acids, trout exhibit strongly homeoviscous unsaturation and cholesterol responses to PCB exposure, or do not respond at all. In particular, the strongest response in trout is in their brains, an organ generally regarded as not having a strong homeoviscous response, indicating that the brain has excess capacity for such adjustments which the combination of 18°C and PCB-153 forces it to use. Gill responds in a similar pattern to goldfish gills, mixing homeoviscous and anti-homeoviscous elements. Heart shows a stronger response than muscle, but both are much more limited than the other tissues, comparable to goldfish muscle. Trout muscle shows a significant response to PCB exposure only while at 18°C, indicating that even resilient muscle must respond, albeit in a manner inconsistent with the other tissues. Most strikingly, the liver shows virtually no response to PCB-153, despite being home to the best-studied homeoviscous response and responding strongly in goldfish, best showing that the trout's homeoviscous machinery is overwhelmed by combined PCB-153 and 18°C in a way that goldfish are not. The heart's notable but comparatively small response to concurrent heat and PCB exposure suggests a

mechanism for heart failure as the reason for temperature-induced mortality, as proposed by Somero, but is not conclusive (Somero, 2004; Somero, 2010).

It is worth acknowledging that the degree of compensation for loss of membrane order cannot be conclusively determined with the experiments presented here. The extent to which the membrane responses demonstrated here restore normal membrane order can be determined with direct measurements of membrane order using 1,3,5-diphenylhexatriene (DPH), Fourier-transform infrared (FTIR) spectroscopy, or another method (Katynski et al., 2004). As membrane order is rarely perfectly compensated (Zehmer and Hazel, 2004), this would be a useful future experiment and a more conclusive demonstration of homeoviscous responses to chemical fluidization. It remains true, however, that the responses observed here are consistent with large-scale and multi-organ efforts to restore and maintain membrane order in the face of stressors that would otherwise disrupt it, even if the degree of membrane order compensation cannot be precisely determined.

The trout's sensitivity to temperature means two things: it must adjust its membranes far more than goldfish do for similar levels of membrane order disturbance in order to maintain protein function, and its capacity for doing so is more limited, forcing it to prioritize membrane order over the other priorities that goldfish membranes can afford to exercise in their gills, livers, and brains (Sandermann Jr., 1983; Sandermann Jr., 2002). Future research can confirm these conclusions by examining membrane order directly, rather than relying on proxy measures, to show conclusively the degree to which PCB-perturbed membrane order is not restored in each tissue in both trout and goldfish.

Figures

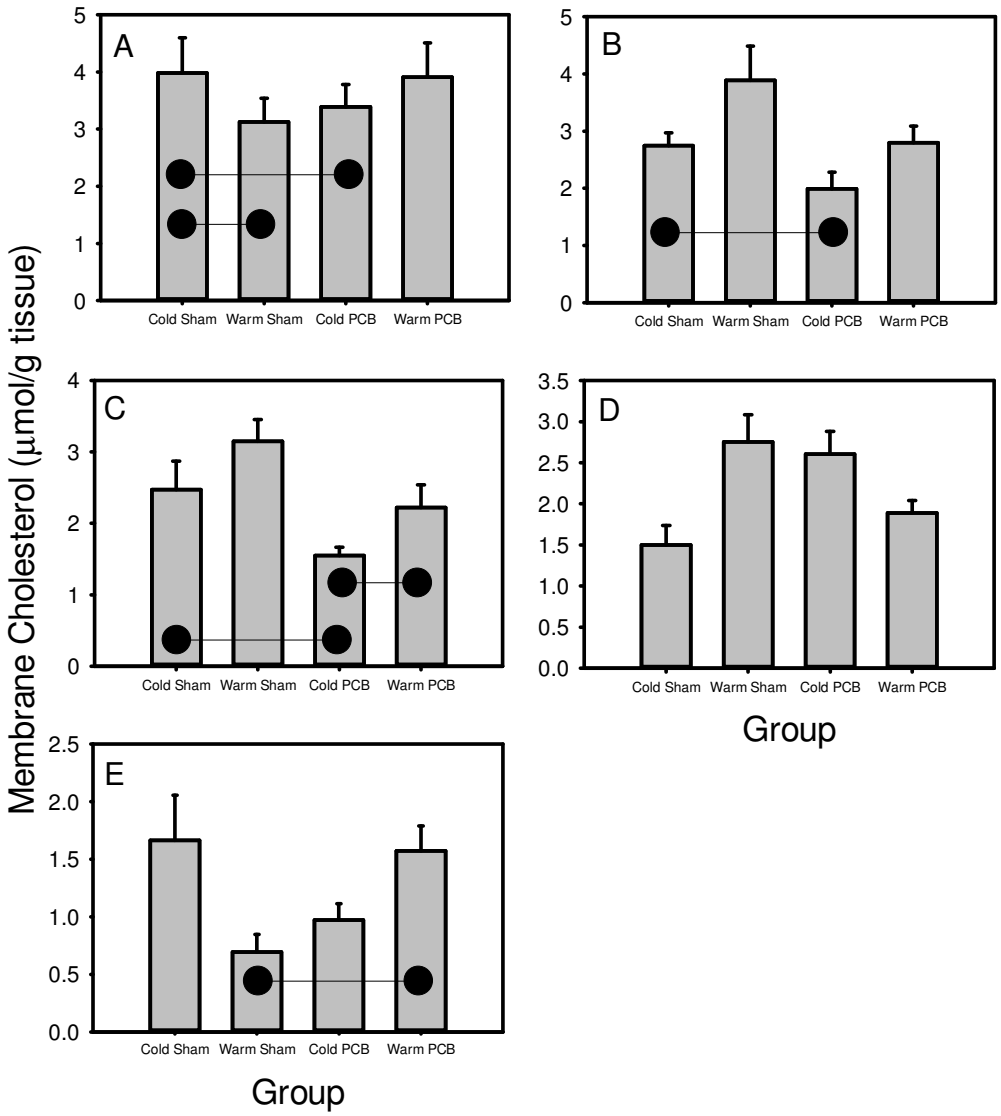

Fig. 4.1. Effects of temperature and PCB-153 exposure on membrane cholesterol concentration (µmol/g tissue) in rainbow trout brain (A), gill (B), heart (C), liver (D), and muscle (E) in four treatment groups. Values are means ± s.e.m. (Brain, gill, heart, and muscle: warm sham n = 12, cold sham n = 17, warm PCB-injected n = 12, cold PCB-injected n = 22. Liver: warm sham n = 12, cold sham n = 17, warm PCB-injected n = 12, cold PCB-injected n = 18.) Pairwise comparisons were performed for temperature (warm vs. cold) within each injection and for injection (PCB vs. sham) within each temperature. Lines connect columns for which pairwise comparisons are significant (Holm-Sidak, $p < 0.05$).

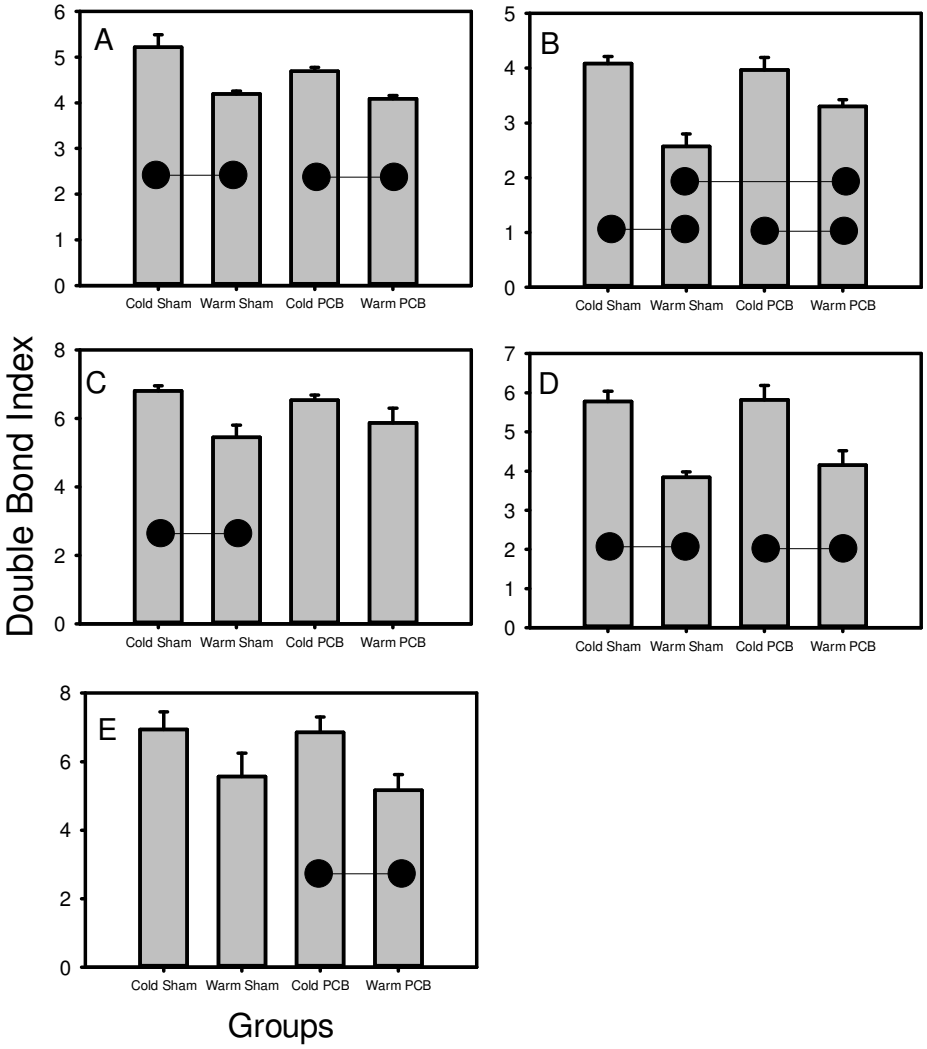

Fig. 4.2. Effects of temperature and PCB-153 exposure on membrane phospholipid double bond index in rainbow trout brain (A), gill (B), heart (C), liver (D), and muscle (E) in four treatment groups. Values are means ± s.e.m. (All tissues: warm sham n = 12, cold sham n = 17, warm PCB-injected n = 12, cold PCB-injected n = 22.) Pairwise comparisons were performed for temperature (warm vs. cold) within each injection and for injection (PCB vs. sham) within each temperature. Lines connect columns for which pairwise comparisons are significant (Holm-Sidak, $p < 0.05$).

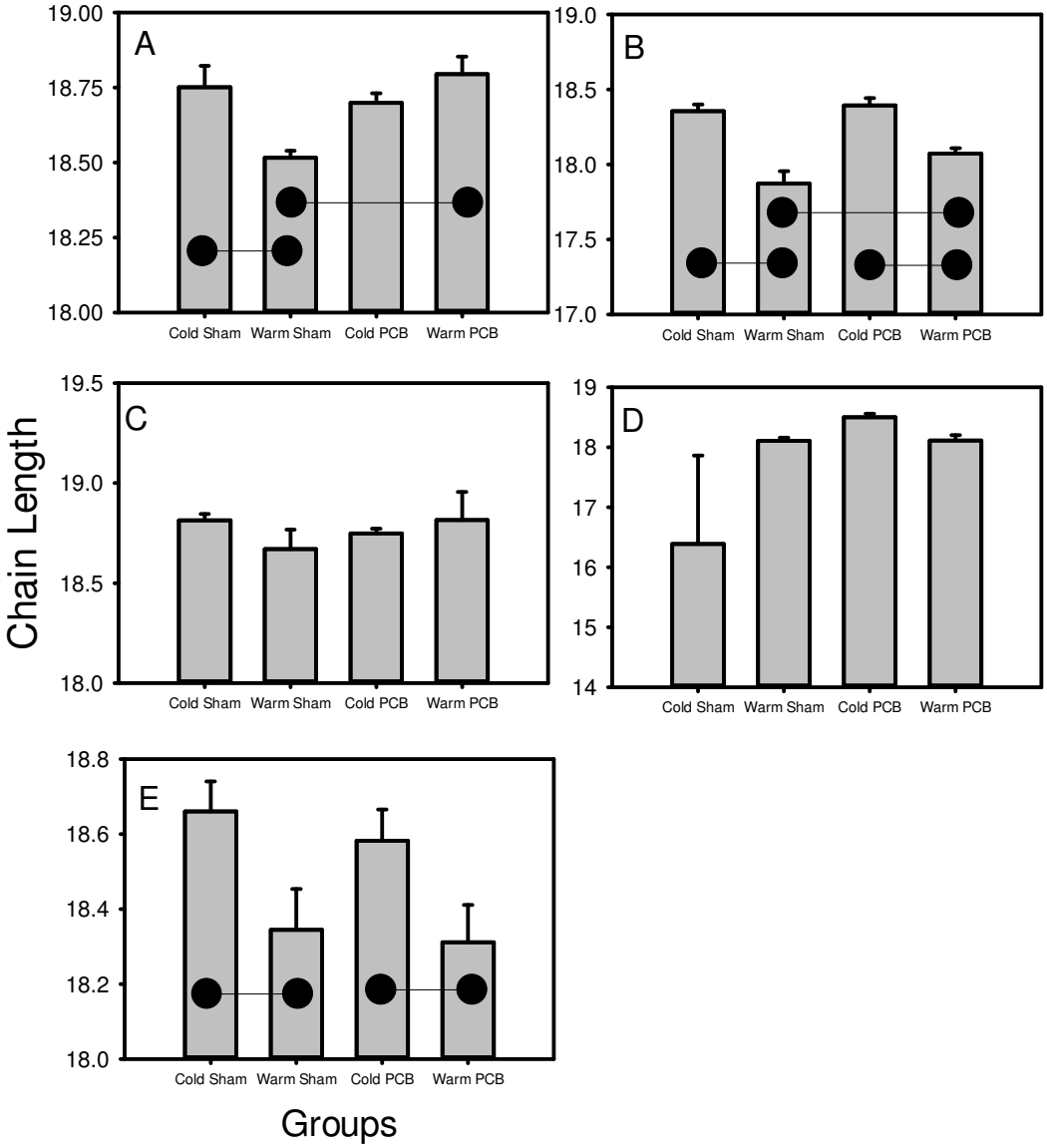

Fig. 4.3. Effects of temperature and PCB-153 exposure on membrane phospholipid fatty acid chain length in rainbow trout brain (A), gill (B), heart (C), liver (D), and muscle (E) in four treatment groups. Values are means ± s.e.m. (All tissues: warm sham n = 12, cold sham n = 17, warm PCB-injected n = 12, cold PCB-injected n = 22.) Pairwise comparisons were performed for temperature (warm vs. cold) within each injection and for injection (PCB vs. sham) within each temperature. Lines connect columns for which pairwise comparisons are significant (Holm-Sidak, $p < 0.05$).

Tables

Table 4.1. p values and effect directions for warmth and PCB exposure on the cholesterol content (µmol/g tissue) of trout membranes. P values derived from separate two-way independent-sample ANOVAs for each tissue. Dir: direction of difference with the cold or sham-injected treatment, respectively.

	Brain		Gill		Heart		Liver		Muscle	
	p	Dir	P	Dir	p	Dir	p	Dir	p	Dir
Warmth	0.267	--	**0.002***	↑	0.053	--	0.526	--	0.923	--
PCB	0.567	--	**0.046***	↓	**<0.001***	↓	0.504	--	0.569	--
Interaction	**0.037***		0.885		0.175		0.078		**0.011***	

Table 4.2. p values and effect directions for warmth and PCB exposure on the double bond index of trout membrane phospholipid fatty acids. P values derived from separate two-way independent-sample ANOVAs for each tissue. Dir, direction of difference with the cold or sham-injected treatment, respectively.

	Brain		Gill		Heart		Liver		Muscle	
	p	Dir	p	Dir	p	Dir	p	Dir	P	Dir
Warmth	**0.001***	↓	**<0.001***	↓	**<0.001***	↓	**<0.001***	↓	**0.007***	↓
PCB	0.074	--	0.148	--	0.770	--	0.607	--	0.0658	--
Interaction	0.223		**0.045***		0.184		0.680		0.776	

Table 4.3. p values and effect directions for warmth and PCB exposure on the chain length of trout membrane phospholipid fatty acids. P values derived from separate two-way independent-sample ANOVAs for each tissue. Dir, direction of difference with the cold or sham-injected treatment, respectively.

	Brain		Gill		Heart		Liver		Muscle	
	p	Dir	p	Dir	p	Dir	p	Dir	P	Dir
Warmth	0.184	**--**	**<0.001***	↓	0.594	**--**	0.431	**--**	**0.003***	↓
PCB	**0.034***	↑	**0.040***	↑	0.582	--	0.211	--	0.556	--
Interaction	**0.002***		0.154		0.144		0.214		0.813	

Table 4.4. Summary of statistical effects on double bond index (DBI), chain length, and cholesterol.

Tissue	Effect of Warmth	Effect of PCB exposure	Interaction
Brain	DBI ↓	CL ↑	CL, Cholesterol
Gill	DBI ↓, CL ↓, Cholesterol ↑	CL ↑, Cholesterol ↓	DBI
Heart	DBI ↓	Cholesterol ↓	
Liver	DBI ↓		
Muscle	DBI ↓, CL ↓		Cholesterol

Table 4.5. Fatty acid composition of membrane phospholipids in the brains of rainbow trout from four treatment groups expressed as molar % of total membrane fatty acids.

	Cold Sham	Warm Sham	Cold PCB	Warm PCB
n	17	12	22	12
Fatty acids				
16:0	19.3 ± 1.0	21.4 ± 0.6	21.5 ± 0.6	23.1 ± 0.5
16:1	2.5 ± 0.2	2.4 ± 0.1	2.8 ± 0.1	3.0 ± 0.1
18:0	8.9 ± 0.3	11.2 ± 0.3	8.2 ± 0.3	8.4 ± 0.3
18:1	38.0 ± 1.1	40.0 ± 1.1	36.0 ± 1.2	31.9 ± 1.6
18:2n-6	2.1 ± 0.2	1.3 ± 0.1	2.2 ± 0.2	-
20:3n-6	1.2 ± 0.1	1.0 ± 0.0	1.2 ± 0.1	1.5 ± 0.1
20:4n-6	1.8 ± 0.2	1.2 ± 0.1	1.4 ± 0.1	1.1 ± 0.0
22:0	4.0 ± 0.2	2.8 ± 0.1	3.8 ± 0.1	3.0 ± 0.1
22:3	1.2 ± 0.1	1.0 ± 0.0	5.1 ± 1.9	17.1 ± 2.2
22:6n-3	17.0 ± 0.9	15.0 ± 0.7	14.5 ± 0.9	8.1 ± 0.5
24:0	-	-	-	1.2 ± 0.2
24:1	3.9 ± 0.2	3.3 ± 0.2	2.1 ± 0.3	1.2 ± 0.3
Total PLFA (µg/g tissue)	9.5 ± 0.8	6.5 ± 0.4	7.8 ± 0.4	8.1 ± 0.8

Values are means ± s.e.m. -, trace component (average < 1% of total). Cold, 5°C. Warm, 20°C. Sham, injected with sunflower oil. PCB, injected with 6 µg/g body mass PCB-153 in sunflower oil.

Table 4.6. Fatty acid composition of membrane phospholipids in the gills of rainbow trout from four treatment groups expressed as molar % of total membrane fatty acids.

	Cold Sham	Warm Sham	Cold PCB	Warm PCB
n	17	12	22	12
Fatty acids				
16:0	29.6 ± 0.5	33.3 ± 0.9	29.0 ± 0.6	31.7 ± 0.5
16:1	5.8 ± 0.3	4.8 ± 0.3	4.7 ± 0.2	5.1 ± 0.1
18:0	5.8 ± 0.3	11.2 ± 1.8	9.2 ± 1.5	7.6 ± 0.4
18:1	21.5 ± 0.3	24.7 ± 1.1	20.8 ± 0.5	24.1 ± 0.4
18:2n-6	5.5 ± 0.1	4.9 ± 0.3	20.5 ± 1.8	5.2 ± 0.1
20:3n-6	4.7 ± 0.1	3.3 ± 0.4	4.4 ± 0.2	3.8 ± 0.1
22:0	5.3 ± 0.2	3.3 ± 0.3	5.1 ± 0.2	3.9 ± 0.1
22:6n-3	20.1 ± 0.6	12.2 ± 1.1	20.2 ± 0.9	16.1 ± 0.6
Total PLFA (µg/g tissue)	2.4 ± 0.2	3.0 ± 0.2	2.3 ± 0.1	2.0 ± 0.2

Values are means ± s.e.m. -, trace component (average < 1% of total). Cold, 5°C. Warm, 20°C. Sham, injected with sunflower oil. PCB, injected with 6 µg/g body mass PCB-153 in sunflower oil.

Table 4.7. Fatty acid composition of membrane phospholipids in the hearts of rainbow trout from four treatment groups expressed as molar % of total membrane fatty acids.

	Cold Sham	Warm Sham	Cold PCB	Warm PCB
N	17	12	22	12
Fatty acids				
16:0	16.4 ± 0.6	20.7 ± 0.7	15.7 ± 0.6	18.8 ± 0.9
16:1	1.4 ± 0.1	1.5 ± 0.2	1.1 ± 0.1	1.6 ± 0.1
18:0	7.9 ± 0.2	8.1 ± 0.3	8.7 ± 0.3	7.7 ± 0.6
18:1	28.2 ± 0.4	27.5 ± 1.1	30.0 ± 0.5	26.6 ± 1.6
18:2n-6	13.4 ± 0.4	11.4 ± 0.6	13.7 ± 0.4	11.3 ± 0.5
18:3n-3	2.3 ± 0.1	1.8 ± 0.1	2.4 ± 0.1	2.0 ± 0.1
20:3n-6	2.8 ± 0.1	2.9 ± 0.1	2.8 ± 0.1	3.2 ± 0.3
22:0	5.2 ± 0.1	5.2 ± 0.1	5.1 ± 0.2	6.6 ± 1.0
22:3	1.1 ± 0.0	1.2 ± 0.1	-	1.3 ± 0.1
22:6n-3	20.8 ± 0.5	18.7 ± 1.6	19.0 ± 0.4	20.0 ± 2.0
Total PLFA (µg/g tissue)	6.9 ± 0.4	6.7 ± 0.4	7.7 ± 0.4	5.9 ± 0.9

Values are means ± s.e.m. -, trace component (average < 1% of total). Cold, 5°C. Warm, 20°C. Sham, injected with sunflower oil. PCB, injected with 6 µg/g body mass PCB-153 in sunflower oil.

Table 4.8. Fatty acid composition of membrane phospholipids in the livers of rainbow trout from the four treatment groups expressed as molar % of total membrane fatty acids.

	Cold Sham	Warm Sham	Cold PCB	Warm PCB
n	17	12	18	12
Fatty acids				
16:0	36.2 ± 1.6	41.5 ± 1.2	35.8 ± 1.2	39.5 ± 2.0
16:1	4.5 ± 0.2	5.1 ± 0.3	4.5 ± 0.2	5.4 ± 0.4
18:0	3.3 ± 0.2	5.0 ± 0.3	3.4 ± 0.2	5.5 ± 0.4
18:1	12.6 ± 0.4	13.3 ± 0.5	13.0 ± 0.4	14.5 ± 0.7
18:2n-6	5.5 ± 0.2	5.2 ± 0.2	5.2 ± 0.2	5.2 ± 0.2
20:4n-6	2.1 ± 0.1	1.4 ± 0.1	2.1 ± 0.2	1.4 ± 0.2
20:5n-3	3.7 ± 0.3	3.0 ± 0.0	3.4 ± 0.3	2.9 ± 0.3
22:6n-3	28.5 ± 0.8	20.0 ± 0.5	28.1 ± 0.7	20.6 ± 0.7
24:1	1.1 ± 0.1	1.3 ± 0.1	1.2 ± 0.0	1.0 ± 0.1
Total PLFA (µg/g tissue)	10.7 ± 0.4	7.7 ± 0.5	10.5 ± 0.5	7.8 ± 0.4

Values are means ± s.e.m. -, trace component (average < 1% of total). Cold, 5°C. Warm, 20°C. Sham, injected with sunflower oil. PCB, injected with 6 µg/g body mass PCB-153 in sunflower oil.

Table 4.9. Fatty acid composition of membrane phospholipids in the muscles of rainbow trout from the four treatment groups expressed as molar % of total membrane fatty acids.

	Cold Sham	Warm Sham	Cold PCB	Warm PCB
n	17	12	22	12
Fatty acids				
16:0	22.1 ± 3.2	28.3 ± 4.2	21.8 ± 2.8	25.4 ± 4.3
16:1	6.1 ± 0.6	2.9 ± 0.6	2.6 ± 0.4	2.8 ± 0.4
18:0	6.1 ± 0.6	7.6 ± 1.7	6.1 ± 0.4	8.2 ± 0.9
18:1	24.6 ± 2.1	22.8 ± 2.8	25.9 ± 2.0	28.0 ± 3.7
18:2n-6	12.0 ± 1.1	9.8 ± 1.3	12.5 ± 1.0	10.5 ± 1.2
18:3n-3	2.0 ± 0.3	1.2 ± 0.3	1.7 ± 0.2	1.2 ± 0.3
20:3n-6	1.3 ± 0.3	-	1.2 ± 0.3	1.1 ± 0.3
20:4n-6	-	1.3 ± 0.3	1.1 ± 0.3	-
20:5n-3	2.7 ± 0.8	3.4 ± 0.8	2.9 ± 0.8	2.1 ± 0.8
22:0	3.8 ± 0.8	1.8 ± 0.8	3.4 ± 0.7	2.5 ± 0.6
22:6n-3	20.5 ± 0.8	18.3 ± 1.1	18.8 ± 0.9	15.8 ± 1.2
Total PLFA (µg/g tissue)	2.8 ± 0.2	3.6 ± 0.9	2.9 ± 0.2	3.2 ± 0.2

Values are means ± s.e.m. -, trace component (average < 1% of total). Cold, 5°C. Warm, 20°C. Sham, injected with sunflower oil. PCB, injected with 6 µg/g body mass PCB-153 in sunflower oil.

Table 4.10. Effect of warmth and PCB exposure on molar percent of individual phospholipid fatty acids in trout membranes. P values derived from separate two-way independent-sample ANOVAs for each fatty acid and tissue using the arcsine of the square root. *Inter*, interaction term. *Direction*, direction of difference with the cold or sham-injected treatment, respectively. Empty cells indicate that a fatty acid was detected at trace levels (<1% of total fatty acids) in that tissue.

		Brain			Gill			Heart			Liver			Muscle		
FA		Warmth	PCB	Inter	Warmth	PCB	Inter	Warmth	PCB	Inter	Warmth	PCB	Inter	Warmth	PCB	Inter
16:0	p	**0.014***	**0.012***	0.693	**<0.001***	0.093	0.0503	**<0.001***	0.082	0.479	**0.006***	0.436	0.608	0.160	0.633	0.724
	Direction	↑	↑		↑	--		↑	--		↑	--		--	--	
16:1	p	0.672	**<0.001***	0.260	0.658	0.469	**0.007***	0.106	0.435	0.108	**0.025***	0.791	0.584	0.431	0.989	0.975
	Direction	--	↑		--	--		--	--		↑	--		--	--	
18:0	p	**<0.001***	**<0.001***	**0.001***	0.050	0.985	**0.005***	0.306	0.751	0.092	**<0.001***	0.309	0.593	0.061	0.562	0.550
	Direction	↑	↓		--	--		--	--		↑	--		--	--	
18:1	p	0.309	**<0.001***	**0.032***	**<0.001***	0.328	0.824	**0.017***	0.705	0.140	**0.035**	0.094	0.394	0.924	0.267	0.533
	Direction	--	↓		↑	--		↓	--		↑	--		--	--	
18:2	p	**<0.001***	**<0.001***	**<0.001***	0.548	0.387	**0.011***	**<0.001***	0.794	0.719	0.440	0.633	0.450	0.080	0.614	0.935
	Direction	↓	↓		--	--		↓	--		--	--		--	--	
18:3	p							**<0.001***	0.140	0.905				0.073	0.801	0.795
	Direction							↓	--					--	--	
20:3	p	0.424	**0.001***	**<0.001***	**<0.001***	0.322	**0.033***	0.060	0.258	0.311				0.343	0.639	0.462
	Direction	--	↑		↓	--		--	--	--				--	--	
20:4	p	**<0.001***	**0.034***	0.077							**<0.001***	0.373	0.459	0.596	0.708	0.257
	Direction	↓	↓								↓	--		--	--	
20:5	p										0.296	0.361	0.985	0.771	0.479	0.268
	Direction										--	--		--	--	
22:0	p	**<0.001***	0.635	0.089	**<0.001***	0.167	**0.027***	0.073	0.125	0.065				0.196	0.567	0.317
	Direction	↓	--		↓	--		--	--					--	--	
22:3	p	**<0.001***	**<0.001***	**<0.001***				**0.033***	0.429	0.109						
	Direction	↑	↓					↑	--							
24:1	p	**<0.001***	**<0.001***	**0.006***							0.965	0.546	**0.038***			
	Direction	↓	↓								--	--				
22:6	p	**<0.001***	**<0.001***	**0.011***	**<0.001***	0.148	**0.045***	0.441	0.788	0.154	**<0.001***	0.877	0.505	**0.014***	**0.039***	0.667
	Direction	↓	↓		↓	--		--	--		↓	--		↓	↓	

CHAPTER 5.
Acclimation to ion-poor water causes remodelling of goldfish membranes

Chapter 5

Acclimation to ion-poor water causes remodelling of goldfish membranes

Based on

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Author Contributions: AG designed this study, performed the exposure, collected the tissues, and analyzed the data. LDF performed the phospholipid extractions and was deeply involved in literature review and interpretation of data. AG and JMW wrote the manuscript.

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Introduction

One of the major functions of cell membranes, in particular those that face water, is osmoregulation. Teleost fish are hyperosmotic to freshwater and hypoosmotic to seawater and in both cases need to prevent their body fluids from equilibrating with the environment. The physiology of ion pumps involved in osmoregulation has been studied extensively (Bourque, 2008). However, the molecular environment of these proteins has received much less attention, even though membrane composition affects permeability to solutes (Hazel and Williams, 1990). Membrane cholesterol and phospholipids are likewise routinely modified in response to environmental stressors (Chapter 3), and membrane composition affects the activity of membrane proteins (Crockett, 1998; Hulbert and Else, 1999; Sandermann Jr, 1983).

Hypoosmotic or ion-poor water conditions are common in nature, despite being studied far less intensively than hyperosmotic conditions (e.g. acclimation of freshwater fish to brackish or sea water). Ion-poor waters are found in ephemeral pools in deserts, streams of the Canadian Shield, rivers in South Asia, the Rio Negro system in Brazil, and many other locales fed primarily by rainwater or where ancient soils have had their minerals depleted over millions of years (Brauner et al., 2012). Hypoosmotic water elicits a characteristic suite of cell- and protein-level responses in fish gills that include a dramatic increase in the surface area of mitochondria-rich-cells (Greco et al., 1996), increased expression of tight-junction protein genes (Chasiotis and Kelly, 2011), and changing which classes of sodium pumps are active (Chasiotis et al., 2009; Ip et al., 2012; Motohashi et al., 2009; Yan et al., 2007). Calcium depletion is a particular concern in these environments (Gorski et al., 2013; Tan et al., 2004; Yang et al., 2005). The lipid composition of fish tissues has been shown to change in response to hyperosmotic conditions, but some studies show decreasing fatty acid saturation (Martínez-Álvarez et al., 2005; Tocher et al., 1995) while others show an increase (Cordier et al., 2002; Hunt et al., 2011). In addition, most studies have examined total tissue lipids, rather than membrane phospholipids or cholesterol specifically (Daikoku et al., 1982; Dantagnan et al., 2007; Khériji et al., 2003). Therefore, they only provide very limited insight

about the potential effects of osmotic stress on membrane composition. Similarly, membrane cholesterol has been shown to increase tolerance for hypoosmotic media and resistance to ion loss in fish cells (Hao et al., 2008; Müller et al., 2008), but the direction of changes in membrane cholesterol in response to membrane-affecting stressors is not universal and, in this case, depends on whether the model is attempting to solidify the membrane to make it less permeable or fluidize it to increase protein activity (Crockett, 1998). Possible changes in membrane fatty acids during acclimation to hypoosmotic conditions remain largely unexplored.

The goal of this study was to examine whether acclimation to hypoosmotic conditions can cause membrane restructuring in freshwater fish. More specifically, my aim was to measure potential changes in the phospholipid fatty acids and cholesterol in the membranes of goldfish gill, intestine, kidney, and white muscle. The goldfish was chosen because of its remarkable ability to tolerate environmental stressors and its native preference for hard-water environments. Muscle was selected as a non-osmoregulatory organ unlikely to show a strong response, to serve as a counterpoint for the three osmoregulatory organs (gill, kidney, and intestine). I hypothesized that phospholipid fatty acid saturation would decrease in response to long-term exposure to ion-poor conditions. Cholesterol is a much less predictable membrane constituent, and whether it will increase to make the membrane less permeable or decrease to make the membrane more fluid is not easily predicted. I also anticipated that the gill, kidney, and intestine would show much stronger responses than muscle, because of their important role in recovering ions that would otherwise be lost to the environment.

Materials and methods

Animals and experimental design

Adult goldfish *Carassius auratus auratus* (Linnaeus) (25.08 ± 13.04 g; n=28) were purchased from Aleongs International (Mississauga, Ontario, Canada) and randomly assigned to two 70-L flow-through tanks containing dechlorinated, well-oxygenated water at 18°C under a 12h:12h light-dark

photoperiod. Fish were from the same batch and therefore the same age. They were fed floating fish pellets (Profishent; Martin Mills; Elmira, Ontario, Canada) daily until satiation. The fish were habituated to these conditions for one month. One of the tanks had standard dechlorinated freshwater as a control (n=14 fish). Beginning at day 0, water subjected to reverse osmosis (RO) was incorporated into the water supply for the ion-poor treatment (n=14). The proportion of incoming water diverted to the RO system and away from the FW source was gradually increased over five days until the water supply consisted exclusively of RO water with a 1/second drip from the freshwater supply. The drip provided a minimal source of ions to prevent mortality. The fish from both groups were euthanized by cervical dislocation 25 days after the osmolarity of the ion-poor water had reached undetectable levels. The gill, intestine, kidney, and white muscle were sampled, quick-frozen in liquid N_2, and stored at -80°C until analyses.

Water quality measurements

Tank water was sampled at days 0-6, 11, 17, 24 and 30. Water was monitored using both a Hach's Model FF-1A Fish Farmer's Water Quality Testing Kit (Hach Company, Loveland, Colorado, USA) as well as an Advanced Instruments Model 3320 osmometer (Norwood, Massachusetts, USA). Total hardness measurements indicated that the freshwater treatment tank remained constant at 102.6 mg/L and the ion-poor treatment exhibited a five-day decline from 102.6mg/mL to the detection limit of 17.1 mg/L. The osmometer results similarly show that the FW treatment remained constant at 7 mOsm/kg. The ion-poor treatment exhibited a five-day decline from 7 mOsm/kg to undetectable levels.

Fatty acid analysis

Total lipids were extracted from ~40 mg of each tissue using chloroform:methanol (2:1 v/v) (Folch et al., 1957). Samples were homogenized (Polytron, Kinematica, Littau, Switzerland) and centrifuged (10 min at 2000 *g*). Supernatants were filtered and 0.25% KCl was added to separate aqueous and organic phases. The aqueous phase was eliminated; the organic phase was evaporated and the lipids resuspended in chloroform before loading on solid-phase extraction columns (Supelclean 3 mL 500 mg LC-NH2; Sigma-Aldrich; St. Louis, MO, USA) to separate the phospholipids. Separation was achieved

by sequential elution of lipid classes—triglycerides (TGs), non-esterified fatty acids (NEFAs), and phospholipids (PLs)—using solvents of increasing polarity: chloroform:isopropanol (3:2 v/v), isopropyl ether:acetic acid (98:2 v/v), and methanol, respectively (Maillet and Weber, 2006). Heptadecanoic acid (17:0; 0.3 mg/mL) was added to each sample as an internal standard (IS) as it is generally absent in animal tissues. The fatty acid composition of PLs was measured after acid transesterification. Fatty acid methyl esters were analyzed on an Agilent Technologies 6890N gas chromatograph (Mississauga, Ontario, Canada) equipped with a split inlet, flame ionization detector and fused silica capillary column (Supelco DB-23, 60 m, 0.25 mm i.d., 0.25 μm film thickness; Sigma-Aldrich) using H_2 as carrier gas, as detailed previously (Magnoni and Weber, 2007). Only the fatty acids accounting for >1% of total fatty acids in membrane phospholipids are reported in this study. Phospholipid recovery could not be calculated.

Membrane cholesterol was measured as non-esterified (free) cholesterol in ~50 mg of tissue. Tissues were homogenized in chloroform:methanol (2:1 v/v). KCl/EDTA (2 M / 5 mM) was added to separate aqueous and organic phases prior to centrifugation (10 min at 2000 *g*). The organic phase was dried, resuspended in 2-methoxyethanol, and stored at -80°C. Cholesterol was measured by fluorometry (SpectraMax Gemini XS, Molecular Devices, Sunnyvale, California, USA) using a commercial assay kit (Cayman Chemical, Ann Arbor, Michigan, USA). This kit was selected because it allows the separate measurement of membrane (free, non-esterified) cholesterol and of cholesterol esters that are only found outside membranes. Cholesterol recovery could not be calculated.

Calculations and statistics

Membrane unsaturation was expressed by the double bond index, calculated as the average number of double bonds divided by the fraction of saturated fatty acids. Data were analyzed using independent-sample t-tests to determine which treatments differed from one another. Statistical analyses were performed using SigmaPlot 12 (Systat, San Jose, CA, USA). All values presented are means ± s.e.m. and a level of significance of $p<0.05$ was used in all tests. Normality of the data was always tested prior

to analyses using the Shapiro-Wilk test, with the Rank Sum test used when normality was not present. All percentages were transformed to the arcsine of their square root before analyses.

Results

Cholesterol

Fig. 5.1 shows the concentration of membrane cholesterol in gill, intestine, kidney, and muscle expressed as µmol per gram of tissue (panel A) and as µmol per µmol phospholipid (panel B). In kidney and muscle, exposure to ion-poor water caused a significant decrease in the concentration of membrane cholesterol ($P<0.05$). In gill and intestine, exposure to ion-poor water had no effect on membrane cholesterol ($P>0.05$).

Double bond index

Fig. 5.2A shows the level of unsaturation of membrane phospholipids in gill, intestine, kidney, and muscle expressed as the double bond index, and Fig. 5.2B shows the chain length. In intestine and muscle, exposure to ion-poor water caused a significant increase in the double bond index ($P<0.05$). In gill and kidney, exposure to ion-poor water had no effect on the double bond index ($P>0.05$). In muscle, exposure to ion-poor water also caused a significant increase in chain length ($P<0.05$), but other tissues were unaffected ($P>0.05$).

Fatty acid composition of membranes

The relative abundance of individual fatty acids in membrane phospholipids of the four tissues is shown in Figs. 5.3-5.6. In gill, ion-poor-water acclimation caused an increase in percent oleate (18:1) and eicosenoate (20:1) and a decrease in percent arachidonate (20:4) ($P<0.05$, Fig. 5.3). In intestine, ion-poor-water acclimation caused a decrease in percent arachidonate and percent behenate (22:0) and an increase in percent eicosenoate (20:1) ($P<0.05$, Fig. 5.4). In kidney, only behenate decreased with ion-poor acclimation ($P<0.05$, Fig. 5.5). In muscle, ion-poor acclimation caused a decrease in percent palmitate

(16:0), palmitoleate (16:1), stearate (18:0), and arachidonate and an increase in eicosenoate and docosahexaenoate (22:6) (Fig. 5.6). All the other membrane fatty acids were not affected by any treatment in any tissue ($P>0.05$). Other indices of fatty acid composition are shown in Table 5.1. In muscle, UFA/SFA and n-3/n-6 are higher in fish exposed to ion-poor water than in control fish ($P<0.05$). In intestine, UFA/SFA and n-3/n-6 are higher in fish exposed to ion-poor water ($P<0.05$).

Discussion

This study demonstrates that key parameters of membrane composition are altered in response to hypoosmotic stress in fish. I show that the membranes of goldfish decrease in saturation and in cholesterol in response to long-term acclimation to hypoosmotic water. I also show that the mechanism and intensity of the response differs between tissues. In kidney, membrane cholesterol greatly decreased, but there was virtually no fatty acid response, with only behenate decreasing. By contrast, intestine showed no cholesterol response, but replaced arachidonate and behenate with eicosenoate. In gill, neither cholesterol nor overall membrane saturation changed in hypoosmotic water, but fatty acid composition still changed, with oleate and eicosenoate replacing arachidonate. Muscle is not directly exposed to the external environment, but muscle membrane cholesterol decreased and muscle palmitate, palmitoleate, stearate, and arachidonate were all replaced with eicosenoate and docosahexaenoate. In keeping with predictions, the overall pattern of membrane fatty acid changes in response to long-term acclimation to hypoosmotic water in all tissues is in the direction of a larger average number of double bonds and decreased membrane order. Rather than increasing saturation to increase membrane order and decrease transcellular permeability, the priority in tissues other than gill is therefore probably to increase the activity of membrane pumps for ion recovery.

Membrane cholesterol consistently decreased in response to hypoosmotic stress, contrary to earlier work in fish cells (Hao et al., 2008; Müller et al., 2008) but consistent with some temperature responses (Crockett and Hazel, 1995). A complicated membrane component, cholesterol serves different

functions in different tissues and often serves as a buffer, increasing membrane order when order is low and decreasing it when it is high (Crockett, 1998). Membrane cholesterol can increase, decrease, or remain unchanged during temperature acclimation in different membranes, indicating that particular membrane stressors do not elicit a consistent cholesterol response across model systems (Crockett and Hazel, 1995; Chapter 3). Isolated cells, particularly spermatozoa used in prior research that have a fixed supply of energy, must prioritize resilience against losing ions to the environment rather than spending energy recovering ions, and therefore increase cholesterol to increase membrane order. Tissues in a whole animal exposed to hypoosmotic water do not have this limitation, and can instead prioritize ion recovery for long-term physiological homeostasis and evolutionary fitness.

The differences in each organ's responses to hypoosmotic conditions indicate that each has a different role in the organism's overall acclimation. The membrane composition of the kidney and intestine both change in ways consistent with the greater ion pump activity observed in those tissues in various fish species, but do so by different mechanisms. Cholesterol decreases in the kidneys, but fatty acid unsaturation increases in the intestine. This most likely serves to retain ions from the bloodstream and dilute the urine (Ip et al., 2012; Motohashi et al., 2009) and to recover ions from the diet (Grosell, 2006), respectively, to make up for losses through other body surfaces. The difference in response may stem from the difference in normal membrane composition. The kidneys have much higher baseline membrane cholesterol and a much higher baseline double bond index than the other three tissues. Under these circumstances, it is easier to reduce membrane order by reducing cholesterol content than by further desaturating the phospholipid fatty acids (Crockett, 1998). Notably, membrane cholesterol reduces membrane permeability (Crockett, 1998), so the cholesterol response in kidney and muscle is a compromise between increasing pump activity and increasing passive ion loss.

The gills' minimal response indicates that ion pump activation is not their primary mode of acclimation. Sodium-potassium pumps do increase in activity during hypoosmotic conditions, but only transiently and only in mitochondria-rich cells, which are a minority of the overall gill surface area (Chasiotis et al., 2012a; Chasiotis et al., 2012b). The primary response to hypoosmotic water in gills must

therefore not be based on membrane fatty acids. Fish gills increase expression of genes associated with tight junction proteins during hypoosmotic stress, which serves to strongly reduce paracellular ion loss and water entry (Chasiotis et al., 2012b). Given that tight-junction proteins do not depend on membrane composition in the same way as ion pumps, the gill can therefore be sealed against losses and avoid much of the permeability-increasing desaturation that characterizes other tissues, leaving ion recovery to the kidney and intestine. This may also serve to prevent disruptions in body fluid pH and respiration rate, as high membrane cholesterol decreases carbon dioxide permeability (Itel et al., 2012; Tsiavaliaris et al., 2015).

The muscle results present a surprise. Muscle shows a much stronger response than any of the other tissues, despite lacking direct contact with the surrounding water. The osmotic stress the muscles experience is due to contact with the fish's body fluids, not to contact with the environment, from which the muscles are sheltered by the rest of the fish's body. This is evidence that the osmoregulatory response of the other three tissues is incomplete and cannot maintain normal osmotic tension in a strongly hypoosmotic environment, as seen in a handful of other fish species (Chasiotis et al., 2009; Ip et al., 2012; Motohashi et al., 2009). An incomplete osmoregulatory response elsewhere means that the muscle is not fully sheltered from ion-poor water and must therefore osmoregulate to protect its internal calcium gradient and prevent loss of function. This suggests that other organs, such as brain or liver, might also change in membrane composition in response to hypoosmotic stress, to likewise protect themselves from hypoosmotic body fluids.

This study demonstrates that hypoosmotic water acclimation, like temperature (Hazel, 1995) and toxin exposure (Chapter 3), is a membrane stressor that leads to changes in membrane composition. Phospholipid fatty acids and cholesterol are both involved, as suggested by earlier work (Hao et al., 2008; Martínez-Álvarez et al., 2005), but are preferentially used in different organs. Intestines decrease membrane saturation to acclimate to ion-poor water, kidneys decrease membrane cholesterol, gills decrease neither, and muscles decrease both. This pattern indicates that the intestine and kidney are both involved in recovering ions from the body fluids (urine and intestinal lumen), but the gill instead

suppresses ion losses. None of these mechanisms are sufficient to maintain normal internal osmolarity against water free of detectable solutes, forcing the muscle to engage in an even more robust membrane response to protect itself from the fish's own body fluids. Future work should combine osmotic stress with temperature or toxin exposure to determine whether one set of membrane changes complicates or neutralizes the other, and should examine other non-osmoregulatory organs to determine whether they behave like muscle during hypoosmotic stress.

Figures

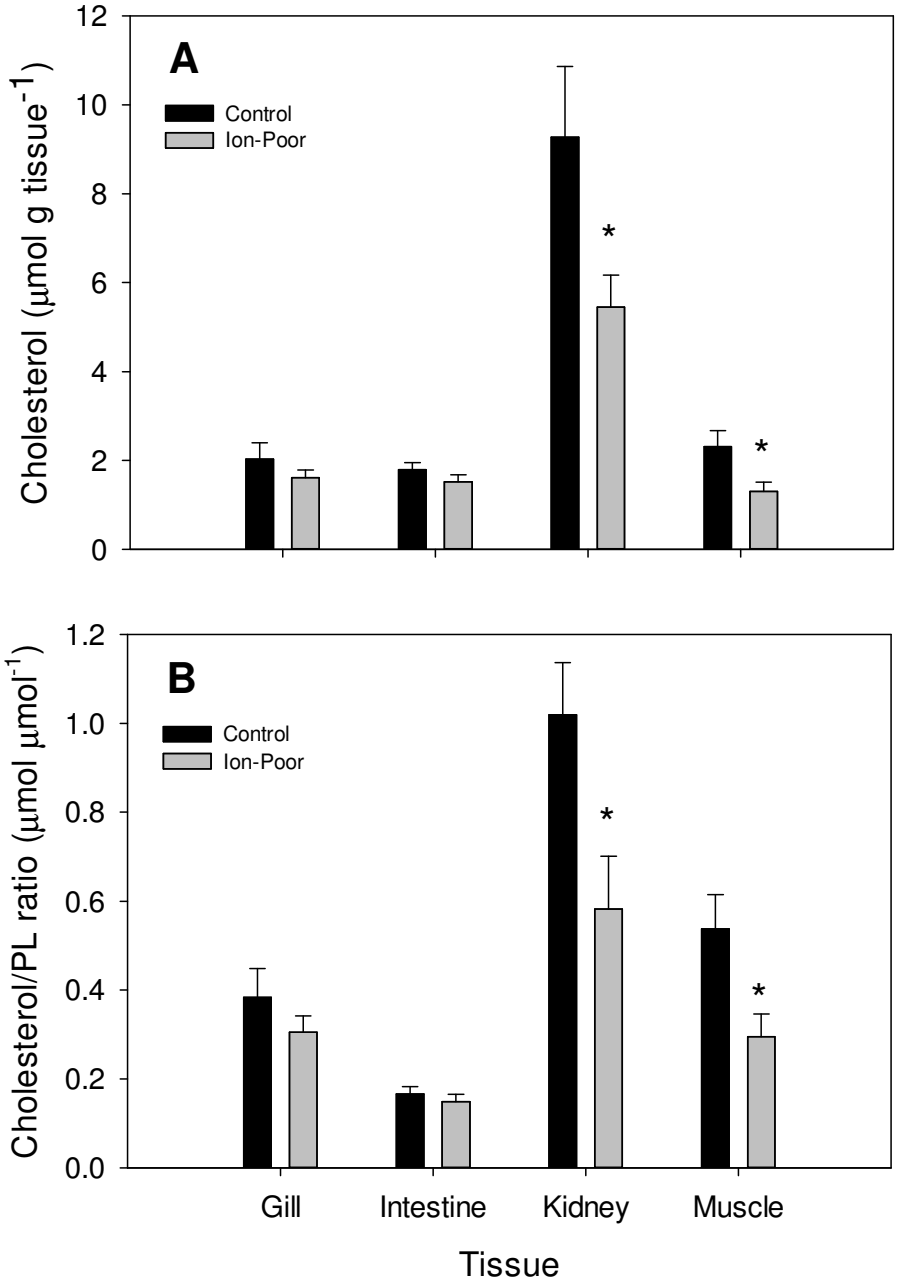

Fig. 5.1. Membrane cholesterol of control and ion-poor-acclimated goldfish in µmol per gram tissue (A) and µmol per µmol phospholipid (B). Values are means ± s.e.m. N = 14 for each group. *P < 0.05.

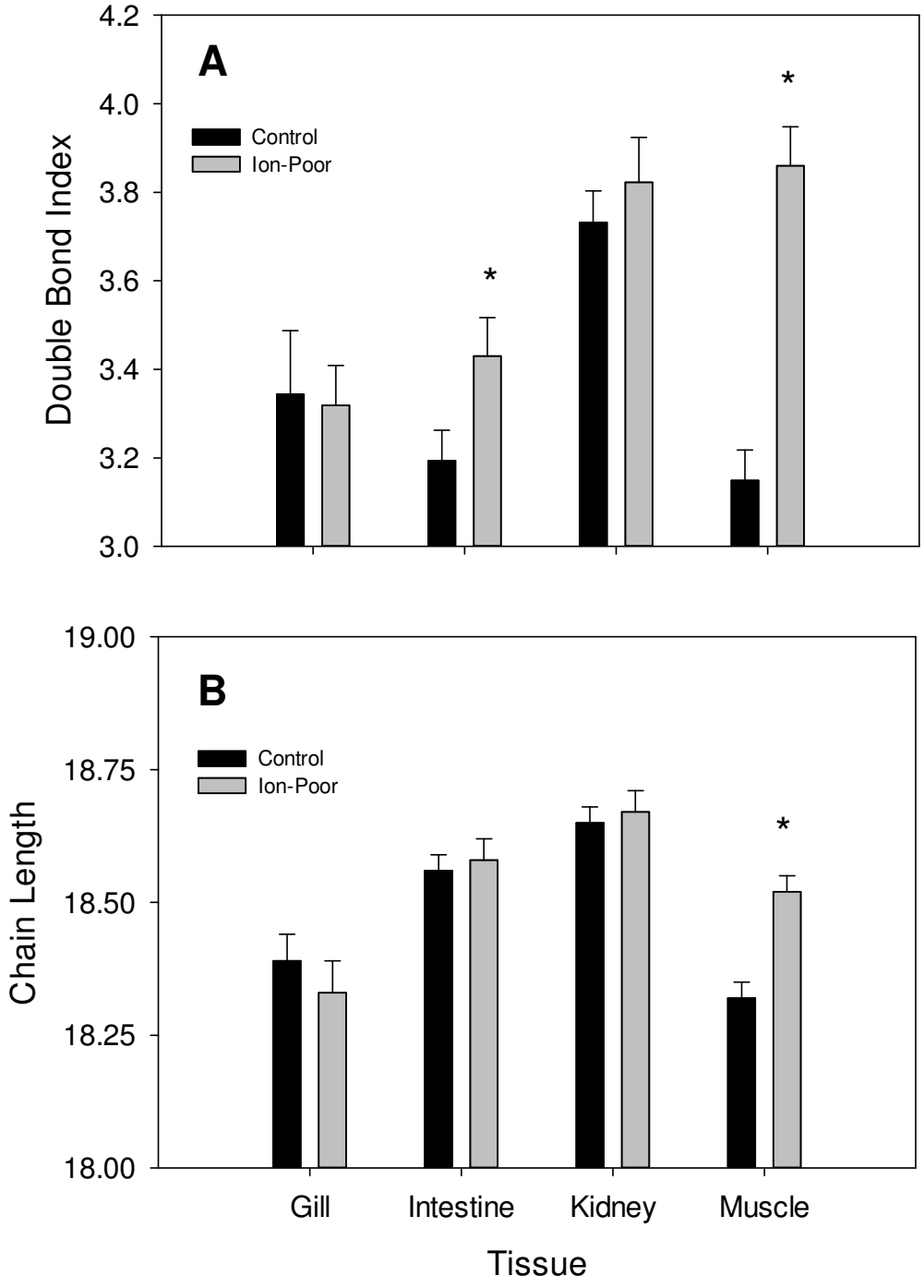

Fig. 5.2. Membrane double bond index (A) and chain length (B) of control and ion-poor-acclimated goldfish. Values are means ± s.e.m. N = 14 for each group. *P < 0.05.

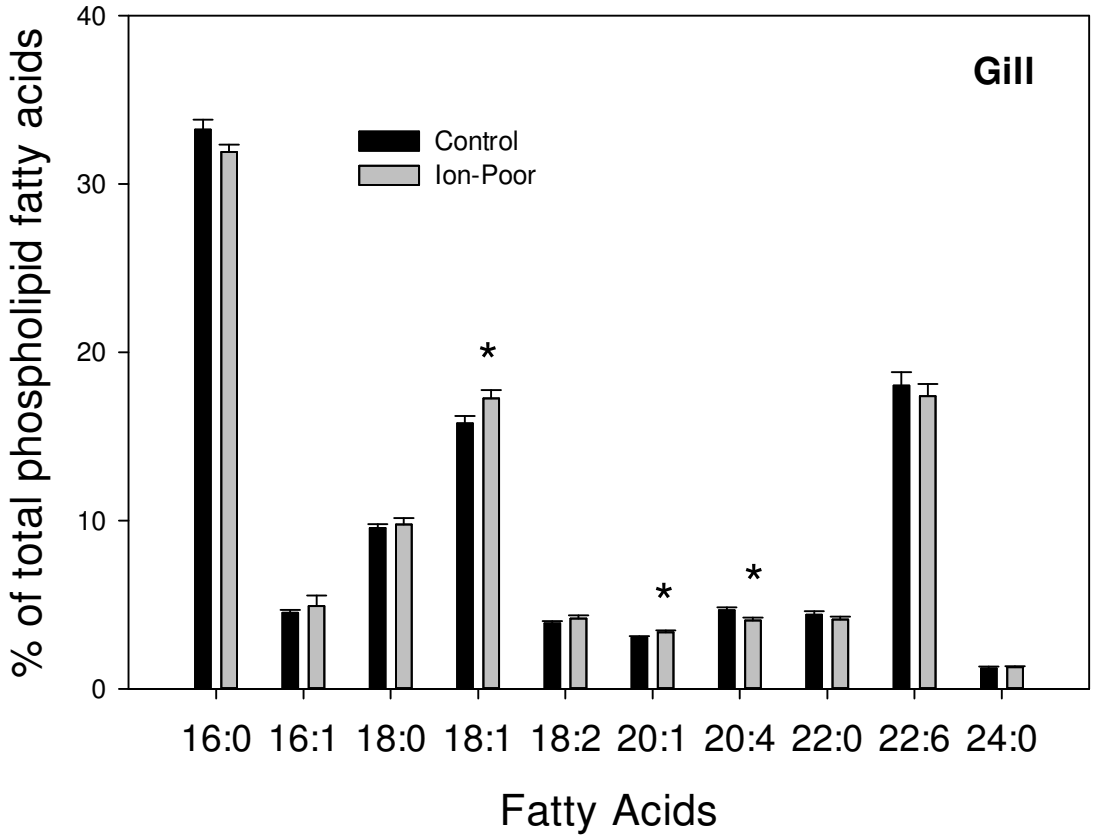

Fig. 5.3. Membrane fatty acid composition of the gills of control (■) and ion-poor-acclimated (■) goldfish in molar percent. Values are means ± s.e.m. N = 14 for each group. *P < 0.05.

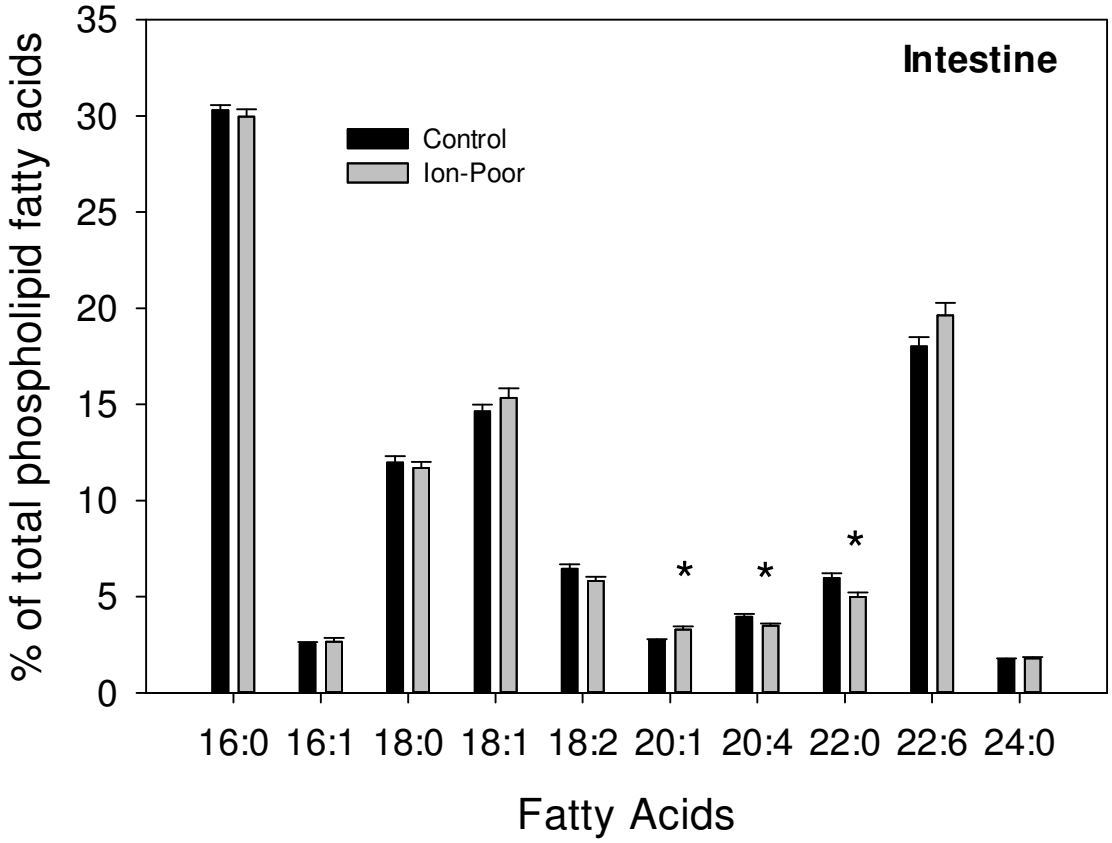

Fig. 5.4. Membrane fatty acid composition of the intestines of control (■) and ion-poor-acclimated (■) goldfish in molar percent. Values are means ± s.e.m. N = 14 for each group. *P < 0.05.

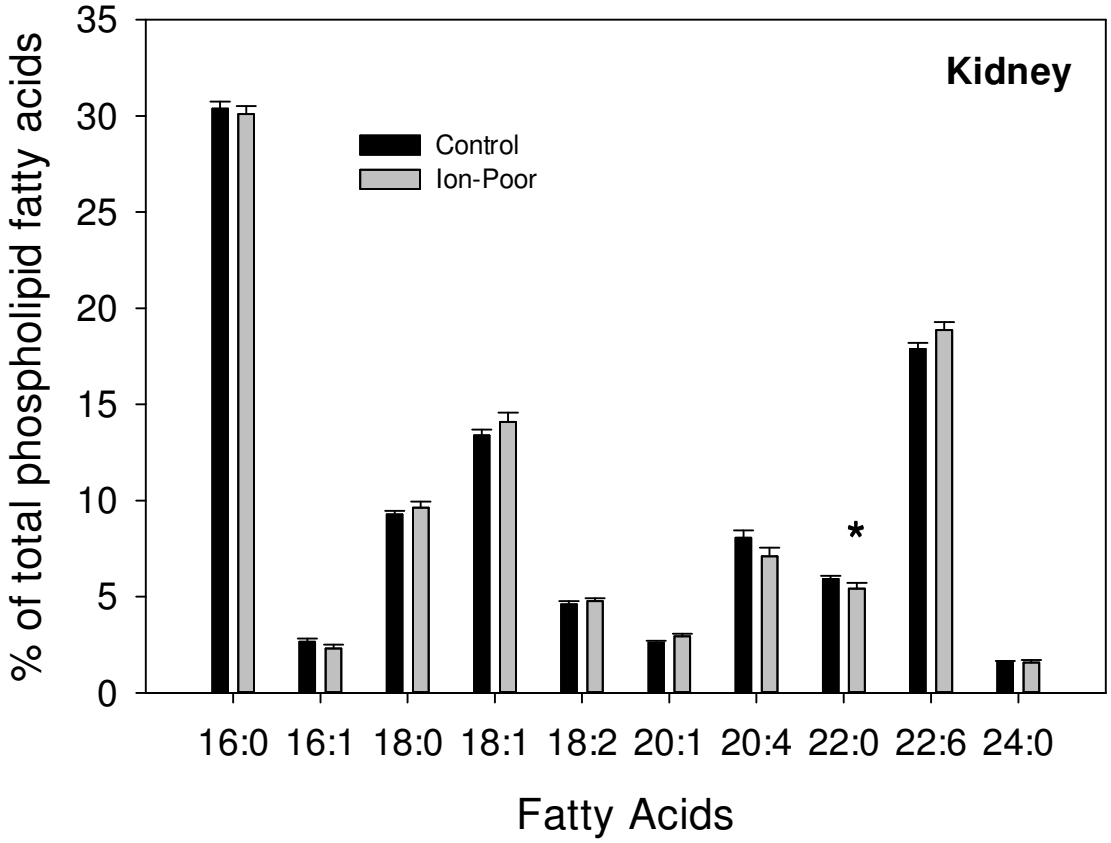

Fig. 5.5. Membrane fatty acid composition of the kidneys of control (■) and ion-poor-acclimated (■) goldfish in molar percent. Values are means ± s.e.m. N = 14 for each group. *P < 0.05.

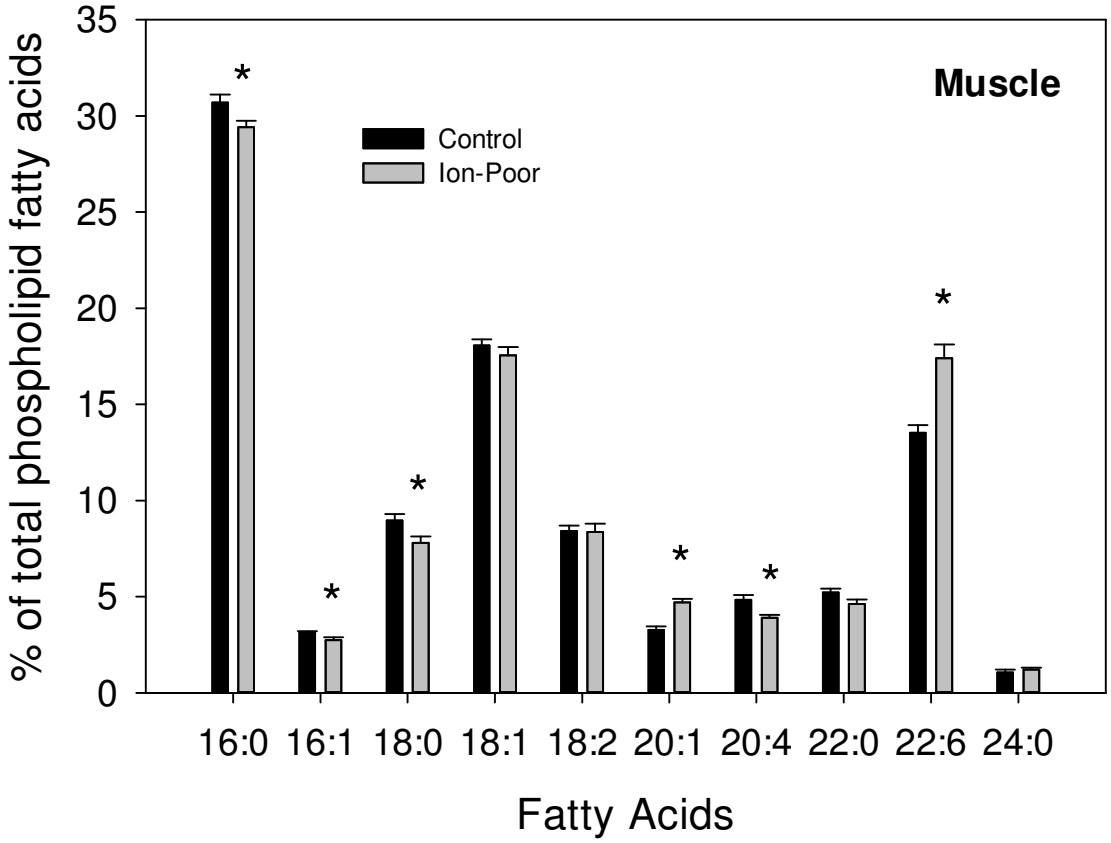

Fig. 5.6. Membrane fatty acid composition of the muscles of control (■) and ion-poor-acclimated (■) goldfish in molar percent. Values are means ± s.e.m. N = 14 for each group. *P < 0.05.

Table

Table 5.1. Indices of membrane fatty acid composition in control and ion-poor-acclimated goldfish. PLFA, phospholipid fatty acids; UFA, unsaturated fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. Values are means±s.e.m. (N=14).

Tissue	Treatment	Total PLFA mass (mg/g tissue)	UFA/SFA	MUFA/PUFA	n-3/n-6
Gill	Control	3.09±0.17	1.06±0.03	0.85±0.04	1.86±0.10
	Ion-poor	3.11±0.13	1.11±0.02	0.99±0.08	1.89±0.10
Intestine	Control	6.50±0.49	0.99±0.02	0.67±0.03	1.60±0.09
	Ion-poor	6.11±0.36	**1.05±0.02**	0.72±0.04	**1.93±0.11**
Kidney	Control	5.67±0.51	1.12±0.02	0.58±0.02	1.19±0.06
	Ion-poor	5.83±0.55	1.14±0.03	0.60±0.04	1.32±0.08
Muscle	Control	2.41±0.10	1.14±0.02	0.86±0.02	0.94±0.04
	Ion-poor	2.64±0.09	**1.31±0.02**	0.79±0.03	**1.27±0.08**

Significant differences between control and ion-poor treatments are indicated in bold ($p<0.05$).

CHAPTER 6.
Conclusions

Overview

A variety of factors and stressors affect membrane composition in ectotherms, and changes in membrane composition feature prominently in many acclimation processes. Similarities and differences in these responses provide insight into common mechanisms of phospholipid modulation as part of acclimation to various stressors and ecological factors. In particular, such similarities will indicate whether the membrane pacemaker concept can serve as a general framework for predicting and understanding membrane composition. Studies were conducted in goldfish (*Carassius auratus*), rainbow trout (*Oncorhynchus mykiss*), and twelve species of wild cyprinid and catostomid fish from southeastern Ontario and southwestern Quebec. The main purpose of this thesis was to investigate the effects of body size, temperature, PCB exposure, and osmotic stress on membrane composition in fish. Specifically, this thesis sought to detect and describe any relationship between membrane composition, size, and phylogeny in cypriniform fish; detect and describe interactions between the homeoviscous response to temperature and any homeoviscous response to PCB-153; compare the response to PCB-153 in more and less thermally sensitive fish; and describe any membrane response to hypoosmotic stress.

To directly test the membrane pacemaker theory's relevance to fish, Chapter 2 is a study of membrane composition and sarco/endoplasmic reticulum calcium ATPase (SERCA) activity from twelve species of wild cypriniform fish. I found the predicted relationship between size and membrane composition, albeit apparently focused on different fatty acids than previously shown in the livers and muscles of mammals (Couture and Hulbert, 1995; Hulbert et al., 2002b), birds (Brand et al., 2003; Hulbert et al., 2002a; Szabó et al., 2010), and rainbow trout (Martin et al., 2013). Phylogeny causes closely related organisms to be more similar to one another independent of size, however, and removing this contribution results in the loss of the relationships between individual fatty acids and size (Fig. 2.6). The membrane pacemaker concept predicts the overall pattern of membrane saturation with increasing size, but not the specific fatty acids involved. I did not detect a relationship between SERCA activity and membrane composition, size, or phylogeny.

In Chapter 3, the effects of PCB-153 exposure were quantified and compared to the effects of temperature in goldfish in a 2×2 factorial design. PCB-153 exposure and temperature both induce homeoviscous responses, demonstrating that the membrane-fluidizing effect known *in vitro* for non-coplanar PCBs also occurs *in vivo* (Bonora et al., 2003; Campbell et al., 2008; Reich et al., 1981). PCB-153 and temperature, however, do not induce the *same* homeoviscous response. Goldfish primarily modulate phospholipids in response to temperature (Fig. 3.3), but respond to PCB exposure in brain and liver by increasing membrane cholesterol (Fig. 3.5). This results in statistically non-interactive responses when heat and PCB-153 occur together, permitting the fish to respond to each stimulus without compromising the response to the other. Additionally, goldfish livers and gills show some anti-homeoviscous membrane changes in response to temperature, decreasing saturation in liver and decreasing cholesterol in gill. This indicates that some membrane changes may cancel each other's effects; that homeoviscous acclimation patterns are species- and tissue-specific; and that membrane changes during temperature acclimation may serve additional priorities as well as maintaining membrane order.

Chapter 4 again examines PCB-153 and temperature, this time in trout. By comparing the response of highly eurythermal goldfish to that of much more sensitive trout, I showed that the importance of cholesterol rather than phospholipids as a major membrane component used to acclimate to PCB exposure is not goldfish-specific. Additionally, trout do not show the reversed cholesterol response or anti-homeoviscous change in liver fatty acids that characterize goldfish. Instead, trout exhibit strongly homeoviscous changes in cholesterol and double bond index in response to PCB exposure, or no response. In particular, the most extensive response in trout is in their brains (Table 4.10), despite the brain's reputation for not exhibiting a strong homeoviscous response. This suggests that, rather than lacking the ability to regulate membrane composition in response to fluidizing stressors, the brain relies on other means to endure or resist membrane perturbation under normal conditions and involves homeoviscous mechanisms only under severe conditions. Effectively, the brain has excess capacity for

such adjustments that the combination of chemical and thermal fluidization forces it to use. Gill responds in a complex pattern of homeoviscous and anti-homeoviscous changes (Figs. 4.1-4.3), rather than the partially anti-homeoviscous pattern seen in goldfish (Figs. 3.3-3.5). Heart shows a stronger response than muscle (Table 4.4), but both are much more limited than the other tissues, comparable to goldfish muscle. Trout muscle shows a significant response to PCB exposure only while at 20°C (Fig. 4.2), indicating that even resilient muscle must respond to this combined stimulus. Most strikingly, trout liver shows virtually no response to PCB-153 (Figs. 4.1, 4.2), despite being home to the best-studied homeoviscous response to temperature and responding strongly in goldfish. Tissues that show small responses to PCB exposure in goldfish show much larger responses in trout, and tissues that show large responses in goldfish show little response in trout, as though the more intense combined stimulus of temperature and PCB exposure pushes unresponsive tissues to protect themselves but cannot induce a larger response in tissues that are already modulating their composition to deal with high temperature. This provides the strongest evidence that the trout's homeoviscous machinery is challenged by combined PCB-153 and temperature acclimation in a way that the goldfish's is not.

Chapter 5 examines an additional stressor, long-term acclimation to hypoosmotic conditions, to characterize its membrane effects. In response to reverse-osmosis water, goldfish gills showed virtually no membrane response (Figs. 5.1, 5.2), while intestines desaturated their membranes (Fig. 5.2), kidneys reduced membrane cholesterol (Fig. 5.1), and muscle showed both effects and additionally increased their chain length (Figs. 5.1, 5.2). These changes increase disorder in membranes, which is associated with higher membrane pump activity and the basis of membrane composition differences in association with temperature, PCB-153, and size. These organs appear to prioritize ion recovery over preventing ion loss. This is despite the kidney's and intestine's ion recovery being ineffective enough that the muscle, not directly exposed to the surrounding medium, must also modify its membranes to recover ions from the blood (Chasiotis et al., 2009; Ip et al., 2012; Motohashi et al., 2009). Effectively, each tissue examined has a unique role in enabling the goldfish to survive extremely low (not detectably different from 0

mOsm) ion concentrations for long periods, and these responses follow similar patterns to those shown during acclimation to temperature and PCB exposure and in association with body size.

Effect of PCB-153

There is a great deal of interest in the effects of polychlorinated biphenyls, particularly those not mediated by the aryl hydrocarbon receptor previously considered organochlorines' primary mode of action. The fluidizing effect of non-dioxin-like PCBs, which are 197 of the 209 PCB congeners, has received growing attention since its discovery (Bonora et al., 2003; Campbell et al., 2008; Reich et al., 1981; Yilmaz et al., 2006) and has the potential to explain many effects of PCB exposure inadequately described by other pathways. Membrane fluidization may also predict new effects, particularly in highly seasonal climates where interaction with homeoviscous acclimation is potentially much more severe. While many changes in membrane composition serve other physiological roles, including modulating membrane permeability (Hao et al., 2008) and maintaining the activity of proteins that require specific fatty acids in their vicinity (Barenholz, 2002), the patterns observed in this thesis indicate that the primary role of the membrane changes induced by these stressors is restoration of membrane order.

I have demonstrated that responses to PCB-induced membrane fluidization are observable *in vivo*. Specifically, I have shown that goldfish and trout exhibit homeoviscous responses to PCB exposure, indicating that PCBs affect membrane fluidity in various tissues in whole organisms (Chapters 3, 4). Homeoviscous responses to PCB exposure further indicate that the intensity of toxic effects induced by PCB-153 is related to the efficacy of the exposed animals' homeoviscous responses. Responses to PCB-153 exposure differ extensively between organs and between species, in keeping with known differences in the extent to which homeoviscous acclimation restores pre-existing membrane order (Hazel et al., 1992; Raynard and Cossins, 1991). Goldfish responses are comparably mild, and include a decrease in membrane unsaturation in gills and an increase in membrane cholesterol in liver and brain (Figs. 3.3, 3.5). Rainbow trout have a much more intense reaction to PCB exposure in most tissues, extensively

modulating brain phospholipids (Fig. 4.3, Table 4.10), decreasing gill and heart cholesterol (Table 4.1), and (counterintuitively) increasing muscle cholesterol in warm fish (Fig. 4.1), but there is no reaction at all in liver. This pattern suggests that the impact of PCB-153 is much higher on thermally-stressed trout than on goldfish at the same temperature, pushing more resilient tissues like brain and muscle to show much stronger responses than they otherwise would and overwhelming any ability to respond in tissues that are already at their limit due to responding to temperature. The extensive changes in the brain proteome of PCB-exposed *Gadus morhua* Atlantic cod (Berg et al., 2011) and interference with the serotinergic system of exposed *Lepomis macrochirus* bluegill (Duffy-Whritenour et al., 2010) may also be best understood as membrane effects. Cod and bluegill are, like rainbow trout, cold-water predators that are likely to be similarly challenged to maintain membrane order and function while contaminated. It therefore stands to reason that PCB-induced membrane fluidization contributes to non-coplanar PCBs' known neurotoxicity (Lilienthal et al., 1990) and may help explain the evidence that the mode of action of certain psychoactive drugs is membrane perturbation (Maruoka et al., 2007). The weak homeoviscous response in heart tissue suggests that failure to maintain membrane order, leading to heart failure, is the major reason for temperature-induced mortality, as proposed by Somero (Somero, 2004; Somero, 2010). Similarly, the total lack of a homeoviscous response to PCB exposure in trout liver, combined with effects on liver functions such as cholesterol metabolism in other organisms (Kato and Yoshida, 1980; Šimečková et al., 2009; Wójtowicz et al., 2005), suggest that widespread metabolic consequences may follow cold-water species exposed to PCB-153 and unable to sufficiently alter their membrane composition to compensate.

The fact that chemical fluidization elicits a homeoviscous response in the tissues of a whole animal has an important implication: that membrane fluidity in fish is sensed and maintained separately from other temperature-sensitive parameters. This is consistent with research in cyanobacteria, which shows that expression of cyanobacterial genes responsible for fatty acid desaturation and phospholipid synthesis is induced during chemical fluidization and hyperosmotic stress (Mikami and Murata, 2003). In

bacteria, evidence suggests that the necessary sensors are histidine kinases (Sakamoto and Murata, 2002; Suzuki et al., 2000), while eukaryotes have transient receptor potential melastatins (TRPMs) that induce thermoregulatory responses and which are partially regulated by membrane thickness, membrane phosphoinositol levels, and associated enzymes (Benedikt et al., 2007; Morenilla-Palao et al., 2009; Yudin and Rohacs, 2012). I have confirmed that fish, and by extension other animals, have a similar ability to respond to PCB-induced membrane fluidization. Further, I have shown that this sensor activates a different suite of downstream responses than bacterial histidine kinases, as PCB exposure frequently does not lead to phospholipid modulation, but rather to cholesterol modulation. This sensor is likely among, or connected to, sterol regulatory-element binding proteins (SREBPs) and in particular SREBP-cleavage-activating protein (SCAP), which are bound to the endoplasmic reticulum membrane in eukaryotes and induce cholesterol synthesis and transport to plasma membranes (Goldstein et al., 2006; Motamed et al., 2011). Some SREBPs are connected to monounsaturated fatty acid synthesis, as well (Pai et al., 1998).

Osmoregulation as a Membrane Stress

In addition to physiological challenges related to salt and water balance, osmotic stresses place membranes under tension as cells swell or shrink (Mikami and Murata, 2003). In turn, cells can detect stretching and respond to it by activating ion pumps (Yang et al., 2005). Organochlorine exposure can disrupt the structure and function of osmoregulatory organs (Costa et al., 2010). Despite these reasons to expect membrane composition to be responsive to osmotic stress, the demonstration of osmotically-induced changes in membrane lipid composition has been surprisingly elusive (Marshall, 2012) and many studies of lipids in the context of osmoregulation have examined total lipids rather than phospholipids, thus limiting their ability to comment on membrane composition (Dantagnan et al., 2007; Hunt et al., 2011; Martínez-Álvarez et al., 2005).

I demonstrated that hypoosmotic stress induces phospholipid and membrane cholesterol changes in goldfish. Goldfish's responses to osmotic stress vary more between organs than responses to temperature or PCB exposure (Chapter 3, 5). Most notably, gill phospholipid fatty acids and cholesterol do not change, despite the gill being the most important and best-known osmoregulatory organ in fish, while kidney, intestine, and muscle respond much more strongly. Temperature and PCB exposure both elicit responses that are relatively similar across tissues, but osmotic stress appears to induce a distinct response in each of the tissues examined. The gill data suggest that goldfish gills do not participate in ion recovery, which would benefit from more fluid membranes that enable higher ion pump activity, but instead gills reduce ion movement to stem losses. Fish tight-junction proteins, which protect against paracellular losses, increase in abundance and expression during acclimation to hypoosmotic water (Chasiotis et al., 2012a). Similarly, the anterior (respiratory) and posterior (osmoregulatory) gills of the *Eriocheir sinensis* mitten crab do not change in composition during acclimation to higher salinity (Chapelle et al., 1976) and Na^+/K^+-ATPase activity decreases in the posterior gills (Chapelle and Zwingelstein, 1984), indicative of an attempt to reduce ion movement across the gill membrane. The estuarine notothenioid fish *Eleginops maclovinus*, likewise, increases Na^+/K^+-ATPase activity primarily in its intestines during acclimation to lower salinity (Vargas-Chacoff et al., 2015). Observing such similar responses in unrelated species suggests that shutting down ion movement without changing membrane composition in some osmoregulatory tissues, and increasing ion movement via changes in membrane composition in others, may be a common osmoregulatory strategy. Desaturating membrane lipids in tissues with enhanced ion pump activity potentially serves another function: to replace fatty acids damaged by reactive oxygen species generated by the increased energy expenditure (Rivera-Ingraham et al., 2015). This suggests that PCB exposure may be of particular concern to creatures simultaneously facing osmotic stress, due to the combined hazard of increased free-radical toxicity and direct membrane perturbation.

Membrane Pacemaker Theory

The membrane pacemaker concept proposes that membrane composition, in particular the relative abundance of polyunsaturated fatty acids, sets metabolic rate via its effects on membrane protein activity (Hulbert and Else, 1999). This concept was originally suggested to connect differences in membrane composition and metabolic rate between species of varying size, based on the positive correlation between levels of docosahexaenoate in phospholipids (DHA; 22:6) and the activity of Na^+/K^+-ATPase (Turner et al., 2005) and calcium-ATPase (Haag et al., 2003; Kearns and Haag, 2002). However, the membrane constituents that vary with body size in mammals and birds are among the constituents affected by various acclimation responses, and these processes serve to maintain the same membrane functions ostensibly preserved by allometric variation in membrane composition (Hazel, 1995). Confirming similarity between allometric and acclimation patterns in membrane composition would show that the membrane pacemaker concept, in addition to providing insight into the challenges posed by body size, can provide a useful framework for understanding metabolism and membrane composition in other contexts.

I have shown that the specific predictions made by the membrane pacemaker theory—that oleate (fatty acid 18:1) increases and docosahexaenoate decreases with body size and that this relates to the activity of prominent membrane proteins—do not hold in cypriniform fish. The specific fatty acids for which I detected an allometric pattern differ from the two identified in birds and mammals; vary between tissues; and reveal phylogenetic rather than allometric patterns, becoming non-significant when corrected for relatedness (Figs. 2.2, 2.3. 2.6). Further, the activity of the important ion pump calcium-ATPase (SERCA) has no relationship to membrane composition or body size in cypriniforms (Fig. 2.5). The signal originally found in mammals has likewise been revealed as an artifact (Valencak and Ruf, 2007). However, overall membrane composition in cypriniforms becomes less unsaturated with size (Fig. 2.2) independent of phylogeny and in keeping with the membrane pacemaker concept's core prediction. The membrane pacemaker concept does not accurately predict the particular cypriniform fatty acids whose

variation reduces membrane order as size increases, nor is membrane composition the main determinant of cypriniform SERCA activity, but membrane composition nevertheless shows a clear relationship with size consistent with the allometric pattern (Clarke and Johnston, 1999) found in fish metabolic rates.

The experimental treatments tested in Chapters 3 through 5 (PCB-153 exposure, a 15°C increase in temperature, and hypoosmotic water) have remarkably similar effects on membrane composition, as shown in Table 6.1. Although PCB-153 rarely induces enough phospholipid modulation to cause a change in double bond index, the fatty acids that respond to PCB-153 exposure are, consistently, a similar assortment to those that respond to temperature and to hypoosmotic conditions in the same tissues. In turn, in the two goldfish tissues for which all three treatments were performed, hypoosmotic stress induces the inverse of the response to high temperature, making hypoosmotic stress effectively analogous to cooling in many respects. The fatty acids identified as varying allometrically in cypriniforms (phylogenetic correction notwithstanding) are extensively represented among those that change with temperature, PCB exposure, and/or hypoosmotic water in goldfish and trout. Increasing size, PCB exposure, and raised temperature almost always induce relative fatty acid changes in the same direction, opposite those induced by hypoosmotic conditions. Despite overwhelming focus on docosahexaenoate in particular as the membrane fatty acid whose rotational motion affects ion pump activity (Candelario and Chachisvilis, 2013; Giroud et al., 2013; Guderley et al., 2008; Haag et al., 2003; Kearns and Haag, 2002; Maixent et al., 2014), DHA is not necessarily modulated in all tissues or in response to all membrane stressors. Rather, a broader series of lipids are increased and decreased to achieve an overall effect on membrane properties. While these relationships do not all hold in any given tissue and the lack of complete symmetry between the experiments makes some comparisons difficult, the overall pattern strongly indicates that the membrane pacemaker concept provides a useful framework for predicting and understanding membrane composition in fish, including during acclimation to membrane-altering stressors. This is particularly so for the double bond index and similar means of combining the contributions of individual fatty acids into a single measure, which removes idiosyncratic fatty acids and

phylogeny as potential confounding factors. It is important to note that, although the membrane pacemaker concept makes no predictions about membrane cholesterol (Hulbert and Else, 1999), cholesterol is a major part of the responses to PCB exposure and osmotic stress in particular, and the utility of the membrane pacemaker concept in predicting membrane behaviour would be greatly increased if cholesterol could be integrated into it.

Future Directions

The pressures that lead to changes in membrane composition interact with each other in a number of ways. Phospholipids and cholesterol respond to body size, osmotic stress, toxin exposure, and temperature in comparable ways that involve the same membrane constituents. This suggests a number of future studies that can build on the findings of this thesis.

Phospholipid fatty acids have a more limited role in determining metabolic rate than previously proposed, showing their previously noted relationship with body size (and therefore with metabolic rate) only when combined into an index, rather than examined individually. Conversely, I have found that cholesterol is a much more important part of homeoviscous acclimation, and particularly the response to PCB exposure and hypoosmotic water, than previously suggested. Cholesterol is frequently modulated in situations in which phospholipid composition is nearly constant and is a prominent, sometimes opposed force in membrane composition even when phospholipids also respond (Chapters 3, 4, 5). It is possible that, while the specific fatty acids that respond to membrane stressors are phylogenetically idiosyncratic, cholesterol participates in a more universal pattern. Examining cholesterol in the context of the membrane pacemaker concept, in particular in fish, would bolster this contention. Integrating cholesterol into a broadened membrane pacemaker concept would increase the predictive power of this concept, or challenge it, particularly in light of the many other physiological roles membrane cholesterol plays.

The shared mechanisms of response between highly varied membrane stressors suggest that these stressors are particularly deleterious in combination. Goldfish respond capably to PCB-153 exposure and

a 15°C temperature difference, but trout show signs of finding this combination overwhelming (Chapters 3, 4). PCB-153 may synergize similarly with osmotic stress. Some crustaceans can osmoregulate in the presence of small quantities of PCBs (Roesljadi et al., 1976), but larger quantities cause massive disruption of osmoregulatory organs in fish (Costa et al., 2010). Given the particular similarities between the changes in membrane composition induced by osmotic stress and those induced by PCB exposure, examining the two in concert would provide additional evidence that membrane stressors can combine to a point that an animal can no longer compensate for their combined burden. This combination would be particularly helpful to examine via direct measurements of membrane order using 1,3,5-diphenylhexatriene (DPH), Fourier-transform infrared (FTIR) spectroscopy, or another method (Katynski et al., 2004). Such measurements could precisely determine the degree of compensation attained for each membrane stressor and their combinations in ways that observing membrane composition itself can only approximate, given that membrane order is rarely perfectly compensated (Zehmer and Hazel, 2004).

With the whole-membrane effects of fluidizing stressors established here, two additional avenues of research become available. Some membranes are larger participants in overall homeoviscous acclimation or allometry than others (Brand et al., 1991; Cossins et al., 1978; Glémet et al., 1997; Szabó et al., 2010), and cholesterol content varies enormously between subcellular compartment membranes (Crockett, 1998). Separating membrane fractions to determine whether they show allometric patterns or responses to temperature, PCB-153, or hypoosmotic water, or similar stresses, will add specificity to the results found here and may connect the membrane pacemaker theory of metabolism to a narrower subset of membranes than previously proposed. This information may be useful for designing future studies that examine the enzymes, transcripts, and genes responsible for maintaining membrane composition and mediating changes thereof, including desaturases, elongases, phosphatidylethanolamine N-methyltransferase, sterol regulatory-element binding proteins, and SREBP-cleavage-activating protein, in the context of these stressors. Further, linking the overall patterns of membrane difference discovered in this thesis to particular membrane fractions would also provide insight into the functions and mechanisms

of enzymes that detect membrane order disturbances, including histidine kinases (Sakamoto and Murata, 2002; Suzuki et al., 2000) and transient receptor potential melastatins (Benedikt et al., 2007; Morenilla-Palao et al., 2009; Yudin and Rohacs, 2012). The membrane order sensor that leads to membrane cholesterol modulation in particular remains uncharacterized, and may best be sought in analyses that exclude cholesterol-poor subcellular fractions.

I have also shown another use for PCB-153. Membrane fluidity can be disrupted *in vitro* by any of various chemicals, whether as a toxic effect or as an intentional manipulation (Friedlander et al., 1987; Lopez-Aparicio et al., 1994; Maruoka et al., 2007; Müller et al., 2008; Yang et al., 2000). PCB-153 is, instead, a membrane fluidizer that induces *in vivo* responses. This chemical therefore shows promise as a means to manipulate membrane order in whole, living organisms without inducing the array of other responses invoked during thermal acclimation, diet modification, osmotic stress, and other means of altering whole-animal membrane composition (Cossins et al., 2002; Guderley et al., 2008). If membrane order can affect metabolic rate, PCB-153 and other fluidizing agents should provide ways to manipulate the metabolic rate of creatures exposed to it. PCB-153 is likely to have other consequences for a whole organism, including biochemical efforts to purge it and free-radical poisoning (Zhou and Zhang, 2005) and its membrane-fluidizing property has not been subjected to dose-response analysis. These consequences will require understanding before PCB-153 can become a tool for membrane perturbation. Access to an in-vivo chemical fluidizing agent with known dose-response relationships also means being able to probe the molecular basis for fluidity-induced membrane composition changes in a whole animal, which has rarely been attempted (Morenilla-Palao et al., 2009) and which may prove particularly helpful in characterizing the regulation of membrane cholesterol in response to fluidizing stressors (Goldstein et al., 2006; Motamed et al., 2011; Pai et al., 1998).

General Conclusions

Membranes are crucial to numerous biological functions, including the creation of concentration gradients, the collection of raw materials, and protecting genetic material from harm. Membranes are composed primarily of lipids, such as phospholipids and cholesterol, which affect the activity of the membrane proteins that perform these functions. Membrane functions constitute the bulk of the cellular activity summarized as metabolic rate, linking membrane composition to whole-animal effects. This thesis investigates body mass (a proxy for metabolic rate), temperature acclimation, PCB exposure, and osmotic stress as factors that affect membrane composition, seeking evidence that these physiological challenges are addressed in similar ways. Experimental evidence demonstrates that overall membrane unsaturation decreases with body size in cypriniform muscles and livers, but the particular fatty acids that comprise this effect differ from those previously demonstrated in mammals and birds and are dependent on phylogeny rather than being universal across species. Further, membrane unsaturation almost always decreases in response to increasing temperature in both trout and goldfish, and increases with hypoosmotic stress in two of four goldfish tissues examined. PCB exposure also induces phospholipid fatty acid responses that are usually consistent with compensating for membrane fluidization, but more often, instead induces increases in membrane cholesterol, and a decrease in membrane cholesterol is a prominent response to hypoosmotic stress in goldfish kidney and muscle. These results all demonstrate that temperature, osmotic stress, PCB exposure, and increasing body size across taxa are all addressed via a similar set of membrane responses in fish, which fit with what the membrane pacemaker theory predicts regarding membrane composition, metabolic rate, and size. Further, they show that some goldfish tissues assist in hypoosmotic acclimation by shutting down ion movement and others participate by actively recovering ions from body fluids to prevent ion loss—in the case of muscle, from the fish's own blood due to the incompleteness of the overall response.

This thesis has also shown that the homeoviscous response of fish varies in strength between more and less thermally sensitive fish. In response to combined PCB exposure and acclimation to 20°C,

goldfish decrease phospholipid unsaturation in gill and cholesterol in liver and brain, showing virtually no interaction between the two stimuli and relatively few individual fatty acids contributing to the change in membrane unsaturation. Goldfish muscle does not respond to PCB exposure. Notably, goldfish gills and liver have partially anti-homeoviscous responses to temperature, decreasing cholesterol and increasing unsaturation and chain length in the service of some other membrane priority. Trout, by contrast, modulate most of the fatty acids identified in their brains in response to PCB exposure, show reduced responses in white muscle and heart, show no liver response to PCB exposure, and show effects that compensate for sub-responses that would be anti-homeoviscous on their own. Additionally, many responses in trout show statistical interaction between temperature and PCB exposure. This reveals that the trout need to activate "reserve capacity" for homeoviscous acclimation in the brain and muscle that can remain dormant in the more eurythermal goldfish in response to a similar stressor, and that trout livers are already at their limit in responding to temperature and cannot respond to simultaneous PCB exposure. These findings provide insight into the regulation of membrane composition in fish by demonstrating that the same membrane parameters are modulated in similar ways in response to various membrane stressors in various tissues. This unified mechanism, further explored, will further clarify and illuminate the role of membranes in stress responses and enable predictions of how other stressors, including other membrane-altering pollutants, might interact to create new hazards for fragile aquatic biomes.

Table 6.1. Summary of effects of experimental treatments on cholesterol and phospholipid fatty acids in Chapters 2-5. DBI, double bond index. Chol, cholesterol. CL, chain length.

Species and organ	Factor	Effect on cholesterol and phospholipids
Goldfish brain	5°C→20°C	DBI ↓, 18:0 ↑, 20:1 ↓, 20:4 ↓
	PCB-153	Chol ↑
Trout brain[1]	5°C→20°C	DBI ↓, 16:0 ↑, 18:0 ↑, 18:2 ↓, 20:4 ↓, 22:0 ↓, 22:3 ↑, 24:1 ↓, 22:6 ↓
	PCB-153	CL ↑, 16:0 ↑, 16:1 ↑, 18:0 ↓, 18:1 ↓, 18:2 ↓, 20:3 ↑, 20:4 ↓, 22:3 ↓, 24:1 ↓, 22:6 ↓
Goldfish gill	5°C→20°C	Chol ↓, DBI ↓, CL ↓, 16:1 ↑, 20:2 ↓
	PCB-153	DBI ↓, 20:2 ↓
	Hypoosmotic water	18:1 ↑, 20:2 ↑, 20:4 ↓
Trout gill[1]	5°C→20°C	DBI ↓, CL ↓, Chol ↑, 16:0 ↑, 18:1 ↑, 20:3 ↓, 22:0 ↓, 22:6 ↓
	PCB-153	CL ↑, Chol ↓
Trout heart	5°C→20°C	DBI ↓, 16:0 ↑, 18:1 ↓, 18:2 ↓, 18:3 ↓, 22:3 ↑
	PCB-153	Chol ↓
Goldfish intestine	Hypoosmotic water	DBI ↑, 20:1 ↑, 20:4 ↑, 22:0 ↓
Goldfish kidney	Hypoosmotic water	Chol ↓, 22:0 ↓
Cypriniform liver[2]	Size	DBI ↓, 16:1 ↓, 18:1 ↓, 18:2 ↓, 18:0 ↑
Goldfish liver	5°C→20°C	DBI ↑, CL ↑, 16:0 ↑, 16:1 ↓, 18:0 ↓, 18:1 ↓, 18:2 ↓,20:1 ↓, 22:6 ↑
	PCB-153	Chol ↑, 16:1 ↓, 20:4 ↑
Trout liver[1]	5°C→20°C	DBI ↓, 16:0 ↑, 16:1 ↑, 18:0 ↑, 18:1 ↑, 20:4 ↓, 22:5 ↑, 22:6 ↓
	PCB-153	--
Cypriniform muscle[2]	Size	DBI ↓, 16:0 ↑, 22:6 ↓
Goldfish muscle	5°C→20°C	DBI ↓, 16:0 ↑, 20:2 ↓
	PCB-153	--
	Hypoosmotic water	Chol ↓, DBI ↑, CL ↑, 16:0 ↓, 16:1 ↓, 18:0 ↓, 20:1 ↑, 20:4 ↓, 22:6 ↑
Trout muscle[1]	5°C→20°C	DBI ↓, CL ↓, 22:6 ↓
	PCB-153	22:6 ↓

[1]Significant interactions between warmth and PCB-153 present but not shown; see Chapter 4.
[2]Specific fatty acid effects shown to be phylogenetic in origin; see Chapter 2.

WORKS CITED

Abdolahpur Monikh, F., Karami, O., Hosseini, M., Karami, N., Bastami, A. A. and Ghasemi, A. F. (2013). The effect of primary producers of experimental aquatic food chains on mercury and PCB153 biomagnification. *Ecotoxicology and Environmental Safety* **94**, 112-115.

Akahane, M., Matsumoto, S., Kanagawa, Y., Mitoma, C., Uchi, H., Yoshimura, T., Furue, M. and Imamura, T. (2015). Comparison of the prevalence of symptoms and medical histories between Yusho patients and healthy controls. *Fukuoka igaku zasshi = Hukuoka acta medica* **106**, 85-118.

Aliche-Djoudi, F., Podechard, N., Collin, A., Chevanne, M., Provost, E., Poul, M., Le Hégarat, L., Catheline, D., Legrand, P., Dimanche-Boitrel, M. T. et al. (2013). A role for lipid rafts in the protection afforded by docosahexaenoic acid against ethanol toxicity in primary rat hepatocytes. *Food and Chemical Toxicology* **60**, 286-296.

Amemiya, C. T., Alfoldi, J., Lee, A. P., Fan, S., Philippe, H., MacCallum, I., Braasch, I., Manousaki, T., Schneider, I., Rohner, N. et al. (2013). The African coelacanth genome provides insights into tetrapod evolution. *Nature* **496**, 311-316.

Andersson, P. L., Berg, A. H., Bjerselius, R., Norrgren, L., Olsén, H., Olsson, P. E., Örn, S. and Tysklind, M. (2001). Bioaccumulation of selected PCBs in zebrafish, three-spined stickleback, and arctic char after three different routes of exposure. *Archives of Environmental Contamination and Toxicology* **40**, 519-530.

Armitage, J. M., Choi, S. D., Meyer, T., Brown, T. N. and Wania, F. (2013). Exploring the role of shelf sediments in the arctic ocean in determining the arctic contamination potential of neutral organic contaminants. *Environmental Science and Technology* **47**, 923-931.

Armstrong, C., Thomas, R. H., Price, E. R., Guglielmo, C. G. and Staples, J. F. (2011). Remodeling mitochondrial membranes during arousal from hibernation. *Physiological and Biochemical Zoology* **84**, 438-449.

Arnold, D. L., Mes, J., Bryce, F., Karpinski, K., Bickis, M. G., Zawidzka, Z. Z. and Stapley, R. (1990). A pilot study on the effects of Aroclor 1254 ingestion by rhesus and cynomolgus monkeys as a model for human ingestion of PCBs. *Food Chemistry and Toxicology* **28**, 847-857.

Aureli, M., Grassi, S., Sonnino, S. and Prinetti, A. (2016). Isolation and analysis of detergent-resistant membrane fractions. *Methods in Molecular Biology* **1376**, 107-131.

Barenholz, Y. (2002). Cholesterol and other membrane active sterols: from membrane evolution to "rafts". *Progress in Lipid Research* **41**, 1-5.

Benedikt, J., Teisinger, J., Vyklicky, L. and Vlachova, V. (2007). Ethanol inhibits cold-menthol receptor TRPM8 by modulating its interaction with membrane phosphatidylinositol 4,5-bisphosphate. *Journal of Neurochemistry* **100**, 211-224.

Berg, K., Puntervoll, P., Klungsøyr, J. and Goksøyr, A. (2011). Brain proteome alterations of Atlantic cod (*Gadus morhua*) exposed to PCB 153. *Aquatic Toxicology* **105**, 206-217.

Bhavsar, S. P., Jackson, D. A., Hayton, A., Reiner, E. J., Chen, T. and Bodnar, J. (2007). Are PCB Levels in Fish from the Canadian Great Lakes Still Declining? *Journal of Great Lakes Research* **33**, 592-605.

Bonora, S., Torreggiani, A. and Fini, G. (2003). DSC and Raman study on the interaction between polychlorinated biphenyls (PCB) and phospholipid liposomes. *Thermochimica Acta* **408**, 55-65.

Bourque, C. W. (2008). Central mechanisms of osmosensation and systemic osmoregulation. *Nature Reviews Neuroscience* **9**, 519-531.

Brand, M. D., Couture, P., Else, P. L., Withers, K. W. and Hulbert, A. J. (1991). Evolution of energy metabolism. Proton permeability of the inner membrane of liver mitochondria is greater in a mammal than in a reptile. *Biochemical Journal* **275**, 81-86.

Brand, M. D., Turner, N., Ocloo, A., Else, P. L. and Hulbert, A. J. (2003). Proton conductance and fatty acyl composition of liver mitochondria correlates with body mass in birds. *Biochemical Journal* **376**, 741-748.

Brauner, C. J., Gonzalez, R. J. and Wilson, J. M. (2012). 9 - Extreme Environments: Hypersaline, Alkaline, and Ion-Poor Waters. In *Fish Physiology*, vol. 32 eds. A. P. F. Stephen D. McCormick and J. B. Colin), pp. 435-476: Academic Press.

Brookes, P. S., Buckingham, J. A., Tenreiro, A. M., Hulbert, A. J. and Brand, M. D. (1998). The Proton Permeability of the Inner Membrane of Liver Mitochondria from Ectothermic and Endothermic Vertebrates and from Obese Rats: Correlations with Standard Metabolic Rate and Phospholipid Fatty Acid Composition. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology* **119**, 325-334.

Broughton, R. E., Betancur-R, R., Li, C., Arratia, G. and Ortí, G. (2013). Multi-locus phylogenetic analysis reveals the pattern and tempo of bony fish evolution. *PLoS Currents*.

Brown, T. M., Kuzyk, Z. Z. A., Stow, J. P., Burgess, N. M., Solomon, S. M., Sheldon, T. A. and Reimer, K. J. (2013). Effects-based marine ecological risk assessment at a polychlorinated biphenyl-contaminated site in Saglek, Labrador, Canada. *Environmental Toxicology and Chemistry* **32**, 453-467.

Brzęk, P., Bielawska, K., Książek, A. and Konarzewski, M. (2007). Anatomic and molecular correlates of divergent selection for basal metabolic rate in laboratory mice. *Physiological and Biochemical Zoology* **80**, 491-499.

Bufalino, A. P. and Mayden, R. L. (2010). Phylogenetic relationships of North American phoxinins (Actinopterygii: Cypriniformes: Leuciscidae) as inferred from S7 nuclear DNA sequences. *Molecular Phylogenetics and Evolution* **55**, 143-152.

Campbell, A. S., Yu, Y., Granick, S. and Gewirth, A. A. (2008). PCB association with model phospholipid bilayers. *Environmental Science and Technology* **42**, 7496-501.

Candelario, J. and Chachisvilis, M. (2013). Activity of Bradykinin B2 Receptor Is Regulated by Long-Chain Polyunsaturated Fatty Acids. *Plos One* **8**, e68151.

Cederholm, C. J., Kunze, M. D., Murota, T. and Sibatani, A. (1999). Pacific salmon carcasses: Essential contributions of nutrients and energy for aquatic and terrestrial ecosystems. *Fisheries* **24**, 6-15.

Cerra, M. C. and Imbrogno, S. (2012). Phospholamban and cardiac function: A comparative perspective in vertebrates. *Acta Physiologica* **205**, 9-25.

Chapelle, S., Dandrifosse, G. and Zwingelstein, G. (1976). Metabolism of phospholipids of anterior or posterior gills of the crab *Eriocheir Sinensis* M. EDW, during the adaptation of this animal to media of various salinities. *International Journal of Biochemistry* **7**, 343-351.

Chapelle, S. and Zwingelstein, G. (1984). Phospholipid composition and metabolism of crustacean gills as related to changes in environmental salinities: Relationship between Na^+-ATPase activity and phospholipids. *Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology* **78**, 363-372.

Chasiotis, H., Effendi, J. C. and Kelly, S. P. (2009). Occludin expression in goldfish held in ion-poor water. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology* **179**, 145-154.

Chasiotis, H. and Kelly, S. P. (2011). Permeability properties and occludin expression in a primary cultured model gill epithelium from the stenohaline freshwater goldfish. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology* **181**, 487-500.

Chasiotis, H., Kolosov, D., Bui, P. and Kelly, S. P. (2012a). Tight junctions, tight junction proteins and paracellular permeability across the gill epithelium of fishes: A review. *Respiratory Physiology and Neurobiology* **184**, 269-281.

Chasiotis, H., Kolosov, D. and Kelly, S. P. (2012b). Permeability properties of the teleost gill epithelium under ion-poor conditions. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* **302**, 727-739.

Chen, W. J. and Mayden, R. L. (2012). Phylogeny of suckers (Teleostei: Cypriniformes: Catostomidae): Further evidence of relationships provided by the single-copy nuclear gene IRBP2. *Zootaxa*, 195-210.

Chen, Y. J. and Hsu, C. C. (1994). Effects of prenatal exposure to PCBs on the neurological function of children: A neuropsychological and neurophysiological study. *Developmental Medicine and Child Neurology* **36**, 312-320.

Clarke, A. and Johnston, N. M. (1999). Scaling of metabolic rate with body mass and temperature in teleost fish. *Journal of Animal Ecology* **68**, 893-905.

Corcoran, M. P., Lamon-Fava, S. and Fielding, R. A. (2007). Skeletal muscle lipid deposition and insulin resistance: Effect of dietary fatty acids and exercise. *American Journal of Clinical Nutrition* **85**, 662-677.

Cordier, M., Brichon, G., Weber, J. M. and Zwingelstein, G. (2002). Changes in the fatty acid composition of phospholipids in tissues of farmed sea bass (*Dicentrarchus labrax*) during an annual cycle. Roles of environmental temperature and salinity. *Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology* **133**, 281-288.

Cossins, A. R. (1976). Homeoviscous Adaptation of Goldfish Brain Membranes. *Journal of General Physiology* **68**, A3-A3.

Cossins, A. R. (1977). Adaptation of Biological-Membranes to Temperature - Effect of Temperature-Acclimation of Goldfish Upon Viscosity of Synaptosomal Membranes. *Biochimica Et Biophysica Acta* **470**, 395-411.

Cossins, A. R., Christiansen, J. and Prosser, C. L. (1978). Adaptation of Biological-Membranes to Temperature - Lack of Homeoviscous Adaptation in Sarcoplasmic-Reticulum. *Biochimica Et Biophysica Acta* **511**, 442-454.

Cossins, A. R., Friedlander, M. J. and Prosser, C. L. (1977). Correlations between Behavioral Temperature Adaptations of Goldfish and Viscosity and Fatty-Acid Composition of Their Synaptic-Membranes. *Journal of Comparative Physiology* **120**, 109-121.

Cossins, A. R., Murray, P. A., Gracey, A. Y., Logue, J., Polley, S., Caddick, M., Brooks, S., Postle, T. and Maclean, N. (2002). The role of desaturases in cold-induced lipid restructuring. *Biochemical Society Transactions* **30**, 1082-6.

Cossins, A. R. and Wilkinson, H. L. (1982). The role of homeoviscous adaptation in mammalian hibernation. *Journal of Thermal Biology* **7**, 107-110.

Costa, P. M., Caeiro, S., Diniz, M. S., Lobo, J., Martins, M., Ferreira, A. M., Caetano, M., Vale, C., DelValls, T. Á. and Costa, M. H. (2010). A description of chloride cell and kidney tubule alterations in the flatfish *Solea senegalensis* exposed to moderately contaminated sediments from the Sado estuary (Portugal). *Journal of Sea Research* **64**, 465-472.

Couture, P. and Hulbert, A. J. (1995). Membrane fatty acid composition of tissues is related to body mass of mammals. *The Journal of Membrane Biology* **148**, 27-39.

Crockett, E. L. (1998). Cholesterol function in plasma membranes from ectotherms: Membrane-specific roles in adaptation to temperature. *American Zoologist* **38**, 291-304.

Crockett, E. L. and Hazel, J. R. (1995). Cholesterol Levels Explain Inverse Compensation of Membrane Order in Brush-Border but Not Homeoviscous Adaptation in Basolateral Membranes from the Intestinal Epithelia of Rainbow Trout. *Journal of Experimental Biology* **198**, 1105-1113.

Daikoku, T., Yano, I. and Masul, M. (1982). Lipid and fatty acid compositions and their changes in the different organs and tissues of guppy, *Poecilia reticulata* on sea water adaptation. *Comparative Biochemistry and Physiology A - Molecular and Integrative Physiology* **73**, 167-174.

Dantagnan, H., Bórquez, A. S., Valdebenito, I. N., Salgado, I. A., Serrano, E. A. and Izquierdo, M. S. (2007). Lipid and fatty acid composition during embryo and larval development of puye *Galaxias maculatus* Jenyns, 1842, obtained from estuarine, freshwater and cultured populations. *Journal of Fish Biology* **70**, 770-781.

de Virville, J. D., Cantrel, C., Bousquet, A. L., Hoffelt, M., Tenreiro, A. M., Pinto, V. V., Arrabaca, J. D., Caiveau, O., Moreau, F. and Zachowski, A. (2002). Homeoviscous and functional adaptations of mitochondrial membranes to growth temperature in soybean seedlings. *Plant, Cell & Environment* **25**, 1289-1297.

Denison, M. S. and Nagy, S. R. (2003). Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annual Review of Pharmacology and Toxicology* **43**, 309-334.

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J. F., Guindon, S., Lefort, V., Lescot, M. et al. (2008). Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res* **36**, W465-9.

Díaz-Uriarte, R. and Garland Jr, T. (1996). Testing hypotheses of correlated evolution using phylogenetically independent contrasts: Sensitivity to deviations from Brownian motion. *Systematic Biology* **45**, 27-47.

Dong, S. J., Yi, C. F. and Li, H. (2015). Changes of *Saccharomyces cerevisiae* cell membrane components and promotion to ethanol tolerance during the bioethanol fermentation. *International Journal of Biochemistry and Cell Biology* **69**, 196-203.

Dowhan, W. (1997). Molecular basis for membrane phospholipid diversity: Why are there so many lipids? In *Annual Review of Biochemistry*, vol. 66, pp. 199-232.

Dragan, Y. P. and Schrenk, D. (2000). Animal studies addressing the carcinogenicity of TCDD (or related compounds) with an emphasis on tumour promotion. *Food Additives & Contaminants* **17**, 289-302.

Drouillard, K. G., Fernie, K. J., Letcher, R. J., Shutt, L. J., Whitehead, M., Gebink, W. and Bird, D. M. (2007). Bioaccumulation and biotransformation of 61 polychlorinated biphenyl and four polybrominated diphenyl ether congeners in juvenile American kestrels (*Falco sparverius*). *Environmental Toxicology and Chemistry* **26**, 313-24.

Duffy-Whritenour, J. E., Kurtzman, R. Z., Kennedy, S. and Zelikoff, J. T. (2010). Non-coplanar polychlorinated biphenyl (PCB)-induced immunotoxicity is coincident with alterations in the serotonergic system. *Journal of Immunotoxicology* **7**, 318-326.

Duhamel, T. A., Green, H. J., Stewart, R. D., Foley, K. P., Smith, I. C. and Ouyang, J. (2007). Muscle metabolic, SR Ca2+-cycling responses to prolonged cycling, with and without glucose supplementation. *Journal of Applied Physiology* **103**, 1986-1998.

Edgar, R. C. (2004). MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* **5**, 1-19.

Else, P. L. and Wu, B. J. (1999). What role for membranes in determining the higher sodium pump molecular activity of mammals compared to ectotherms? *Journal of Comparative Physiology - B Biochemical, Systemic, and Environmental Physiology* **169**, 296-302.

Farkas, T., Dey, I., Buda, C. and Halver, J. E. (1994). Role of phospholipid molecular species in maintaining lipid membrane structure in response to temperature. *Biophysical Chemistry* **50**, 147-155.

Farkas, T., Fodor, E., Kitajka, K. and Halver, J. E. (2001). Response of fish membranes to environmental temperature. *Aquaculture Research* **32**, 645-655.

Farkas, T., Kitajka, K., Fodor, E., Csengeri, I., Lahdes, E., Yeo, Y. K., Krasznai, Z. and Halver, J. E. (2000). Docosahexaenoic acid-containing phospholipid molecular species in brains of vertebrates. *Proceedings of the National Academy of Sciences USA* **97**, 6362-6.

Felsenstein, J. (1985). Phylogenies and the comparative method. *American Naturalist* **125**, 1-15.

Ficko, S. A., Luttmer, C., Zeeb, B. A. and Reimer, K. (2013). Terrestrial ecosystem recovery following removal of a PCB point source at a former pole vault line radar station in Northern Labrador. *Science of The Total Environment* **461–462**, 81-87.

Friedlander, G., Le Grimellec, C., Giocondi, M.-C. and Amiel, C. (1987). Benzyl alcohol increases membrane fluidity and modulates cyclic AMP synthesis in intact renal epithelial cells. *Biochimica et Biophysica Acta - Biomembranes* **903**, 341-348.

Froese, R. and Pauly, D. (2016). FishBase: www.fishbase.org.

Furuya, H., Yamada, T., Ohyagi, Y., Ikezoe, K., Miyoshi, T., Fujii, N. and Kira, J. i. (2005). Neurological signs and symptoms in patients with chronic PCB poisoning (Yusho accident) for more than 36 years. *Journal of Dermatological Science, Supplement* **1 SUPPL.**, S39-S44.

Garcia Fernandez, M. I., Ceccarelli, D. and Muscatello, U. (2004). Use of the fluorescent dye 10-*N*-nonyl acridine orange in quantitative and location assays of cardiolipin: a study on different experimental models. *Analytical Biochemistry* **328**, 174-180.

Garland, T., Jr., Harvey, P. H. and Ives, A. R. (1992). Procedures for the Analysis of Comparative Data Using Phylogenetically Independent Contrasts. *Systematic Biology* **41**, 18-32.

Garland, T., Midford, P. E. and Ives, A. R. (1999). An introduction to phylogenetically based statistical methods, with a new method for confidence intervals on ancestral values. *American Zoologist* **39**, 374-388.

Gaspar-Ramírez, O., Pérez-Vázquez, F. J., Salgado-Bustamante, M., González-Amaro, R., Hernandez-Castro, B. and Pérez-Maldonado, I. N. (2015). DDE and PCB 153 independently induce aryl hydrocarbon receptor (AhR) expression in peripheral blood mononuclear cells. *Journal of Immunotoxicology* **12**, 266-272.

Giroud, S., Frare, C., Strijkstra, A., Boerema, A., Arnold, W. and Ruf, T. (2013). Membrane Phospholipid Fatty Acid Composition Regulates Cardiac SERCA Activity in a Hibernator, the Syrian Hamster (*Mesocricetus auratus*). *Plos One* **8**, e63111.

Glémet, H. C., Gerrits, M. F. and Ballantyne, J. S. (1997). Membrane lipids of red muscle mitochondria from land-locked and sea-run Arctic char, *Salvelinus alpinus*. *Marine Biology* **129**, 673-679.

Goldstein, J. L., DeBose-Boyd, R. A. and Brown, M. S. (2006). Protein Sensors for Membrane Sterols. *Cell* **124**, 35-46.

Gorski, P. A., Glaves, J. P., Vangheluwe, P. and Young, H. S. (2013). Sarco(endo)plasmic reticulum calcium ATPase (SERCA) inhibition by sarcolipin is encoded in its luminal tail. *Journal of Biological Chemistry* **288**, 8456-8467.

Greco, A. M., Fenwick, J. C. and Perry, S. F. (1996). The effects of soft-water acclimation on gill structure in the rainbow trout *Oncorhynchus mykiss*. *Cell and Tissue Research* **285**, 75-82.

Grosell, M. (2006). Intestinal anion exchange in marine fish osmoregulation. *Journal of Experimental Biology* **209**, 2813-2827.

Guderley, H., Kraffe, E., Bureau, W. and Bureau, D. P. (2008). Dietary fatty acid composition changes mitochondrial phospholipids and oxidative capacities in rainbow trout red muscle. *Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology* **178**, 385-399.

Guderley, H., Pierre, J. S., Couture, P. and Hulbert, A. J. (1997). Plasticity of the properties of mitochondria from rainbow trout red muscle with seasonal acclimatization. *Fish Physiology and Biochemistry* **16**, 531-541.

Guindon, S., Dufayard, J.-F., Lefort, V., Anisimova, M., Hordijk, W. and Gascuel, O. (2010). New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0. *Systematic Biology* **59**, 307-321.

Haag, M., Magada, O. N., Claassen, N., Böhmer, L. H. and Kruger, M. C. (2003). Omega-3 fatty acids modulate ATPases involved in duodenal Ca absorption. *Prostaglandins Leukotrienes and Essential Fatty Acids* **68**, 423-429.

Haggerty, C., Hoggard, N., Brown, D. S., Clapham, J. C. and Speakman, J. R. (2008). Intra-specific variation in resting metabolic rate in MF1 mice is not associated with membrane lipid desaturation in the liver. *Mechanisms of Ageing and Development* **129**, 129-137.

Hanks, S. K., Quinn, A. M. and Hunter, T. (1988). The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* **241**, 42-52.

Hao, S. J., Hou, J. F., Jiang, N. and Zhang, G. J. (2008). Loss of membrane cholesterol affects lysosomal osmotic stability. *General Physiology and Biophysics* **27**, 278-283.

Hazel, J. R. (1972). The effect of temperature acclimation upon succinic dehydrogenase activity from the epaxial muscle of the common goldfish (*Carassius auratus* L)-I. Properties of the enzyme and the effect of lipid extraction. *Comparative Biochemistry and Physiology -- Part B: Biochemistry and* **43**, 837-861.

Hazel, J. R. (1979). Influence of thermal acclimation on membrane lipid composition of rainbow trout liver. *American Journal of Physiology* **236**, R91-101.

Hazel, J. R. (1984). Effects of temperature on the structure and metabolism of cell membranes in fish. *American Journal of Physiology* **246**, R460-R470.

Hazel, J. R. (1995). Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation? *Annual Reviews in Physiology* **57**, 19-42.

Hazel, J. R. and Landrey, S. R. (1988). Time course of thermal adaptation in plasma membranes of trout kidney. II. Molecular species composition. *American Journal of Physiology* **255**, R628-34.

Hazel, J. R., McKinley, S. J. and Williams, E. E. (1992). Thermal adaptation in biological membranes - interacting effects of temperature and pH. *Journal of Comparative Physiology B* **162**, 593-601.

Hazel, J. R. and Williams, E. E. (1990). The Role of Alterations in Membrane Lipid-Composition in Enabling Physiological Adaptation of Organisms to Their Physical-Environment. *Progress in Lipid Research* **29**, 167-227.

Hazel, J. R., Williams, E. E., Livermore, R. and Mozingo, N. (1991). Thermal adaptation in biological membranes: functional significance of changes in phospholipid molecular species composition. *Lipids* **26**, 277-82.

Helgen, K. M. (2011). The Mammal Family Tree. *Science* **334**, 458-459.

Hill, J. E., Lawson, L. L. and Hardin, S. (2014). Assessment of the Risks of Transgenic Fluorescent Ornamental Fishes to the United States Using the Fish Invasiveness Screening Kit (FISK). *Transactions of the American Fisheries Society* **143**, 817-829.

Hulbert, A. J. (2007). Membrane fatty acids as pacemakers of animal metabolism. *Lipids* **42**, 811-819.

Hulbert, A. J. and Else, P. L. (1999). Membranes as possible pacemakers of metabolism. *Journal of Theoretical Biology* **199**, 257-274.

Hulbert, A. J. and Else, P. L. (2000). Mechanisms underlying the cost of living in animals. *Annual Reviews in Physiology* **62**, 207-235.

Hulbert, A. J. and Else, P. L. (2005). Membranes and the setting of energy demand. *Journal of Experimental Biology* **208**, 1593-1599.

Hulbert, A. J., Faulks, S., Buttemer, W. A. and Else, P. L. (2002a). Acyl composition of muscle membranes varies with body size in birds. *Journal of Experimental Biology* **205**, 3561-3569.

Hulbert, A. J., Rana, T. and Couture, P. (2002b). The acyl composition of mammalian phospholipids: an allometric analysis. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **132**, 515-527.

Hunt, A. Ö., Özkan, F., Engin, K. and Tekelioğlu, N. (2011). The effects of freshwater rearing on the whole body and muscle tissue fatty acid profile of the European sea bass (*Dicentrarchus labrax*). *Aquaculture International* **19**, 51-61.

Ibarz, A., Blasco, J., Beltran, M., Gallardo, M. A., Sanchez, J., Sala, R. and Fernandez-Borras, J. (2005). Cold-induced alterations on proximate composition and fatty acid profiles of several tissues in gilthead sea bream (*Sparus aurata*). *Aquaculture* **249**, 477-486.

Iftikar, F. I. and Hickey, A. J. R. (2013). Do Mitochondria Limit Hot Fish Hearts? Understanding the Role of Mitochondrial Function with Heat Stress in *Notolabrus celidotus*. *Plos One* **8**, e64120.

Ip, Y. K., Loong, A. M., Kuah, J. S., Sim, E. W. L., Chen, X. L., Wong, W. P., Lam, S. H., Delgado, I. L. S., Wilson, J. M. and Chew, S. F. (2012). Roles of three branchial Na^+-K^+-ATPase α-subunit isoforms in freshwater adaptation, seawater acclimation, and active ammonia excretion in *Anabas testudineus*. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* **303**, R112-R125.

Itel, F., Al-Samir, S., Oberg, F., Chami, M., Kumar, M., Supuran, C. T., Deen, P. M., Meier, W., Hedfalk, K., Gros, G. et al. (2012). CO_2 permeability of cell membranes is regulated by membrane cholesterol and protein gas channels. *FASEB Journal* **26**, 5182-91.

Jones, K. C. and de Voogt, P. (1999). Persistent organic pollutants (POPs): state of the science. *Environmental Pollution* **100**, 209-221.

Kato, N. and Yoshida, A. (1980). Effect of dietary PCB on hepatic cholesterogenesis in rats. *Nutrition Reports International* **21**, 107-112.

Katynski, A. L., Vijayan, M. M., Kennedy, S. W. and Moon, T. W. (2004). 3,3',4,4',5-Pentachlorobiphenyl (PCB 126) impacts hepatic lipid peroxidation, membrane fluidity and beta-

adrenoceptor kinetics in chick embryos. *Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology* **137**, 81-93.

Kearns, S. D. and Haag, M. (2002). The effect of omega-3 fatty acids on Ca-ATPase in rat cerebral cortex. *Prostaglandins Leukotrienes and Essential Fatty Acids* **67**, 303-308.

Kemp, P. and Smith, M. W. (1970). Effect of temperature acclimatization on the fatty acid composition of goldfish intestinal lipids. *Biochemical Journal* **117**, 9-15.

Khériji, S., El Cafsi, M., Masmoudi, W., Castell, J. D. and Romdhane, M. S. (2003). Salinity and temperature effects on the lipid composition of mullet sea fry (*Mugil cephalus*, Linne, 1758). *Aquaculture International* **11**, 571-582.

Kim, Y.-m., Farrah, S. R. and Baney, R. H. (2007). Membrane damage of bacteria by silanols treatment. *Electronic Journal of Biotechnology* **10**.

Kolomiytseva, I. K. (2011). Lipids in mammalian hibernation and artificial hypobiosis. *Biochemistry (Moscow)* **76**, 1291-1299.

Kolomiytseva, I. K., Perepelkina, N. I., Zharikova, A. D. and Popov, V. I. (2008). Membrane lipids and morphology of brain cortex synaptosomes isolated from hibernating Yakutian ground squirrel. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology* **151**, 386-391.

Kraffe, E., Marty, Y. and Guderley, H. (2007). Changes in mitochondrial oxidative capacities during thermal acclimation of rainbow trout *Oncorhynchus mykiss*: roles of membrane proteins, phospholipids and their fatty acid compositions. *Journal of Experimental Biology* **210**, 149-165.

Kvist, L., Giwercman, A., Weihe, P., Kold Jensen, T., Grandjean, P., Halling, J., Skaalum Petersen, M. and Lundberg Giwercman, Y. (2014). Exposure to persistent organic pollutants and sperm sex chromosome ratio in men from the Faroe Islands. *Environment International* **73**, 359-364.

Lee, H. J., Balasubramanian, S. V., Murer, H., Biber, J. and Morris, M. E. (1999). Modulation of sulfate renal transport by alterations in cell membrane fluidity. *Journal of Pharmaceutical Sciences* **88**, 976-980.

Lee, Y. J. and Yang, J. H. (2012). Chlorination of ortho-position on polychlorinated biphenyls increases protein kinase C activity in neuronal cells. *Toxicological Research* **28**, 107-112.

Lilienthal, H., Neuf, M., Munoz, C. and Winneke, G. (1990). Behavioral effects of pre- and postnatal exposure to a mixture of low chlorinated PCBs in rats. *Fundamental and Applied Toxicology* **15**, 457-467.

Lopez-Aparicio, P., Recio, M. N., Prieto, J. C. and Perez-Albarsanz, M. A. (1994). Role of lindane in membranes. Effects on membrane fluidity and activity of membrane-bound proteins. *Bioscience Reports* **14**, 131-138.

Lung, S.-C. C., Guo, Y.-L. L. and Chang, H.-Y. (2005). Serum concentrations and profiles of polychlorinated biphenyls in Taiwan Yu-cheng victims twenty years after the incident. *Environmental Pollution* **136**, 71-79.

Maddison, W. P. and Maddison, D. R. (2011). Mesquite: a modular system for evolutionary analysis: http://mesquiteproject.org.

Magnoni, L. and Weber, J. M. (2007). Endurance swimming activates trout lipoprotein lipase: plasma lipids as a fuel for muscle. *Journal of Experimental Biology* **210**, 4016-4023.

Maillet, D. and Weber, J. M. (2006). Performance-enhancing role of dietary fatty acids in a long-distance migrant shorebird: the semipalmated sandpiper. *Journal of Experimental Biology* **209**, 2686-2695.

Maixent, J. M., Fares, M., François, C., Delmotte, A. and Rigoard, P. (2014). Docosahexaenoic acid (DHA) injection in spinal cord transection stimulates Na^+,K^+-ATPase in skeletal muscle via β 1 subunit. *Cellular and molecular biology (Noisy-le-Grand, France)* **60**, 22-29.

Marshall, W. S. (2012). 8 - Osmoregulation in Estuarine and Intertidal Fishes. In *Fish Physiology*, vol. Volume 32 eds. A. P. F. Stephen D. McCormick and J. B. Colin), pp. 395-434: Academic Press.

Martin, N., Bureau, D. P., Marty, Y., Kraffe, E. and Guderley, H. (2013). Dietary lipid quality and mitochondrial membrane composition in trout: Responses of membrane enzymes and

oxidative capacities. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology* **183**, 393-408.

Martin, N., Kraffe, E., Le Grand, F., Marty, Y., Bureau, D. P. and Guderley, H. (2015). Dietary fatty acid composition and the homeostatic regulation of mitochondrial phospholipid classes in red muscle of rainbow trout (*Oncorhynchus mykiss*). *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology* **323**, 60-71.

Martínez-Álvarez, R. M., Sanz, A., García-Gallego, M., Domezain, A., Domezain, J., Carmona, R., del Valle Ostos-Garrido, M. and Morales, A. E. (2005). Adaptive branchial mechanisms in the sturgeon *Acipenser naccarii* during acclimation to saltwater. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology* **141**, 183-190.

Maruoka, N., Murata, T., Omata, N., Takashima, Y., Tanii, H., Yonekura, Y., Fujibayashi, Y. and Wada, Y. (2007). Effects of chlorpromazine on plasma membrane permeability and fluidity in the rat brain: A dynamic positron autoradiography and fluorescence polarization study. *Progress in Neuro-Psychopharmacology and Biological Psychiatry* **31**, 178-186.

McKinley, S. J. and Hazel, J. R. (2000). Does membrane fluidity contribute to thermal compensation of beta-adrenergic signal transduction in isolated trout hepatocytes? *Journal of Experimental Biology* **203**, 631-640.

Meng, M. S., West, G. C. and Irving, L. (1969). Fatty acid composition of caribou bone marrow. *Comparative Biochemistry And Physiology* **30**, 187-191.

Mikami, K. and Murata, N. (2003). Membrane fluidity and the perception of environmental signals in cyanobacteria and plants. *Prog Lipid Res* **42**, 527-43.

Mirarab, S., Bayzid, M. S., Boussau, B. and Warnow, T. (2014). Statistical binning enables an accurate coalescent-based estimation of the avian tree. *Science* **346**.

Mitoma, C., Uchi, H., Tsukimori, K., Yamada, H., Akahane, M., Imamura, T., Utani, A. and Furue, M. (2015). Yusho and its latest findings-A review in studies conducted by the Yusho Group. *Environment International* **82**, 41-48.

Morenilla-Palao, C., Pertusa, M., Meseguer, V., Cabedo, H. and Viana, F. (2009). Lipid raft segregation modulates TRPM8 channel activity. *The Journal of Biological Chemistry* **284**, 9215-9224.

Motamed, M., Zhang, Y., Wang, M. L., Seemann, J., Kwon, H. J., Goldstein, J. L. and Brown, M. S. (2011). Identification of luminal loop 1 of scap protein as the sterol sensor that maintains cholesterol homeostasis. *Journal of Biological Chemistry* **286**, 18002-18012.

Motohashi, E., Hasegawa, S., Mishiro, K. and Ando, H. (2009). Osmoregulatory responses of expression of vasotocin, isotocin, prolactin and growth hormone genes following hypoosmotic challenge in a stenohaline marine teleost, tiger puffer (*Takifugu rubripes*). *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology* **154**, 353-359.

Müller, K., Müller, P., Pincemy, G., Kurz, A. and Labbe, C. (2008). Characterization of sperm plasma membrane properties after cholesterol modification: Consequences for cryopreservation of rainbow trout spermatozoa. *Biology of Reproduction* **78**, 390-399.

Nakanishi, H., Tsuda, T., Fukui, S. and Hirayama, T. (1987a). Comparative studies on additive effects of sodium dodecylbenzensulfonate and sodium stearate on uptake of chemicals by willow shiner (*Gnathopogon caerulescens*). *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology* **86**, 361-364.

Nakanishi, H., Tsuda, T., FuKui, S. and Hirayama, T. (1987b). Correlative studies on changes in lipid composition of gills and uptake of chemicals of willow shiner fish (*Gnathopogon caerulescens*) by exposure to detergent. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology* **86**, 339-341.

Nebert, D. W., Dalton, T. P., Okey, A. B. and Gonzalez, F. J. (2004). Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer. *Journal of Biological Chemistry* **279**, 23847-23850.

Nei, M., Xu, P. and Glazko, G. (2001). Estimation of divergence times from multiprotein sequences for a few mammalian species and several distantly related organisms. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 2497-2502.

Nylander, J. A. A., Ronquist, F., Huelsenbeck, J. P. and Nieves-Aldrey, J. L. (2004). Bayesian phylogenetic analysis of combined data. *Systematic Biology* **53**, 47-67.

Oliveira, F. S. T., Vieira-Filho, L. D., Cabral, E. V., Sampaio, L. S., Silva, P. A., Carvalho, V. C. O., Vieyra, A., Einicker-Lamas, M., Lima, V. L. M. and Paixão, A. D. O. (2012). Reduced cholesterol levels in renal membranes of undernourished rats may account for urinary Na^+ loss. *European Journal of Nutrition* **52**, 1233-1242.

Overgaard, J., Tomcala, A., Sorensen, J. G., Holmstrup, M., Krogh, P. H., Simek, P. and Kostal, V. (2008). Effects of acclimation temperature on thermal tolerance and membrane phospholipid composition in the fruit fly *Drosophila melanogaster*. *Journal of Insect Physiology* **54**, 619-629.

Pai, J. T., Guryev, O., Brown, M. S. and Goldstein, J. L. (1998). Differential stimulation of cholesterol and unsaturated fatty acid biosynthesis in cells expressing individual nuclear sterol regulatory element-binding proteins. *Journal of Biological Chemistry* **273**, 26138-26148.

Polley, S. D., Tiku, P. E., Trueman, R. T., Caddick, M. X., Morozov, I. Y. and Cossins, A. R. (2003). Differential expression of cold- and diet-specific genes encoding two carp liver Delta 9-acyl-CoA desaturase isoforms. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* **284**, R41-R50.

Pronko, A. J., Perlman, B. M. and Ashley-Ross, M. A. (2013). Launches, squiggles and pounces, oh my! the water-land transition in mangrove rivulus (*Kryptolebias marmoratus*). *Journal of Experimental Biology* **216**, 3988-3995.

Rao, X., Huang, X., Zhou, Z. and Lin, X. (2013). An improvement of the 2ˆ(–delta delta CT) method for quantitative real-time polymerase chain reaction data analysis. *Biostatistics, bioinformatics and biomathematics* **3**, 71-85.

Raynard, R. S. and Cossins, A. R. (1991). Homeoviscous adaptation and thermal compensation of sodium-pump of trout erythrocytes. *American Journal of Physiology* **260**, R916-R924.

Reich, T., Depew, M. C., Marks, G. S., Singer, M. A. and Wan, J. K. S. (1981). Effect of polychlorinated biphenyls (PCBs) on phospholipid membrane fluidity. *Journal of Environmental Science and Health Part A -*

Toxic/Hazardous Substances and Environmental Engineering **16**, 65-71.

Revell, L. J. (2012). phytools: an R package for phylogenetic comparative biology (and other things). *Methods in Ecology and Evolution* **3**, 217-223.

Rice, D. C. and Hayward, S. (1997). Effects of postnatal exposure to a PCB mixture in monkeys on nonspatial discrimination reversal and delayed alternation performance. *NeuroToxicology* **18**, 479-494.

Rivera-Ingraham, G. A., Barri, K., Boël, M., Farcy, E., Charles, A.-L., Geny, B. and Lignot, J.-H. (2015). Osmoregulation and salinity-induced oxidative stress: is oxidative adaptation determined by gill function? *Journal of Experimental Biology*.

Roberts, M. E., Burr, B. M., Whiles, M. R. and Santucci, V. J., Jr. (2006). Reproductive ecology and food habits of the blacknose shiner, *Notropis heterolepis*, in Northern Illinois. *American Midland Naturalist* **155**, 70-83.

Robinson, T., Ali, U., Mahmood, A., Chaudhry, M. J. I., Li, J., Zhang, G., Jones, K. C. and Malik, R. N. (2016). Concentrations and patterns of organochlorines (OCs) in various fish species from the Indus River, Pakistan: A human health risk assessment. *Science of The Total Environment* **541**, 1232-1242.

Roesljadi, G., Anderson, J. W., Petrocelli, S. R. and Giam, C. S. (1976). Osmoregulation of the grass shrimp *Palaemonetes pugio* exposed to polychlorinated biphenyls (PCBs). I. Effecton chloride and osmotic concentrations and chloride-and water-exchange kinetics. *Marine Biology* **38**, 343-355.

Rolfe, D. F. S. and Brown, G. C. (1997). Cellular energy utilization and molecular origin of standard metabolic rate in mammals. *Physiological reviews* **77**, 731-758.

Ronquist, F. and Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572-1574.

Rovito, D., Giordano, C., Vizza, D., Plastina, P., Barone, I., Casaburi, I., Lanzino, M., De Amicis, F., Sisci, D., Mauro, L. et al. (2013). Omega-3 PUFA ethanolamides DHEA and EPEA induce autophagy through PPARγ activation in MCF-7 breast cancer cells. *Journal of Cell Physiology* **228**, 1314-1322.

Roy, R., Das, A. B. and Ghosh, D. (1997). Regulation of membrane lipid bilayer structure during seasonal variation: a study on the brain membranes of *Clarias batrachus*. *Biochimica et Biophysica Acta - Biomembranes* **1323**, 65-74.

Ruf, T., Valencak, T., Tataruch, F. and Arnold, W. (2006). Running speed in mammals increases with muscle n-6 polyunsaturated fatty acid content. *Plos One* **1**, e65.

Safe, S. H. (1994). Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic responses, and implications for risk assessment. *Critical Reviews in Toxicology* **24**, 87-149.

Saint-Amour, D., Roy, M. S., Bastien, C., Ayotte, P., Dewailly, E., Després, C., Gingras, S. and Muckle, G. (2006). Alterations of visual evoked potentials in preschool Inuit children exposed to methylmercury and polychlorinated biphenyls from a marine diet. *NeuroToxicology* **27**, 567-578.

Saitoh, K., Sado, T., Doosey, M. H., Bart Jr, H. L., Inoue, J. G., Nishida, M., Mayden, R. L. and Miya, M. (2011). Evidence from mitochondrial genomics supports the lower Mesozoic of South Asia as the time and place of basal divergence of cypriniform fishes (Actinopterygii: Ostariophysi). *Zoological Journal of the Linnean Society* **161**, 633-662.

Sakamoto, T. and Murata, N. (2002). Regulation of the desaturation of fatty acids and its role in tolerance to cold and salt stress. *Current Opinion in Microbiology* **5**, 206-210.

Salonen, A., Ahola, T. and Kaariainen, L. (2005). Viral RNA replication in association with cellular membranes. *Curr Top Microbiol Immunol* **285**, 139-73.

Sandermann Jr, H. (1983). Lipid solvation and kinetic cooperativity of functional membrane proteins. *Trends in Biochemical Sciences* **8**, 408-411.

Schäfer, P., Müller, M., Krüger, A., Steinberg, C. E. W. and Menzel, R. (2009). Cytochrome P450-dependent metabolism of PCB52 in the nematode *Caenorhabditis elegans*. *Archives of Biochemistry and Biophysics* **488**, 60-68.

Scheider, W. A., Cox, C., Hayton, A., Hitchin, G. and Vaillancourt, A. (1998). Current status and temporal trends in concentrations of persistent toxic substances in sport fish and juvenile forage fish in the Canadian waters of the Great Lakes. *Environmental Monitoring and Assessment* **53**, 57-76.

Schmidt-Nielsen, K. (1984). Scaling. Why is animal size so important? Cambridge: Cambridge University Press.

Schmidt-Nielsen, K. (1990). Animal Physiology. New York: Cambridge University Press.

Schünke, M. and Wodtke, E. (1983). Cold-induced increase of δ9- and δ6-desaturase activities in endoplasmic membranes of carp liver. *Biochimica et Biophysica Acta - Biomembranes* **734**, 70-75.

Seebacher, F., Murray, S. A. and Else, P. L. (2009). Thermal acclimation and regulation of metabolism in a reptile (*Crocodylus porosus*): the importance of transcriptional mechanisms and membrane composition. *Physiological and Biochemical Zoology* **82**, 766-775.

Shen, K., Shen, C., Yu, J., Yu, C., Chen, L., Shi, D. and Chen, Y. (2011). PCB congeners induced mitochondrial dysfunction in Vero cells. *Journal of Hazardous Materials* **185**, 24-28.

Šimečková, P., Vondráček, J., Procházková, J., Kozubík, A., Krčmář, P. and Machala, M. (2009). 2,2′,4,4′,5,5′-Hexachlorobiphenyl (PCB 153) induces degradation of adherens junction proteins and inhibits β-catenin-dependent transcription in liver epithelial cells. *Toxicology* **260**, 104-111.

Sinensky, M. (1974). Homeoviscous adaptation--a homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli*. *Proceedings of the National Academy of Sciences USA* **71**, 522-5.

Singer, S. J. and Nicolson, G. L. (1972). The fluid mosaic model of the structure of cell membranes. *Science* **175**, 720-31.

Snyder, R. J. and Hennessey, T. M. (2003). Cold tolerance and homeoviscous adaptation in freshwater alewives (*Alosa pseudoharengus*). *Fish Physiology and Biochemistry* **29**, 117-126.

Somero, G. N. (2004). Adaptation of enzymes to temperature: searching for basic "strategies". *Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology* **139**, 321-333.

Somero, G. N. (2010). The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine 'winners' and 'losers'. *Journal of Experimental Biology* **213**, 912-920.

Sørensen, S., Lund, K. H., Cederberg, T. L. and Ballin, N. Z. (2016). Identification of Baltic Sea salmon based on PCB and dioxin profiles. *Food Control* **61**, 165-171.

Squadrone, S., Favaro, L., Prearo, M., Vivaldi, B., Brizio, P. and Abete, M. C. (2013). NDL-PCBs in muscle of the European catfish (*Silurus glanis*): an alert from Italian rivers. *Chemosphere* **93**, 521-525.

Stubbs, C. D. and Smith, A. D. (1984). The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. *Biochimica Et Biophysica Acta* **779**, 89-137.

Suwalsky, M., Benites, M., Villena, F., Aguilar, F. and Sotomayor, C. P. (1997). The organochlorine pesticide heptachlor disrupts the structure of model and cell membranes. *Biochimica et Biophysica Acta - Biomembranes* **1326**, 115-123.

Suzuki, I., Los, D. A. and Murata, N. (2000). Perception and transduction of low-temperature signals to induce desaturation of fatty acids. *Biochemical Society Transactions* **28**, 628-630.

Szabó, A., Mézes, M., Romvári, R. and Fébel, H. (2010). Allometric scaling of fatty acyl chains in fowl liver, lung and kidney, but not in brain phospholipids. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **155**, 301-308.

Tan, Y. S., Chen, C. H., Lawrence, D. and Carpenter, D. O. (2004). Ortho-substituted PCBs kill cells by altering membrane structure. *Toxicological Sciences* **80**, 54-59.

Tang, C. H., Wu, W. Y., Tsai, S. C., Yoshinaga, T. and Lee, T. H. (2010). Elevated Na^+/K^+-ATPase responses and its potential role in triggering ion reabsorption in kidneys for homeostasis of marine euryhaline milkfish (*Chanos chanos*) when acclimated to hypotonic fresh water. *Journal of Comparative Physiology - B Biochemical, Systemic, and Environmental Physiology* **180**, 813-824.

Team, R. C. (2016). R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

Tocher, D. R., Castell, J. D., Dick, J. R. and Sargent, J. R. (1995). Effects of salinity on the fatty acid compositions of total lipid and individual glycerophospholipid classes of Atlantic salmon (*Salmo salar*) and turbot (*Scophthalmus maximus*) cells in culture. *Fish Physiology and Biochemistry* **14**, 125-137.

Trueman, R. J., Tiku, P. E., Caddick, M. X. and Cossins, A. R. (2000). Thermal thresholds of lipid restructuring and δ9-desaturase expression in the liver of carp (*Cyprinus carpio* L.). *Journal of Experimental Biology* **203**, 641-650.

Tsiavaliaris, G., Itel, F., Hedfalk, K., Al-Samir, S., Meier, W., Gros, G. and Endeward, V. (2015). Low CO_2 permeability of cholesterol-containing liposomes detected by stopped-flow fluorescence spectroscopy. *The FASEB Journal* **29**, 1780-1793.

Tupling, A. R., Bombardier, E., Gupta, S. C., Hussain, D., Vigna, C., Bloemberg, D., Quadrilatero, J., Trivieri, M. G., Babu, G. J., Backx, P. H. et al. (2011). Enhanced Ca^{2+} transport and muscle relaxation in skeletal muscle from sarcolipin-null mice. *American Journal of Physiology - Cell Physiology* **301**, C841-C849.

Turk, H. F. and Chapkin, R. S. (2013). Membrane lipid raft organization is uniquely modified by n-3 polyunsaturated fatty acids. *Prostaglandins Leukotrienes and Essential Fatty Acids* **88**, 43-47.

Turner, N., Haga, K. L., Else, P. L. and Hulbert, A. J. (2006). Scaling of Na^+,K^+-ATPase molecular activity and membrane fatty acid composition in mammalian and avian hearts. *Physiological and Biochemical Zoology* **79**, 522-533.

Turner, N., Haga, K. L., Hulbert, A. J. and Else, P. L. (2005). Relationship between body size, Na^+-K^+-ATPase activity, and membrane lipid composition in mammal and bird kidney. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* **288**, R301-R310.

Vagner, M. and Santigosa, E. (2011). Characterization and modulation of gene expression and enzymatic activity of delta-6 desaturase in teleosts: A review. *Aquaculture* **315**, 131-143.

Vaish, V. and Sanyal, S. N. (2011). Non steroidal anti-inflammatory drugs modulate the physicochemical properties of plasma membrane in experimental colorectal cancer: a fluorescence spectroscopic study. *Molecular and Cellular Biochemistry* **358**, 161-171.

Valencak, T. G. and Ruf, T. (2007). N-3 polyunsaturated fatty acids impair lifespan but have no role for metabolism. *Aging Cell* **6**, 15-25.

Vance, E. D., Audubert, F. and Pritchard, H. P. (1982). The conversion of phosphatidylethanolamine to phosphatidylcholine in animal cells. In *Biochemistry of S-Adenosylmethionine and Related Compounds: Proceedings of a Conference held at the Lake of the Ozarks (Missouri) on October 26–29, 1981*, pp. 119-128. London: Palgrave Macmillan UK.

Vargas-Chacoff, L., Saavedra, E., Oyarzún, R., Martínez-Montaño, E., Pontigo, J. P., Yáñez, A., Ruiz-Jarabo, I., Mancera, J. M., Ortiz, E. and Bertrán, C. (2015). Effects on the metabolism, growth, digestive capacity and osmoregulation of juvenile of sub-Antarctic notothenioid fish *Eleginops maclovinus* acclimated at different salinities. *Fish Physiology and Biochemistry* **41**, 1369-1381.

Veld, G. I., Driessen, A. J. and Konings, W. N. (1993). Bacterial solute transport proteins in their lipid environment. *FEMS Microbiol Rev* **12**, 293-314.

Venkataraman, P., Selvakumar, K., Krishnamoorthy, G., Muthusami, S., Rameshkumar, R., Prakash, S. and Arunakaran, J. (2010). Effect of melatonin on PCB (Aroclor 1254) induced neuronal damage and changes in Cu/Zn superoxide dismutase and glutathione peroxidase-4 mRNA expression in cerebral cortex, cerebellum and hippocampus of adult rats. *Neuroscience Research* **66**, 189-197.

Vladkova, R., Koynova, R., Teuchner, K. and Tenchov, B. (2010). Bilayer structural destabilization by low amounts of chlorophyll a. *Biochimica et Biophysica Acta - Biomembranes* **1798**, 1586-1592.

Wang, J., Bi, Y., Henkelmann, B., Pfister, G., Zhang, L. and Schramm, K. W. (2016). PAHs and PCBs accumulated by SPMD-based virtual organisms and feral fish in Three Gorges Reservoir, China. *Science of The Total Environment* **542**, 899-907.

Warnock, D. E., Roberts, C., Lutz, M. S., Blackburn, W. A., Young, W. W., Jr. and Baenziger, J. U. (1993). Determination of plasma membrane lipid mass and composition in cultured Chinese hamster ovary cells using high gradient magnetic affinity chromatography. *Journal of Biological Chemistry* **268**, 10145-53.

White, C. R., Phillips, N. F. and Seymour, R. S. (2006). The scaling and temperature dependence of vertebrate metabolism. *Biology Letters* **2**, 125-127.

White, C. R. and Seymour, R. S. (2005). Allometric scaling of mammalian metabolism. *Journal of Experimental Biology* **208**, 1611-1619.

Wodtke, E. and Cossins, A. R. (1991). Rapid cold-induced changes of membrane order and Δ9-desaturase activity in endoplasmic reticulum of carp liver: A time-course study of thermal acclimation. *Biochimica et Biophysica Acta - Biomembranes* **1064**, 343-350.

Wójtowicz, A. K., Goch, M. and Gregoraszczuk, E. L. (2005). Polychlorinated biphenyls (PCB 153 and PCB 126) action on conversion of 20-hydroxylated cholesterol to progesterone, androstenedione to testosterone, and testosterone to estradiol 17β. *Experimental and Clinical Endocrinology & Diabetes* **113**, 464-470.

Wone, B. W. M., Donovan, E. R., Cushman, J. C. and Hayes, J. P. (2013). Metabolic rates associated with membrane fatty acids in mice selected for increased maximal metabolic rate. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology* **165**, 70-78.

Wu, B. J., Hulbert, A. J., Storlien, L. H. and Else, P. L. (2004). Membrane lipids and sodium pumps of cattle and crocodiles: An experimental test of the membrane pacemaker theory of metabolism. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* **287**, R633-R641.

Yan, J. J., Chou, M. Y., Kaneko, T. and Hwang, P. P. (2007). Gene expression of Na^+/H^+ exchanger in zebrafish H^+-ATPase-rich cells during acclimation to low-Na^+ and acidic environments. *American Journal of Physiology - Cell Physiology* **293**, C1814-C1823.

Yang, L., Zhang, G. J., Zhong, Y. G. and Zheng, Y. Z. (2000). Influence of membrane fluidity modifiers on lysosomal osmotic sensitivity. *Cell Biology International* **24**, 699-704.

Yang, M., Li, X.-l., Xu, H.-y., Sun, J.-b., Mei, B., Zheng, H.-f., Piao, L.-h., Xing, D.-g., Li, Z.-l. and Xu, W.-x. (2005). Role of arachidonic acid in hyposmotic membrane stretch-induced increase in calcium-activated potassium currents in gastric myocytes. *Acta Pharmacologica Sinica* **26**, 1233-1242.

Yilmaz, B., Sandal, S., Chen, C.-H. and Carpenter, D. O. (2006). Effects of PCB 52 and PCB 77 on cell viability, [Ca2+]i levels and membrane fluidity in mouse thymocytes. *Toxicology* **217**, 184-193.

Yudin, Y. and Rohacs, T. (2012). Regulation of TRPM8 channel activity. *Molecular and Cellular Endocrinology* **353**, 68-74.

Zehmer, J. K. and Hazel, J. R. (2003). Plasma membrane rafts of rainbow trout are subject to thermal acclimation. *Journal of Experimental Biology* **206**, 1657-1667.

Zehmer, J. K. and Hazel, J. R. (2004). Membrane order conservation in raft and non-raft regions of hepatocyte plasma membranes from thermally acclimated rainbow trout. *Biochimica et Biophysica Acta - Biomembranes* **1664**, 108-116.

Zehmer, J. K. and Hazel, J. R. (2005). Thermally induced changes in lipid composition of raft and non-raft regions of hepatocyte plasma membranes of rainbow trout. *Journal of Experimental Biology* **208**, 4283-4290.

Zhang, X. C., Liu, M. and Zhao, Y. (2015). How does transmembrane electrochemical potential drive the rotation of F(o) motor in an ATP synthase? *Protein & Cell* **6**, 784-791.

Zhou, C. and Zhang, C. (2005). Protective effects of antioxidant vitamins on Aroclor 1254-induced toxicity in cultured chicken embryo hepatocytes. *Toxicology in Vitro* **19**, 665-673.