

Electrospray ionisation mass spectrometric analysis of lipid restructuring in the carp (*Cyprinus carpio* L.) during cold acclimation

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Summary

Cold acclimation of carp from 30°C to 10°C causes a restructuring of liver microsomal phospholipids characterised by increased proportions of monounsaturated fatty acid in phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Here, we have used electrospray ionisation mass spectrometry (ESI-MS) to determine the patterns of alteration to individual molecular species compositions of PC, PE and phosphatidylinositol (PI) in response to gradually decreasing temperature. The results demonstrate that cold induces precise changes to a limited number of phospholipid species, and that these changes are distinct and different for each phospholipid class. The major change for PC was increased 16:1/22:6, but for PE the species that increased was 18:1/22:6. By contrast, the PI

species that increased during cold acclimation were characterised by an *sn*-1 monounsaturated fatty acid in combination with arachidonoyl or eicosapentaenoyl fatty acid at the *sn*-2 position. Analysis of acyl distribution indicates that cold only caused the accumulation of monounsaturated fatty acids at the *sn*-1 and not at the *sn*-2 position of phospholipids. These results highlight the tight and restricted range of modifications that membranes make to their phospholipid composition in response to thermal stress.

Key words: cold acclimation, carp, *Cyprinus carpio*, phospholipid, phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, monounsaturated fatty acid, ESI-MS.

Introduction

One of the most consistent responses to cold in poikilothermic organisms is the restructuring of cellular membranes in response to changing environmental temperature. Many poikilotherms have been shown to adjust the compositions of their cellular membranes to preserve physical properties such as membrane fluidity within a relatively tight physiological range. One mechanism to achieve this is to increase the proportion of unsaturated fatty acids in membrane phospholipids, thereby causing a disordering of the hydrocarbon interior of the membrane, which compensates for the cold-induced ordering (Cossins, 1994; Hazel, 1995). Maintenance of membrane structure is critical for survival, and the molecular composition of membrane phospholipids appears to be rapidly modified at different temperatures to keep the physical properties of the membrane relatively constant. Trueman et al. (2000) reported that endoplasmic reticulum phospholipids from carp liver showed substantial changes in fatty acid composition, with linear decreases in the proportion of saturates with temperature over a cooling regime of 30°C to 10°C. The plasma membrane of trout hepatocytes has also been

shown to be capable of a continuous lipid restructuring and turnover, responding rapidly upon cold exposure by adjusting membrane phospholipid composition (Williams and Hazel, 1994, 1995; Hazel and Williams, 1990).

Traditional methods of analysing the phospholipid components of biological membranes have tended to concentrate upon their component fatty acids, rather than examining the biologically relevant molecules in their entirety, thereby oversimplifying the processes occurring. Changes in lipid composition during temperature-induced membrane restructuring are extremely complex. The structure of membrane phospholipids is itself complicated, the physical properties of each molecular species being dependent upon headgroup, fatty acid chain length, unsaturation of individual fatty acids and the positional distribution of the two fatty acids within the molecule (Stubbs and Smith, 1984; Hazel and Williams, 1990). Inclusion of the first unsaturated bond causes the greatest change in the physical properties of any fatty acid, with subsequent unsaturation having progressively less effect (Coolbear et al., 1983; Stubbs and Smith, 1984).

The results from Trueman et al. (2000), which stimulated this further analysis, showed that cooling-induced lipid restructuring in carp liver microsomes was accompanied by co-ordinated expression of various lipid biosynthetic enzymes. Fatty acyl $\Delta 9$ -desaturase activity (EC No. 1.14.99.5) was an integral part of this response; $\Delta 9$ -desaturase is responsible for incorporating the first double bond into fatty acids at the C9–C10 position, causing maximum disordering. Previous studies have primarily tended to examine this conversion of the saturated fatty acids palmitate (16:0) and stearate (18:0) into their monounsaturated derivatives palmitoleate (16:1) and oleate (18:1). Analysis solely of the desaturase pathways, although important, ignores the complexity of the molecular species structure of membrane phospholipids and the mechanisms by which membrane fluidity is maintained. In addition to $\Delta 9$ - and other desaturases, a number of additional mechanisms contribute to the regulation of membrane phospholipid composition, including fatty acid elongases, acyltransferases, *de novo* synthesis, intracellular transport processes and phospholipase activities.

Previous analyses of the effects of temperature on the molecular structures of membrane phospholipids have been hampered by the laborious nature of the analytical procedures involved. These have generally involved sequential steps of thin layer chromatography, enzymatic hydrolysis by phospholipase C, formation of dinitrobenzoyl (DNB)-diacylglycerol derivatives, and resolution of DNB-diacylglycerol species by high-performance liquid chromatography (HPLC). In addition to the errors inherent in any multi-step procedure, identification of eluted species is often problematic, relying on relative retention time. Moreover, co-elution of multiple species has meant that complete identification of individual species has required repeat analyses using mobile phases of different polarities (Bell and Tocher, 1989).

Recently, the application of electrospray ionisation mass spectrometry (ESI-MS) to the analysis of molecular species compositions of membrane phospholipids has attracted great interest. ESI-MS provides a rapid and sensitive quantitative technique for the detailed characterisation of phospholipid molecular structures, and a variety of tandem MS/MS procedures can provide unequivocal assignments of both composition and positional assignment of fatty acids within each phospholipid species (Brügger et al., 1997; Han and Gross, 1995). In the present study, we have used ESI-MS to determine detailed changes to the molecular species of carp liver microsomal phospholipids over five days after a change in environmental temperature. The results demonstrate clearly both the relatively restricted nature and the precise regulation of the adaptive process involved.

Materials and methods

Animals

Carp (*Cyprinus carpio* L.) 400–500 g in mass, obtained from

Westlake Fisheries, Fiddlers Ferry, Widnes, UK, were maintained in 2000 l aquaria at Liverpool University for at least 3 months prior to the experiment. Temperature was kept constant at $30 \pm 1^\circ\text{C}$, with a photoperiod of 12 h:12 h light:dark, and fish were fed to satiation once a day on a commercial trout diet [size 50 pellet: protein 45%, oil 21%, ash 10%, fibre 1%; Trouw (UK) Ltd, Longridge, Preston, UK].

Cooling regime

Animals were gradually cooled from 30°C to the specific temperatures of 23°C , 17°C and 10°C and were maintained at those temperatures for five days (see Trueman et al., 2000, for full details of the stepwise cooling programme). All temperature changes were at a rate of 1°C h^{-1} for a maximum rate of change of 7°C day^{-1} (Schunke and Wodtke, 1983). One group of fish was maintained at 30°C throughout the experiment and sampled on day 5. For each sampling temperature, replicate fish were killed by pithing, their livers were removed and liver microsomal fractions were isolated.

Isolation of liver microsomes

Liver microsomes were prepared at Liverpool University using a modification of the method of Wodtke and Cossins (1991). All procedures were carried out at 0 – 4°C . Liver tissue was weighed, minced and homogenised in four volumes (w/v) buffer (250 mmol l^{-1} sucrose, 20 mmol l^{-1} Hepes, pH 7.4) using eight passes in a glass–Teflon homogeniser. The homogenate was centrifuged for 30 min at $10\,000g$, the supernatant removed and CsCl added to give a final concentration of 15 mmol l^{-1} before centrifuging again at $120\,000g$ for 80 min. The resulting pellet was gently resuspended in a saline solution (150 mmol l^{-1} NaCl, 0.1 mmol l^{-1} EDTA, 20 mmol l^{-1} Hepes, pH 7.4), taking care not to disturb the glycogen portion of the pellet, and was centrifuged at $120\,000g$ for 60 min. After centrifuging, the pellet was resuspended in a small volume of saline solution and frozen at -80°C .

Mass spectrometric analysis of phospholipids

Total lipids were extracted from $50 \mu\text{l}$ of liver microsomes using chloroform and methanol, according to Bligh and Dyer (1959). After drying under nitrogen gas, lipid was dissolved either in methanol:chloroform:water (7:2:1 v:v:v) for single-stage MS analysis and tandem MS analysis of PC, PI and PS or in the same solvent containing 0.5% (w/v) sodium iodide for tandem MS analysis of PE. All electrospray ionisation mass spectrometric (ESI-MS) analysis was performed using a triple-quadrupole tandem mass spectrometer (Quattro Ultima, Micromass, Manchester, England) equipped with a Z-spray electrospray ionization interface. Samples were infused with a syringe pump (Model 11, Harvard Apparatus, South Natick, MA, USA) at a flow rate of $5 \mu\text{l min}^{-1}$ directly into the mass spectrometer. Dry heated nitrogen was used as both the cone gas (70 l h^{-1}) and the desolvation gas (600 l h^{-1}), while dry argon was used as the collision gas (0.35 Pa).

Table 1. Summary of class-specific tandem mass spectrometric experiments performed on total phospholipid extract of carp liver microsomes

Phospholipid class	Ionisation	Scan mode	Reconstituted ions
Phosphatidylcholine	Positive	Parents of m/z 184	[M+H] ⁺
Phosphatidylethanolamine	Positive	Constant Neutral Loss of m/z 141	[M+Na] ⁺
Phosphatidylserine	Negative	Constant Neutral Loss of m/z 87	[M-H] ⁻
Phosphatidylinositol	Negative	Parents of m/z 241	[M-H] ⁻

Single-stage ESI-MS

Samples were analysed in both positive and negative ionisation modes with applied capillary voltages of +3kV and -3kV and cone voltages of +90V and -100V, respectively. Positive ionisation spectra were dominated by the PC species, while negative ionisation spectra contained peaks for all phospholipids present. All data were collected with a scan time of 2.5 s over a mass:charge (m/z) range of 600–950 and were recorded as a signal average of 10 scans at atomic resolution.

Tandem mass spectrometry

Although all phospholipids could be detected by single-stage MS, quantification can be problematic owing to signal overlap, differential ionisation and adduct formation. Tandem MS of phospholipids after electrospray ionisation can be used to confirm the identity of ions and can be diagnostic for a class of phospholipid within a complex mixture.

Tandem MS is accomplished by passing the precursor ion of selected m/z into the second quadrupole (collision cell). Collision-induced dissociation (CID) of the precursor ion with argon gas generates product ions, which are subsequently analysed in the third quadrupole. For unambiguous identification of individual phospholipids, the mass-selected precursor ion in negative ionisation is subjected to CID and the resulting product ion spectrum is routinely dominated by peaks corresponding to the fatty acid chains. Moreover, depending on the collision voltage used, preferential loss of fatty acyl products from either the *sn*-1 or the *sn*-2 position of the glycerol backbone can also provide evidence for the stereospecificity of individual phospholipids (Han and Gross, 1995). Such analysis, based on detection of fatty acyl product ions and of the acyl ketene moieties formed by loss of acyl groups (Han and Gross, 1995; Hsu and Turk, 2000), was performed to characterise all the molecular species assignments presented in this paper. Under more specific conditions, molecular ions of certain classes of phospholipids will generate diagnostic product ions (Brugger et al., 1997). Subsequent precursor scans that search for these specific product ions will then create a quantitative mass spectrum containing only peaks pertaining to that particular class. Such class-specific fragmentation and subsequent scanning modes established in our laboratory are outlined in Table 1.

Precursor scan data were recorded with a scan time of 12 s over m/z ranges of 675–860 (PC) and 750–925 (PI). Neutral loss spectral data were recorded with a scan time of 2.5 s over an m/z range of 625–900.

The spectra obtained were processed using Masslynx software

(Micromass, Manchester, UK). Preliminary studies (results not shown) established that samples were sufficiently diluted for all responses to be linear with concentration for each phospholipid class. Each phospholipid generated either a protonated [M+H]⁺ or sodiated [M+22]⁺ ion in positive ionisation and a deprotonated [M-H]⁻ molecular ion in negative ionisation, together with associated ¹³C-containing ions. Molecular species compositions of individual ion peaks were assigned on the basis of a combination of product, precursor and neutral loss scans as appropriate. Results are presented in terms of the predominant molecular species determined for each ion peak and are expressed as mol % of the total in each phospholipid class.

Statistical analysis

Data were analysed for significant differences between warm-acclimated control and cooled fish using one-way analysis of variance (ANOVA), adjusting for multiple comparisons with Bonferroni's test. $P \leq 0.05$ was used to establish statistical significance.

Results

Phospholipid composition of carp liver microsomes

Analysis of carp liver microsomes acclimated at 30°C showed that the major phospholipid groups present were phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) (Fig. 1. Table 2). The PC, PE and PI from carp liver microsomes were all dominated by molecular species containing polyunsaturated fatty acids (PUFA), with low proportions of species containing combinations of saturated and monounsaturated fatty acids. The spectra of all three phospholipid classes were relatively simple, comprising essentially 10 PC (Fig. 1A), 9 PE (Fig. 1C) and 8 PI (Fig. 1D) molecular species. Docosahexaenoate (22:6) was the major PUFA component within the PC and PE fractions, while arachidonate (20:4) was present in larger amounts in PI. There were also smaller amounts of phosphatidylserine (Fig. 1B; PS16:0/22:6, $m/z=806.5$; PS18:0/22:6, $m/z=834.6$) and phosphatidylglycerol (Fig. 1B; PG16:0/18:1, $m/z=747.6$) present within the total lipid extract. Surprisingly, given the previous report of a relatively high abundance of eicosapentaenoic acid (20:5 n -3) in carp microsomal phospholipid (Gracey, 1996), this fatty acid could not be detected in any PC or PE molecular species and was only present in PI as PI18:1/20:5. This lack of 20:5 in PC and PE was confirmed by fragmentation product analysis by tandem MS/MS of all ion peaks that could possibly contain 20:5.

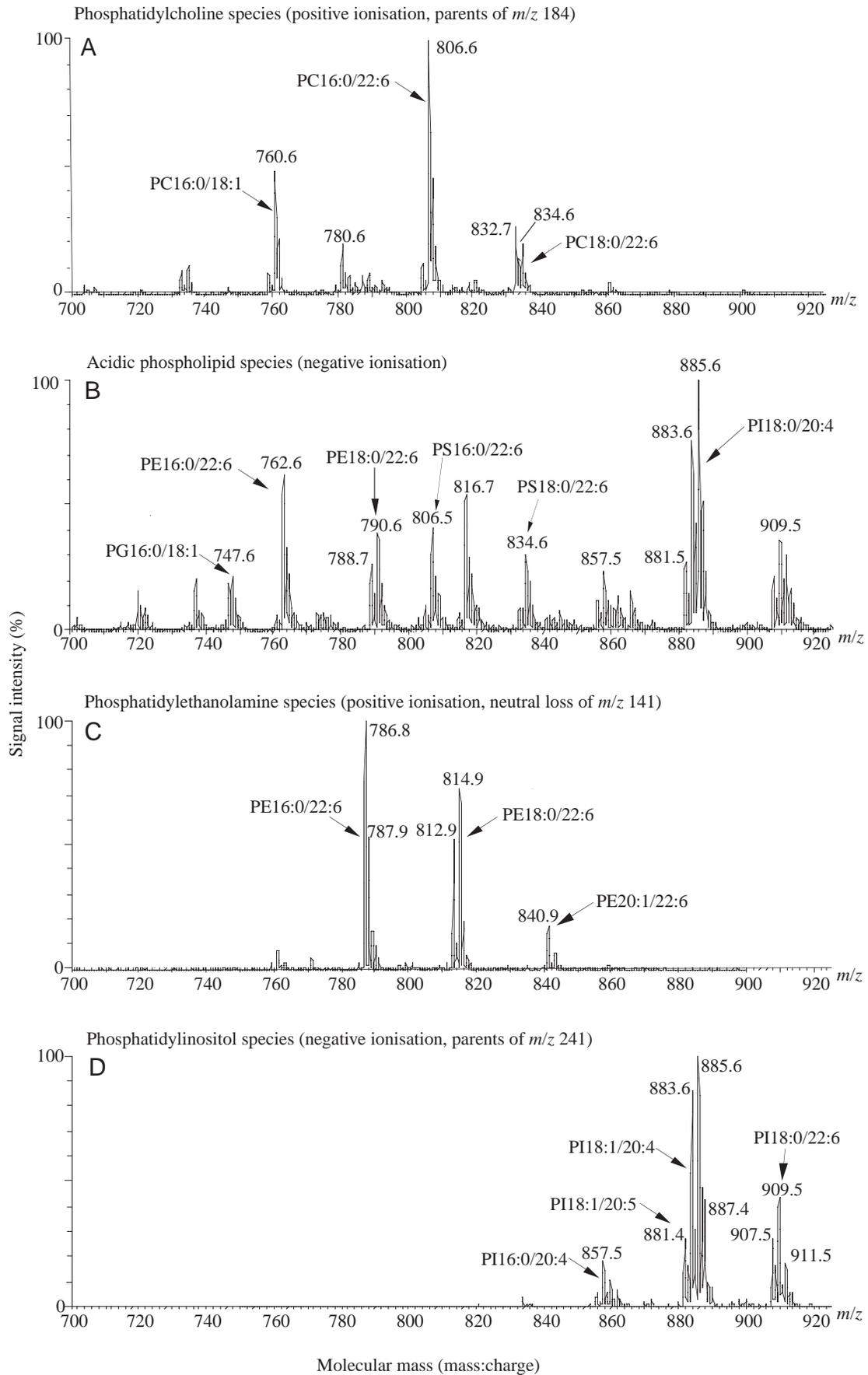


Fig. 1. Electrospray ionisation mass spectra showing molecular species composition of carp liver microsome phospholipids. As phosphatidylcholine (PC) species were partially sodiated under positive ionisation conditions (spectra not shown), they were analysed from precursor scans of m/z 184 (A) that selectively detected $[M+H]^+$ molecular ions. Acidic phospholipids were detected as molecular ions under negative ionisation conditions (B). As plasmalogen phosphatidylethanolamine (PE) species were not detected in this spectrum, PE species were quantified as sodium adducts from constant neutral loss scans of m/z 141 under positive ionisation conditions (C) after the addition of 5 mmol l^{-1} NaI. Such neutral loss scans selectively detected diacyl but not alkenylacyl PE species. Phosphatidylinositol (PI) species were identified from precursor scans of m/z 241 (D), while phosphatidylserine (PS) and phosphatidylglycerol (PG) were identified from constant neutral loss scans of m/z 87 and precursor scans of m/z 153, respectively (data not shown).

Table 2. Molecular species compositions of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) from microsomes prepared from livers of 30°C-acclimated carp

Molecular species	Composition (mol %, mean \pm S.D.)*		
	PC (N=4)	PE (N=4)	PI (N=3)
16:0/16:0	1.28 \pm 0.41 (734)	ND	ND
16:0/16:1	1.67 \pm 0.27 (732)	ND	ND
16:0/18:1	8.51 \pm 1.59 (760)	ND	ND
16:1/18:1	1.28 \pm 0.31 (758)	ND	ND
16:0/20:4	1.99 \pm 0.35 (782)	1.13 \pm 0.47 (738)	11.59 \pm 3.98 (857)
16:1/20:4	5.39 \pm 1.64 (780)	2.36 \pm 1.32 (736)	3.69 \pm 0.76 (855)
16:0/22:6	61.19 \pm 2.50 (806)	62.09 \pm 3.54 (762)	ND
16:1/22:6	4.03 \pm 0.79 (804)	1.47 \pm 0.77 (760)	ND
18:0/18:0	ND	2.17 \pm 1.75 (746)	ND
18:0/18:1	ND	0.93 \pm 0.66 (744)	ND
18:0/20:4	ND	ND	52.23 \pm 9.14 (885)
18:1/20:4	ND	ND	16.89 \pm 2.74 (883)
18:1/20:5	ND	ND	6.33 \pm 2.01 (881)
18:0/22:6	5.91 \pm 1.06 (834)	19.49 \pm 3.64 (790)	3.93 \pm 2.77 (909)
18:1/22:6	8.74 \pm 0.78 (832)	7.57 \pm 2.69 (788)	2.90 \pm 1.82 (907)
20:1/20:4	ND	ND	2.45 \pm 0.74 (911)
20:1/22:6	ND	2.81 \pm 0.57 (816)	ND

*Molecular mass:charge (m/z) is given in brackets below the mol % composition of each fatty acid. ND, not detected.

The molecular species analysis presented in Table 2 shows very clearly that, despite the restricted number of molecular species present, the compositions of PC, PE and PI were maintained very precisely and independently of each other. For instance, polyunsaturated molecular species containing 16:0 at the sn -1 position were predominant for PC and PE, while PI contained a higher proportion of species with sn -1 18:0. All monounsaturated PC species contained 16:0 or 16:1 at their sn -1 position, while monounsaturated PE species contained sn -1 18:0.

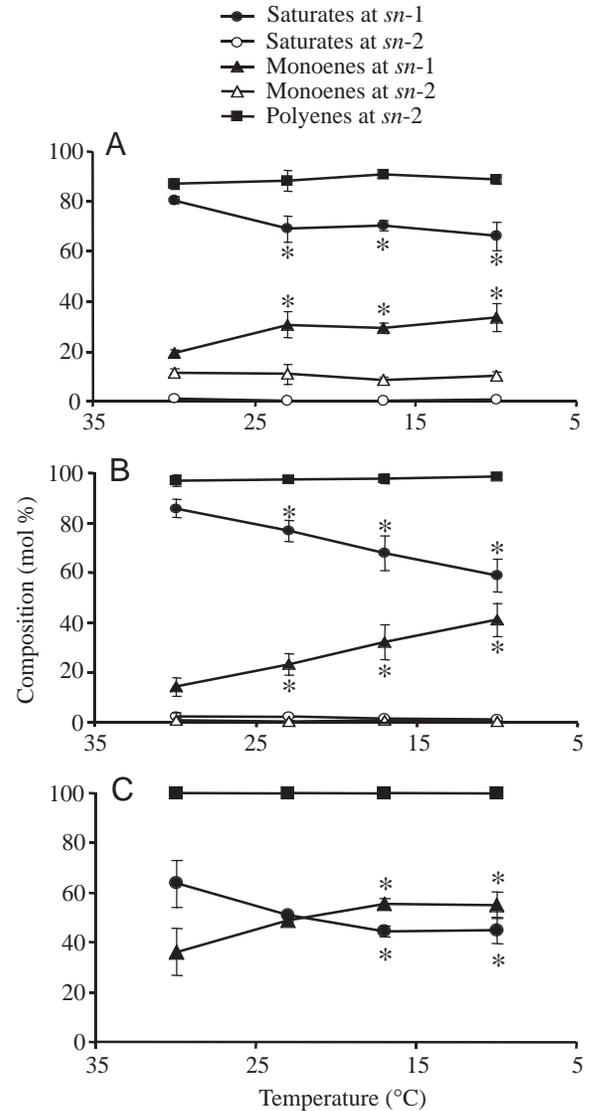


Fig. 2. Changes in fatty acid saturation of the molecular species components of the three phospholipid classes, (A) phosphatidylcholine (PC), (B) phosphatidylethanolamine (PE) and (C) phosphatidylinositol (PI), isolated from carp liver microsomes. Fish were kept at each temperature for five days before being killed. Values represent means \pm S.D. ($N=3-4$ individuals). A significant difference from the control value (30°C) is indicated by an asterisk [$P \leq 0.05$; one-way analysis of variance (ANOVA)].

Effects of cooling on microsome phospholipid composition Phosphatidylcholine

The total proportion of all PC species possessing saturated fatty acids at the sn -1 position was significantly higher in the control fish maintained at 30°C than in any of the cooled fish (Fig. 2A). By contrast, PC from fish kept at the three lower temperatures exhibited a greater proportion of monounsaturated fatty acids at the sn -1 position than did PC from 30°C control fish (Fig. 2A). There were no significant compositional differences observed at the sn -2 position of any of the molecular species measured. The proportion of PCs

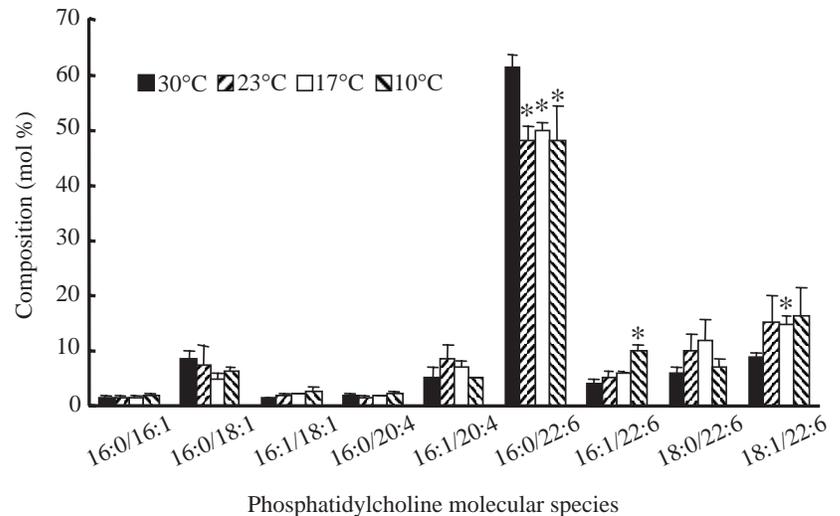


Fig. 3. The effect of temperature acclimation on the individual molecular species composition of the phosphatidylcholine fraction from carp liver microsomes. Results are expressed as mol % total phosphatidylcholine (means \pm S.D., $N=3-4$ individuals). A significant difference from the control value (30°C) is indicated by an asterisk [$P \leq 0.05$; one-way analysis of variance (ANOVA)].

possessing saturated fatty acids, monounsaturated fatty acids or polyunsaturated fatty acids at this position was not affected by temperature (Fig. 2A).

Phosphatidylethanolamine

The composition of PE molecular species altered in response to temperature with a very similar pattern to that of PC. Again, the fatty acid distribution at the *sn-2* position was not influenced by temperature (Fig. 2B), while the proportion of PE species with a saturated fatty acid at the *sn-1* position was significantly greater at 30°C than at the lower temperatures. Conversely, the proportion of PE species with a monounsaturated fatty acid at the *sn-1* position was significantly lower in the control fish (Fig. 2B) compared with fish maintained at all three lower temperatures.

Phosphatidylinositol

In contrast to both PC and PE, no PI species was detected with either a saturated fatty acid or a monounsaturated fatty acid at the *sn-2* position; all of the PI species contained a polyunsaturated fatty acid at the *sn-2* position. As with PC and PE, the proportion of saturated or monounsaturated fatty acids at the *sn-1* position of PI changed significantly with temperature. At 30°C, the PI species contained significantly more saturated fatty acids than at the lower temperature (Fig. 2C), while the reverse was observed for PI species containing monounsaturated fatty acids.

Individual molecular species

Phosphatidylcholine

The modulation of PC molecular species present in liver microsomes in response to temperature was relatively restricted and modest. The proportions of the three monounsaturated PC species, PC16:0/16:1, PC16:0/18:1 and PC16:1/18:1, did not change significantly with acclimation temperature (Fig. 3). By contrast, the already low proportion of PC16:0/16:0 at 30°C ($1.80 \pm 0.57\%$) was significantly lower

at 23°C ($0.77 \pm 0.01\%$) and 17°C ($0.67 \pm 0.22\%$). The proportions of the major polyunsaturated PC species changed in response to temperature, but the temperature dependence of such change varied for individual species. For instance, the proportion of PC16:0/22:6 decreased between 30°C ($61.19 \pm 2.50\%$) and 23°C ($47.78 \pm 2.82\%$) and then remained constant down to 10°C, while the proportion of PC16:1/22:6 only increased significantly between 17°C ($5.80 \pm 0.40\%$) and 10°C ($9.92 \pm 1.23\%$) (Fig. 3).

Phosphatidylethanolamine

Significant temperature-dependent changes were observed for proportions of three of the four major PE species, but for none of the five minor PE species (PE18:0/18:0, PE18:0/18:1, PE16:0/20:4, PE16:1/20:4, PE16:1/22:6; Fig. 4). As for PC, the proportion of the predominant PE species (PE16:0/22:6) decreased significantly between 30°C ($62.09 \pm 3.54\%$) and 23°C ($46.31 \pm 9.23\%$) and then remained constant down to 10°C. By contrast, proportions of the two PE species containing both 22:6 and a monounsaturated fatty acid both increased incrementally between 30°C and 10°C (Fig. 4); the proportion of PE18:1/22:6 increased from $7.57 \pm 2.69\%$ at 30°C to $21.49 \pm 5.63\%$ at 10°C, while corresponding values for PE20:1/22:6 were $2.81 \pm 0.57\%$ and $14.43 \pm 3.47\%$ at 30°C and 10°C, respectively. It is important to note that the proportion of PE species containing 18:1 only changed with temperature when that fatty acid was at the *sn-1* and not the *sn-2* position. This was shown clearly by the progressive increased proportion of PE18:1/22:6 with decreasing temperature in contrast to the unaltered concentration of PE18:0/18:1.

Phosphatidylinositol

The composition of liver microsomal PI altered in response to temperature to a greater extent than that of either PC or PE. As with PC and PE though, there was a consistent pattern to these changes, with increased proportions of PI species containing *sn-1* monounsaturated fatty acids and decreased proportions of PI

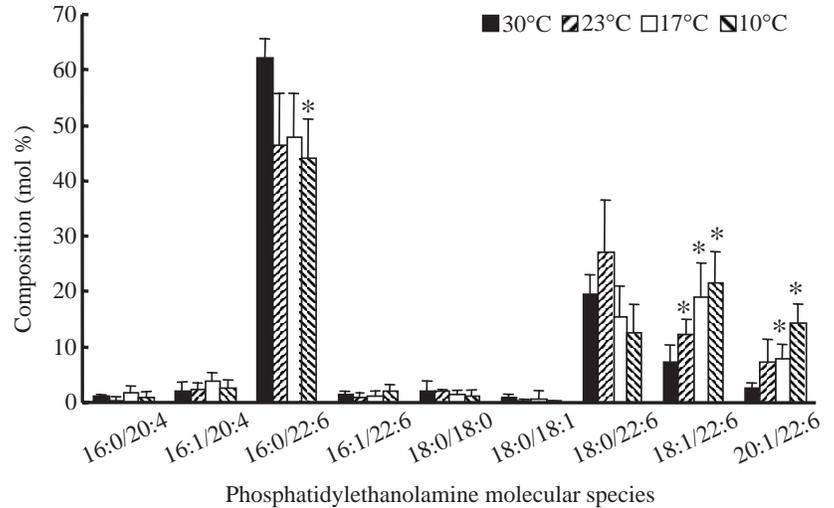


Fig. 4. The effect of temperature acclimation on the individual molecular species composition of the phosphatidylethanolamine fraction from carp liver microsomes. Results are expressed as mol % total phosphatidylethanolamine (means \pm S.D., $N=3-4$ individuals). A significant difference from the control value (30°C) is indicated by an asterisk [$P \leq 0.05$; one-way analysis of variance (ANOVA)].

species containing *sn*-1 saturated fatty acids (Fig 5) at lower temperatures. This was most apparent for the considerable and progressive decreased proportion of PI18:0/20:4 from 52.2 \pm 9.1% at 30°C to 29.99 \pm 5.72% at 10°C. The parallel decreased proportion of PI16:0/20:4 was of smaller magnitude and was only significant at 23°C and 17°C. By contrast, cooling from 30°C to 10°C significantly and progressively increased the proportions of PI18:1/20:4 (from 16.9 \pm 2.7% to 22.85 \pm 2.34%), PI18:1/20:5 (from 6.3 \pm 2% to 11.30 \pm 1.80%) and PI20:1/20:4 (from 2.45 \pm 0.7% to 10.16 \pm 2.13%). Proportions of PI species containing 22:6 (PI18:0/22:6 and PI18:1/22:6) did not alter systematically with variation in temperature, although that of PI18:1/22:6 was significantly higher at 17°C. The only PI species containing a monounsaturated fatty acid not to respond to cooling at any temperature was PI16:1/20:4.

Discussion

There is substantial evidence to suggest that poikilothermic organisms, including several fish species, possess the ability to modify the fatty acid composition of their membrane

phospholipids in response to changes in temperature (Logue et al., 1995; Tiku et al., 1996). Increased concentrations of unsaturated fatty acids in membrane phospholipids at lower temperatures have been linked with retaining membrane fluidity, a process termed homeoviscous adaptation (Macartney et al., 1994). In this study, ESI-MS was used to define the precise nature of the changes to phospholipid molecular species of carp liver microsomes that occur in response to lower environmental temperature. ESI-MS resolves intact molecular species and provides unambiguous positional assignment of the fatty acid distribution at the *sn*-1 or *sn*-2 position of individual phospholipid molecular species (Han and Gross, 1995; Hsu and Turk, 2000). We chose to study effects over the first five days after initiation of the cooling regime because the lipid response was essentially completed during this time (Trueman et al., 2000) and this period related to induced activity of the $\Delta 9$ -acyl CoA desaturase (Tiku et al., 1996).

ESI-MS analysis facilitated the analysis of the effects of decreasing temperature on PI as well as on PC and PE molecular species, and the different responses of these three

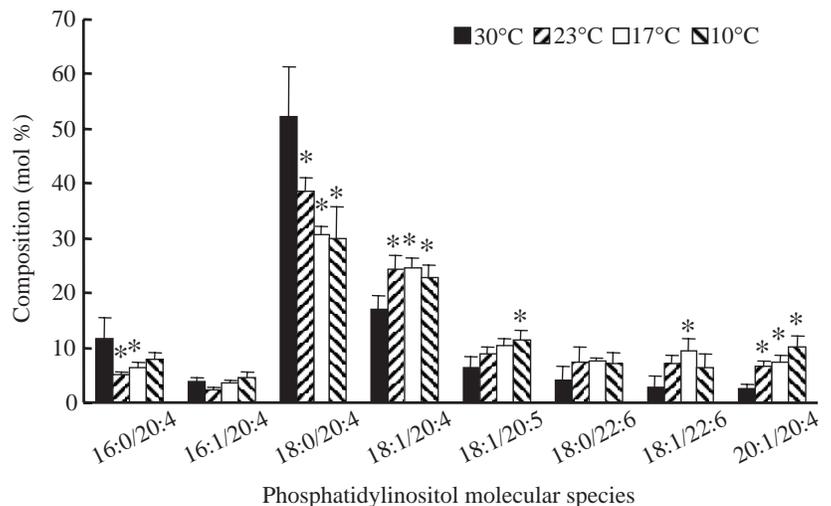


Fig. 5. The effect of temperature acclimation on the individual molecular species composition of the phosphatidylinositol fraction from carp liver microsomes. Results are expressed as mol % total phosphatidylinositol (means \pm S.D., $N=3-4$ individuals). A significant difference from the control value (30°C) is indicated by an asterisk [$P \leq 0.05$; one-way analysis of variance (ANOVA)].

phospholipid classes to temperature change are potentially important for maintenance of membrane function. The fundamental principles defining these changed compositions were essentially similar for all three phospholipid classes, with increased proportions of species containing monounsaturated fatty acids at the expense of species containing saturated fatty acids. The extent of these changes, however, both in magnitude and in the number of species affected, was greatest for PI, then for PE and finally for PC. Unfortunately, the poor availability of synthetic individual molecular species of PI has precluded comparison of the liquid crystal to gel transition temperatures of PI, PE and PC species. Consequently, it is not clear whether modulation of PI species composition in response to temperature will exert a disproportionate effect on membrane structure and function.

It is not surprising that cooling was not associated with any change to the proportions of PC, PE or PI species containing polyunsaturated fatty acids esterified at the *sn*-2 position of the glycerol backbone of the molecule. As all three phospholipid classes already contained high proportions of these fatty acids at 30°C, increasing their content still further at lower temperatures would have exerted little effect on membrane structure or fluidity. The greatest effect on the physical properties of a fatty acid is apparent on introduction of the first unsaturated double bond into a saturated molecule (Coolbear et al., 1983; Stubbs and Smith, 1984). This is reflected in the strategy adopted by carp liver microsomes to adapt to lower temperatures, with saturated fatty acids being replaced by monounsaturated fatty acids in all phospholipid classes. It is important to recognise, however, that this response is highly regulated and co-ordinated and cannot be simply a consequence of increased $\Delta 9$ -desaturase activity providing more monounsaturated fatty acids for esterification into phospholipids at lower temperature. While induction of the $\Delta 9$ -desaturase contributes to the overall temperature response (Trueman et al., 2000), it cannot explain why the response is restricted to selected individual molecular species of phospholipids containing monounsaturated fatty acids or why such changes were only apparent for fatty acids esterified at the *sn*-1 and not at the *sn*-2 position.

This selectivity of the response to temperature can be seen most clearly for the composition of microsomal PC (Fig. 3), which was enriched specifically with PC16:1/22:6 and PC18:1/22:6 at lower temperatures at the expense of PC16:0/22:6. The lack of change to other PC species containing either 16:1, 18:1 or 22:6 emphasises the precision of the response. For instance, over the same temperature range that modulated the proportions of PC16:1/22:6 and PC16:0/22:6, no response was apparent for PC18:1/22:6, PC18:0/22:6, PC16:1/20:4 or PC16:1/18:1 proportions. Contrasting selectivity was also apparent for the response of PE composition to lower temperatures (Fig. 4), as increased proportions were apparent only for PE18:1/22:6 and PE20:1/22:6 and not for PE16:1/22:6 or any other PE species. The observation that PE18:0/18:1 concentration did not alter with temperature also demonstrates the positional selectivity of

the response, clearly showing that the lower temperatures only stimulated incorporation of the 18:1 monounsaturated fatty acid into the *sn*-1 and not into the *sn*-2 position of PE.

The effect of temperature acclimation in fish on the molecular species compositions of liver PI has not previously been examined. While temperature selectivity of the PI response was initially less obvious, this may reflect the very different composition of PI from PC and PE molecular species in carp liver microsomes (Table 2). PC and PE compositions were dominated by species containing 22:6 n -3, but PI was predominantly composed at all temperatures of species containing 20:4 n -6 at the *sn*-2 position. The details of the PI response, however, are intriguing and differ considerably from those of either PC or PE. The proportion of PI18:0/20:4, which remained the predominant PI species at all temperatures, decreased by over 40% between 30°C and 10°C (Fig. 5). The large magnitude of this decrease was not accompanied by any correspondingly large increased proportion of an individual PI species containing monounsaturated fatty acids. Instead, proportions of three such PI species, PI18:1/20:4, PI18:1/20:5 and PI20:1/20:4, increased in parallel with decreasing temperature. PI was the only phospholipid class where significant modification to the proportions of fatty acids esterified at the *sn*-2 position was observed, shown by the increased proportions of PI18:1/22:6 at 17°C and PI18:1/20:5 at 10°C.

These results confirm and extend those in the earlier study by Trueman et al. (2000), which examined the effects of differential cooling on the fatty acid composition of the phospholipid classes PC and PE. We demonstrate the enhanced analysis possible by analysis of individual molecular species instead of total fatty acid compositions. For instance, inspection of the PC fraction shows clearly that the significant increased proportion of total 16:1 n -9 in PC after cooling for a period of five days (Trueman et al., 2000) reflected the increased contribution of a single PC species (PC16:1/22:6) with no alteration to other PC species containing 16:1 (Fig. 3). Similarly, Trueman et al. (2000) found transient changes in the total 16:1, 18:1 and 20:1 contents of the PE fraction depending upon acclimation temperature and length of acclimation. Molecular species analysis shows that the increased total 20:1 reflected solely the greater quantities of PE20:1/22:6 at lower temperatures (Fig. 4) and also explained the transient nature of the temperature response of total 18:1 n -9 in PE. Lower temperatures (Fig. 4) were associated with a range of non-significant changes to the contents of a range of PE species containing 18:1 n -9. The variation in these changes was sufficient to mask the significant increased proportion of PE18:1/22:6 when combined for total fatty acid analysis.

While considerable attention has been given to the effects of temperature acclimation on membrane phospholipid compositions (Stubbs and Smith, 1984; Cossins, 1994; Hazel, 1995; Hazel and Williams, 1990), the basic mechanisms that regulate membrane phospholipid compositions have not been clearly identified. By comparison, extensive studies in rat liver have demonstrated a complex interaction of phospholipase

and acyltransferase activities that regulates dynamic processes of acyl remodelling. For instance, analysis of phospholipid synthetic pathways using radiochemical HPLC has consistently demonstrated an initial synthesis of PC16:0/18:2 by rat liver *in vivo* (Burdge et al., 1994) and in isolated rat hepatocytes (Tijburg et al., 1991), with subsequent conversion to other PC species by processes of acyl exchange. Such acyl remodelling is responsible for the incorporation of 20:4n-6 into rat liver PC, while 22:6n-3 is introduced into rat liver PC by *N*-methylation of PE. One study that followed the incorporation pattern of exogenously added radiolabelled phospholipids has indicated that a complex interaction of deacylation/reacylation, elongation and desaturation reactions is similarly involved in the early restructuring of PC molecular species from plasma membrane fractions of isolated trout hepatocytes (Williams and Hazel, 1995). The precise details of such acyl remodelling of phospholipids in fish liver, however, is still unknown. Such information will be essential to determine the mechanisms regulating membrane adaptations to temperature change. In this context, the great sensitivity and selectivity of phospholipid molecular species analysis by ESI-MS should prove especially valuable. ESI-MS cannot only provide detailed compositional information but is ideally designed for metabolic studies of phospholipid synthesis and turnover using deuteriated substrates (Hunt et al., 2001).

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