Characterisation of the circulating and tissue-specific alterations to the lipidome in response to moderate and major cold stress in mice

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Abstract

This study analysed the effects of 24 hours of cold stress (22°C or 5°C *vs*. mice maintained at 30°C) on the plasma, brown adipose tissue (BAT), subcutaneous (SubQ) and epididymal (Epi) white adipose tissue (WAT), liver, and skeletal muscle lipidome of mice. Using mass spectrometry-lipidomics 624 lipid species were detected, of which 239 were significantly altered in plasma, 134 in BAT, and 51 in the liver. In plasma, acylcarnitines and free fatty acids were markedly increased at 5°C. Plasma triacylglycerols (TGs) were reduced at 22°C and 5°C. We also identified ether lipids as a novel, cold-induced lipid class. In BAT, TGs were the principal lipid class affected by cold stress, being significantly reduced at both 22°C and 5°C. Interestingly, while BAT TG species were uniformly affected at 5°C, at 22°C we observed species-dependent effects, with TGs containing longer and more unsaturated fatty acids particularly sensitive to the effects of cold. In the liver, TGs were the most markedly affected lipid class, increasing in abundance at 5°C. TGs containing longer and more unsaturated fatty acids accumulated to a greater degree. Our work demonstrates: (1) Acute exposure to moderate (22°C) cold stress alters the plasma and BAT lipidome; although this effect is markedly less pronounced than at 5°C. (2) Cold stress at 5°C dramatically alters the plasma lipidome, with ether lipids identified as a novel lipid class altered by cold exposure. (3) That cold-induced alterations in liver and BAT TG levels are not uniform, with changes being influenced by acyl chain composition.

Keywords: Lipids; metabolism; environmental temperature; cold; mass spectrometry

Introduction

Cold stress is a significant challenge to organismal homeostasis. In mammals, brown adipose tissue (BAT) has evolved to offer protection during times of either cold stress or arousal from hibernation (2). While the importance of the thermogenic capacity of BAT has long been recognised as crucial in adaptive responses to cold in rodents, the re-discovery and definitive identification of metabolically active BAT in adult humans (4, 26, 31, 32, 36) has stimulated a resurgence in the interest in the control of thermogenesis. A principal reason for this is because BAT is a metabolic sink for glucose, several types of lipids, and branched chain amino acids (BCAAs) (1, 28, 34). Accordingly, targeting the thermogenic potential of BAT is suggested as a potential therapy to treat obesity and its associated metabolic complications (28).

Another reason for the renewed interest in the physiological effects of cold is because the majority of pre-clinical studies house mice at 20-22°C, significantly below their thermoneutral temperature of ~30°C (3). Such conditions will induce cold stress and concomitant BAT activity and may limit the translational potential of pre-clinical studies, particularly in mouse models of metabolic disease where elevations in metabolites such as fatty acids, glucose, and BCAA, all of which are key thermogenic substrates for activated BAT, contribute to disease progression (7, 25). Currently, however, little is known about how the moderate cold stress associated with housing at 20-22°C impacts circulating and tissue metabolites.

Several previous studies have used mass spectrometry-based approaches to examine how cold influences circulating and adipose tissue lipids. Specifically, it has been shown that cold exposure at 4°C for 3 to 7 days alters BAT TG composition (18, 19), although effects in WAT were relatively modest (18). Decreased circulating phosphatidic acid species were identified as a signature of cold exposure at 4°C for 7 days (18). Excitingly, Villanueva and

co-workers showed that exposure at 4°C for 24 hrs increased plasma acylcarnitine levels which subsequently fuelled BAT thermogenesis (29).

These previous studies have focused on the effects of cold exposure on lipids in either the adipose tissue (BAT and WAT) or plasma. Global profiling of the lipid changes in other metabolically important organs, such as the skeletal muscle and liver, may identify unrecognised effects of cold exposure. Moreover, previous studies have only examined lipid changes following cold stress at 4°C (18, 19, 29). How the moderate cold stress typical of standard mouse vivariums (20-22°C) impacts tissue lipids, both in relation to thermoneutrality (~30°C) or a more severe cold challenge (~4°C), is unknown. To address these gaps in our knowledge, we used mass spectrometry to assess the plasma, BAT, liver, subcutaneous (SubQ) and epididymal (Epi) WAT, and skeletal muscle (gastrocnemius) lipidome of mice housed at either 5, 22, or 30°C for 24 hours.

Materials and Methods

Mice

Male wildtype C57BL/6J mice (n=19) were bred and sourced from a colony housed at the Alfred Medical Research and Education Precinct Animal Services (AMREP AS). All animals were fed a normal chow irradiated rodent diet for the duration of the study (14.0MJ/kg, 75.2% kJ from carbohydrate, 4.8% from fat, 20% from protein; Specialty Feeds, Glen Forrest, Western Australia, Australia). Mice had free access to food and water (except for in the fasting period before endpoint) and maintained in an environmental setting set at 22±1°C with a 12 h light/dark cycle. The experiments were approved by the Alfred Medical Research Education Precinct (AMREP) Animal Ethics Committee and conducted according to the guidelines of the National Health and Medical Research Council (NHMRC) of Australia for animal experimentation.

Study Design

Mice were transferred to a room set at 30°C to acclimatise to thermoneutrality where they were group housed for one week. Following this, they were randomly divided into three groups to be singly housed at either: (1) 30°C (n=7), (2) 22°C (n=6), or (3) 5°C (n=6) for the next 24 hrs. Before and after the 24 hr period, mice were weighed and their body composition assessed. For the last 5 hrs of the 24 hr period, mice were fasted. At the end of this 24 hr period, mice were euthanised and interscapular brown adipose tissue (BAT), liver, SubQ and Epi WAT, skeletal muscle (gastrocnemius) and blood collected (plasma separated) and stored at -80°C for later processing. An overview of the study procedures and a consolidated standards of animal experiment reporting (CONSAERT) flow diagram are shown in are shown in Supplementary Figures 1A and B, respectively.

104 **Body Composition Analysis**

105 Fat mass and lean mass of the mice were measured using a 4-in-1 EchoMRI body
106 composition analyser (EchoMRI^{TM}, Houston, TX, USA) as previously described in full (17)
107 and standard scales were used for measuring total body mass (Mettler Toledo, Greifensee,
108 Switzerland).

109 **Lipidomics**

110 Tissues (20-40 mg wet weight) were homogenised in PBS (without calcium or magnesium)
111 with either a motorised homogeniser (gastrocnemius) or a Pestle motor (liver, BAT, SubQ
112 WAT; Chemglass LifeSciences; CLS-5001;). Extracts were then sonicated (20 s at 17%
113 amplitude; Misonix ultrasonix liquid processor with Q Sonica CL5). Protein concentrations
114 of the tissue samples were determined and samples were diluted in PBS to 2.5µg of protein/µl
115 for liver or 5µg/µl for all other tissues. Lipids were extracted from 10µl of this tissue lysate
116 (25µg of protein for liver and 50µg for all other tissue) and 10µl of plasma using a single
117 phase chloroform methanol extraction, as described previously (33), and analysed by liquid
118 chromatography (LC) ESI-MS/MS using an Agilent 1290 LC system and Agilent 6490 triple
119 quadrupole mass spectrometer. The mass spectrometer conditions used here have been
120 described previously (13, 14). Owing to their complex nature, individual triglyceride species
121 were measured using two approaches to obtain more quantitative results. Initially, each
122 triglyceride sum composition was measured using single ion monitoring [SIM] where no
123 fragmentation was performed. Then to obtain better information regarding each triglyceride
124 sum composition, multiple reaction monitoring was used, where fragments corresponding to
125 the neutral loss (NL) of a fatty acid was monitored. In general the SIM analysis provides the
126 most quantitative result, as the NL measurement would result in both differing signal
127 responses and overlapping signals between isomeric species due to the number of acyl

combinations. Overall TG saturation status was determined by summing SIM TG species with a given number of double bonds, regardless of acyl chain composition. Overall SIM TG acyl chain composition was determined by summing TG species with a given number of carbon atoms within the fatty acyl chains, regardless of their saturation status.

Statistical Analysis

Statistical analysis was initially performed in R. A 1-way analysis of variance (ANOVA) was performed on each of the 624 lipids to determine if statistically significant differences were present between any of the 3 groups (30, 22, and 5°C). The Benjamini-Hochberg correction was applied to control the false discovery rate (10%). Tukey's honestly significant difference test was applied to determine which groups statistically significantly differed from each other. This analysis was performed in GraphPad Prism version 7. One sample from the liver 5°C group was excluded as the majority of the individual TG species as well as total TG were greater than 2 SDs away from the mean of the group. No other samples were omitted from any of the analysis. The phenotypic data shown in Figure 1 was also analysed by 1-way ANOVA and Tukey's HSD. Box and whisker plots show the median, 25^{th} and 75^{th} percentiles (hinges of the box), the min and max values (whiskers), as well as all data points. Statistical significance was set at $P<0.05$.

Results

Body weight characteristics following 24 hr housing at 30, 22, or 5°C

After 1 week of housing at 30°C, mice were randomly assigned to either remain at 30°C for 24 hr, or be exposed to an acute cold stress, 22°C or 5°C, for 24 hr. No differences in body weight, fat mass or lean mass were observed between the groups prior to the 24 hr acute cold challenge (data not shown). As expected, mice housed at 5°C for 24 hr had a significant decrease in body weight, lean mass, and fat mass compared with mice housed at either 30°C or 22°C (Figure 1A-C). Housing at 22°C did not affect either body weight, lean mass, or fat mass (Figure 1).

Acute cold exposure alters the plasma lipidome

Firstly, we determined how moderate (22°C) and major (5°C) cold stress affected the abundance of lipid species in the circulation. Lipid species from numerous lipid classes were altered by acute cold exposure, with 239 being significantly different between at least 2 of the experimental groups out of the 624 lipids detected (including 41 SIM TGs and SIM TG(O)s) (Figure 2A and Supplementary Table 1; all Supplementary Figures are available at the following link: https://doi.org/10.6084/m9.figshare.12298976). As expected, relative to mice housed at 30°C, alterations in the plasma lipidome were more marked in mice housed at 5°C compared with 22°C (Figure 2). Consistent with previous findings, the most significantly affected lipids classes in plasma at 5°C were acylcarnitines (12 significantly changed/12 measured) and free fatty acids (16/17) (Supplementary Figure 2). Notably, however, cold exposure at 22°C did not affect any free fatty acid or acylcarnitine species (Figure 2B-C).

Plasma TGs were markedly affected by acute cold exposure, with 46/77 (60%) specific NL TGs and 23/30 (70%) SIM TGs being significantly reduced at 5°C (Figure 2A and D). This decrease was similar for TGs of varying total acyl chain length and saturation status (Figure 2D). Notably, of the 39 lipids in plasma that were significantly different between mice housed at 30°C and 22°C, 14 were specific NL TGs and a further 13 were SIM TGs (6 representative SIM TGs are shown in Figure 2D). These changes led to a modest, but statistically significant decrease in total plasma TG between mice housed at 22 and 30°C (Figure 2D; first panel). While we did not observe a decrease in total DGs, several DG species (8/25) were reduced following acute cold exposure, in particular at 5°C (Supplementary Figure 3A). Several lysophospholipids, in particular LPCs (16/25), were reduced in mice housed at 5°C relative to those housed at either 22°C or 30°C (Figure 2A and Supplementary Figure 3B). A number of sphingolipid species (24/134) were also altered in mice housed at 5°C compared with mice housed at either 22°C or 30°C (Figure 2A and Supplementary Figure 3C).

Numerous phospholipids were affected by cold exposure (78/205) (Figure 2A). Interestingly, a large proportion of these changes were within the ether lipid class of phospholipids (Figure 3A; 48/84 (58%)) relative to conventional diacyl phospholipids (30/121 (25%)). Total levels of PC(O) and PE(O), in which an alkyl chain is attached via an ether bond to the sn-1 position of the glycerol backbone, and PC(P), in which an alkenyl chain is attached via an ether bond to the sn-1 position of the glycerol backbone, were all significantly increased following acute exposure at 5°C (Figure 3B). While total PE(P) levels were not significantly altered, numerous individual PE(P) species were significantly increased following acute exposure at 5°C (Figure 3A). The sn-1 position alcohols are typically either 16:0, 18:0, or 18:1; we noted that PE(P) containing an 18:0 alkenyl chain at the sn-1 position tended to be the significantly increased PE(P) species. The total levels of

PE(P) containing an 18:0 sn-1 position fatty-alcohol, but not 16:0 or 18:1, was significantly increased at 5°C (Figure 3C). At the individual species level, 2/9 PE(P-16:0), 7/9 PE(P-18:0), and 0/9 PE(P-18:1) species were altered by acute cold exposure at 5°C. While we do not have full compositional data for all PC(O), PC(P), and PE(O) species, we did observe significant increases in PC(O), PC(P), and PE(O) species containing 16:0 and 18:0 fatty alcohols following cold exposure (Figure 3A and D). Although these effects were most marked at 5°C, significant increases were also observed at 22°C (Figure 3D).

The effect of acute cold exposure on the BAT lipidome

BAT plays a key role in the response to cold stress and, therefore, we next examined how moderate (22°C) and major (5°C) cold stress affected the abundance of lipid species in BAT. Of the 624 lipids detected in BAT (including 41 SIM TGs and SIM TG(O)s), 134 were significantly different between at least 2 of the experimental groups (Figure 4A and Supplementary Table 2). Of these, 80/134 were TGs (56 specific NL TGs and 24 SIM TGs), the overall effect of which was to significantly decrease total TG following acute exposure at 5°C (Figure 4B). There was a tendency for decreased total TG at 22°C, but the changes were variable (Figure 4B). To provide a more detailed assessment of the impact of acute cold exposure on BAT TGs, we grouped SIM TGs (i) by the total number of acyl-chain carbons; and (ii) by their saturation status. With regards to TG total acyl chain length, an interesting pattern of changes was observed: (1) TGs with the fewest acyl chain carbons (48-51) were markedly reduced at 5°C, and somewhat reduced at 22°C (Figure 4C). TGs with 52 to 54 acyl chain carbons were only modestly reduced at 5°C, and were unaffected at 22°C (Figure 4C). (3) TGs with either 56 or 58 acyl chain carbons were markedly reduced at both 22°C and 5°C (Figure 4C). With regards to acyl chain saturation status, the most saturated TGs containing

0-2 doubles bonds were reduced at 5°C, while those containing 3-5 double bonds were only modestly affected by cold exposure at either 22°C or 5°C (Figure 4D). TGs containing 6-10 double bonds were markedly reduced at both 22°C and 5°C, with no significant differences observed between these two cold conditions (Figure 3D). These effects are exemplified in TGs with a total acyl chain length of 54, for which numerous different saturation statuses exist (Supplementary Figure 4A) and are indicative of preferential BAT TG utilisation in response to cold stress. A small number of phospholipid and sphingolipid species were also affected by cold exposure (Figure 4A and Supplementary Figure 4B).

The effect of acute cold exposure on the liver lipidome

While cold stress is known to affect liver lipid metabolism, how cold stress affects the liver lipidome has not previously been addressed. Of the 624 lipids detected in liver (including 41 SIM TGs and SIM TG(O)s), 51 were significantly different between at least 2 of the experimental groups (Figure 5A and Supplementary Table 3). Of these, 39/51 were TGs (26 specific NL TGs and 13 SIM TGs), with some DG species also altered (7/51). Despite an increase in a large number of TGs, the total TG level within the liver was not significantly increased, although a trend towards an increase at 5°C was evident (Figure 5B). To further interrogate the liver TG changes we grouped SIM TG species by saturation status. This revealed a clear effect of saturation status on the degree to which acute cold exposure affected TGs (Figure 5C). Specifically, TGs containing 0 or 1 double bond were not altered by acute cold exposure, TGs with 2-4 double bonds were significantly increased, although relatively modestly, while those TGs containing more than 5 double bonds, in particular those TG species with more than 7 double bonds, were very markedly increased by acute cold exposure (Figure 5C). Furthermore, while no differences in liver TGs containing 0-5 double

bonds was observed when comparing 22°C *vs* 5°C, TGs containing 6-10 double bonds were greatly increased at 5°C compared with 22°C (Figure 5C). Representative SIM TG species with varying saturation status are shown in Figure 5D. While TGs tended to be increased at 22°C relative to 30°C, (Figure 5B and C), these differences did not reach statistical significance. A similar pattern of effects was observed for DGs. Specifically, while total DGs and those containing saturated and/or monounsaturated fatty acids were unaffected by acute cold exposure, DG species containing a polyunsaturated fatty acid were significantly increased at 5°C (Figure 5E).

The effect of acute cold exposure on the skeletal muscle and subcutaneous and epididymal white adipose tissue lipidome

Skeletal muscle plays an important role in thermogenesis via shivering-induced heat production. How, or indeed if, acute cold exposure influences the skeletal muscle lipidome is has not been addressed. Moderate or major cold stress had very limited effects on lipid levels within the gastrocnemius, with none of the 624 lipids assessed being significantly different between the groups following FDR correction (Supplementary Tables 4-6). Some TG species showed a trend towards being increased following cold exposure at 5°C (Supplementary Figure 5A). With regards to DGs, several DG species tended to increase following acute cold exposure at 5°C, however, substantial variability in the data was observed, notably within the 22°C group C (Supplementary Figure 5B). While the changes in the gastrocnemius are marginal, they were largely restricted to TGs and DGs, both of which tended to increase following acute cold exposure at 5°C, similar to what was observed in the liver. Finally, we examined the effects of acute cold exposure on SubQ and Epi WAT. After FDR correction, no lipids in either of these WAT depots were significantly changed (Supplementary Tables 4-

6). While not significant, a trend towards decreased total TG levels and TG species following acute cold exposure at 5°C was evident for SubQ WAT (Supplementary Figure 5C). Total TG levels within the Epi WAT were completely unaltered following acute cold exposure (Supplementary Figure 5D). Given the marked increase in plasma free fatty acids (Figure 2B) and the decrease in fat mass (Figure 1C) observed at 5°C, a decrease in SubQ and/or Epi WAT TG levels might have been expected. The lack of difference in adipose TGs is probably because we extract a standardised amount of sample during the lipid extraction procedure. This paucity of significantly altered lipid species in SubQ WAT following acute cold exposure is relatively consistent with a previous report, who likewise observed far fewer lipid changes in WAT relative to BAT in response to cold exposure at 4°C (18).

Our results demonstrate that lipid changes in response to acute cold stress occur principally in the plasma, BAT, and liver. We performed a Venn diagram analysis to determine the commonalities and distinctions in the lipid changes in plasma, BAT, and liver in the following conditions: 30°C *vs.* 22°C (Supplementary Figure 6), 30°C *vs.* 22°C (Supplementary Figure 7), and 22°C *vs.* 5°C (Supplementary Figure 8). While numerous lipid species were significantly altered at 22°C compared with 30°C (75 in BAT, 39 in plasma, 2 in liver), very few of these changes were shared between BAT, plasma, and liver. Indeed, while the majority of the significantly altered lipids in BAT and plasma were TGs, there was minimal overlap observed in the significantly altered TG species (Supplementary Figure 6). Specifically, while the BAT TGs affected were of varying saturation status and acyl chain length, those TGs that were significantly altered in the plasma primarily contained 15-18 carbon long fatty acyl chains (Supplementary Figure 6). Similarly, there was little overlap in the significantly altered lipids in the 22°C *vs*. 5°C analysis (Supplementary Figure 7). However, in the 30°C *vs*. 5°C analysis there was considerable commonality in the lipid changes between the plasma, liver, and BAT, particularly within TGs (Supplementary Figure

291 8). This was most marked for TGs that contained long chain (>20 carbon acyl chains) poly-
292 unsaturated fatty acids, the changes in which were shared between plasma, liver and BAT, or
293 between BAT and liver (Supplementary Figure 8). Some of these findings are discussed in
294 more detail in the succeeding section.

295

Discussion

This study demonstrates the following: (i) That acute exposure to moderate (22°C) cold stress, the typical housing temperature in experimental studies in mice, significantly alters aspects of the plasma and BAT lipidome, most notably BAT TGs; although this effect is markedly less pronounced than that observed in response to major (5°C) cold stress. (ii) That cold stress at 5°C dramatically alters the plasma lipidome, with ether lipids identified as a novel lipid class altered by cold exposure. (iii) That cold-induced alterations in liver and BAT TG levels are not uniform, with changes being heavily influenced by acyl chain composition.

Similar to a previous report (29), our results identify acylcarnitines and free fatty acids as being markedly altered by cold stress at 5°C. These authors subsequently demonstrated the existence of a thermogenic circuit in which cold-induced increases in free fatty acids serve as substrates for acylcarnitine synthesis in the liver that when released into the systemic circulation fuel BAT thermogenesis (29). We did not detect any increase in hepatic acylcarnitine levels, suggesting that the newly synthesised acylcarnitines are rapidly released from the liver. Importantly, we observed no increase in circulating acylcarnitine or free fatty acid species in mice housed at 22°C relative to those housed at 30°C, indicating that acylcarnitines are unlikely to fuel BAT thermogenesis under the moderate cold stress imposed by housing at 22°C. In other work, Tseng and co-workers examined the impact of 5°C cold exposure for 7 days, relative to housing at 30°C, on the plasma, BAT, and WAT lipidome (18). Our data had minimal overlap with the top 10 cold-regulated lipid species identified by Lynes and co-workers (18). Specifically, 6 of the 10 most significantly affected lipids were phosphatidic acid (PA) species (18). Of the 12 measured PA species in the present study, only 2 were significantly altered, and the changes were modest. Nonetheless, we did observe a significant decrease in PA 40:4, likely equivalent to PA 18:0/22:4, one of the top 10 cold affected lipid species in the study by Lynes and co-workers. The remaining 4 lipids

identified by Tseng and co-workers in their top 10 cold affected lipids were either not assessed in our platform (PG 22:1/22:6 and SM d18:2/20:4) or were not altered by cold exposure in our study (PS 18:0/20:4 and monohexosyl ceramide d18:2/24:0). In contrast to our findings and that of Simcox and co-workers (29), Lynes and co-workers (18) did not observe changes in acylcarnitine species. These difference may relate to the duration of the cold stress, 5 and 24 hrs employed by Simcox and co-workers (29) and herein, respectively, and 7 days by Lynes and co-workers (18). The differences observed between these studies may reflect the dynamic nature of the cold-induced changes in the plasma lipidome and, furthermore, suggest that increased acylcarnitine production may be an interim thermogenic mechanism.

As expected, cold exposure had a major impact on plasma TGs, reducing total plasma TG levels as a result of decreases in numerous individual TG species. While these effects were far more pronounced at 5°C, robust decreases were also observed at 22°C. Cold exposure did not appear to affect specific TG species, instead reducing plasma TG levels in an acyl chain length and saturation status independent manner. These results are consistent with a previous finding showing reduced total plasma TGs following cold exposure (4°C for 24 hrs) (10). Decreases in plasma TGs are likely due to greater TG clearance from the circulation as a result of increased usage of TG-derived fatty acids by BAT (1, 12, 16), which is required to fuel thermogenesis. While the lipolysis of BAT TG stores is essential for thermogenesis, recent studies highlight the importance of exogenous fatty acids delivered via the circulation from either the lipolysis of WAT TG stores or from circulating lipoproteins for BAT thermogenesis (12, 27). Importantly, relative to 30°C, housing at 22°C significantly reduced total and numerous individual plasma TG species, indicating that even under conditions of relatively mild cold stress BAT uses circulating TGs to fuel continuing thermogenic activity. Indeed, it is now well appreciated that housing mice at 20-22°C, typical

346 conditions for animal experimentation, causes significant thermal stress and thereby leads to
347 activated thermoregulatory thermogenesis (15).

348 It has recently been shown that housing mice at their thermoneutral temperature,
349 typically 30°C, exacerbates the development of atherosclerosis, fatty liver disease, and
350 obesity (8, 9, 30). Elevated circulating lipids, in particular TG, play key roles in the
351 development of these conditions and, therefore, our findings provide some insight as to why
352 housing at thermoneutrality exacerbates the development of chronic metabolic diseases. A
353 limitation of the present study is that we only housed mice at 22°C for 24 hrs and,
354 accordingly, whether the changes in plasma TG that we observed are sustained during
355 chronic housing is uncertain. However, given that (i) mice housed at 22°C have chronically
356 activated BAT, (ii) that chronic housing at 30°C dramatically reduces BAT activity and alters
357 BAT morphology (5), and (iii) that circulating TGs are a major source of free fatty acids for
358 thermogenesis, differences in plasma TGs may be expected under chronic housing
359 conditions.

360 A novel finding from the present work is the finding that cold exposure increases the
361 levels of circulating ether lipids. Ether lipids are abundant membrane lipids and are
362 compositionally and structurally similar to their conventional diacyl
363 phosphatidylethanolamine (PE) and phosphatidylcholine (PC) counterparts (6, 22). However,
364 rather than an ester linked fatty acid at the sn-1 position of the glycerol backbone, as is the
365 case for PE and PC, ether lipids contain either an ether-linked (i.e. PC(O) and PE(O)) or vinyl
366 ether-linked (i.e. PC(P) and PE(P)), in the case of plasmalogens, hydrocarbon at the sn-1
367 position. These subtle chemical differences confer upon ether lipids specific functional
368 attributes (6, 22). While the relevance of increased circulating ether lipids following cold
369 exposure to thermogenesis is unclear, recent evidence does support a role for ether lipids in
370 regulating thermogenesis. Firstly, alkylglycerols, ether lipids that contain only the sn-1 ether

linked hydrocarbon, have been shown to promote the thermogenic capacity of white adipose tissue depots (35). Secondly, cold exposure increases the synthesis of ether lipids in BAT, which is suggested to be required for mitochondrial fission and thermogenesis (20, 21). While we did not observe any significant increases in PC or PE ether lipids in BAT following cold exposure, the relatively short nature of our study may have precluded us from making such an observation. Nonetheless, an intriguing finding was a significant increase in a number of TG(O) species, i.e. TG species with an ether linked chain at the sn-1 position of the glycerol backbone, within BAT. Whether this reflects increased endogenous BAT synthesis of ether lipids or uptake of ether lipids from the circulation is unclear. Our data are supportive of a potential role for ether lipids in thermogenesis and the possible contribution of increased circulating ether lipids to thermogenesis warrants further investigation.

TG catabolism within BAT is essential to fuel thermogenesis (2) and, consistently, we observed decreased TG levels within BAT following cold exposure at both 5°C and 22°C, relative to 30°C. However, while exposure at 5°C had a relatively uniform effect, reducing TG species of varying acyl chain length and saturation status similarly, exposure at 22°C had very modest effects on shorter and intermediate (48-54 carbons) acyl chain length TGs but markedly reduced the longest (56-58 carbons) TG species. This effect was even more pronounced when examining TGs by saturation status, with modest reductions in TG species containing 0 to 5 double bonds, but marked reductions in TGs containing 6 to 10 double bonds. These data suggest that TGs within BAT are catabolised in a selective manner in response to cold stress. These findings are consistent with previous research showing that fatty acid mobilisation from TG stores within WAT occurs in a selective manner (23, 24), with fatty acids of 16-20 carbon atoms in length and containing 4 or 5 double bonds having the highest mobilisation rate (23, 24). Similar effects have been observed in fasting-induced lipolysis in BAT (11).

Previous studies have reported a significant increase in liver TGs following acute cold exposure at 4°C (10) (10, 12) and, although not reaching statistical significance, we did observe a trend towards increased total liver TGs following cold exposure at 5°C. Cold exposure at 22°C did not result in any changes in the liver lipidome. While housing at thermoneutrality was recently shown to exacerbate liver TG accumulation, this only occurred when animals were fed the high-calorie, Western-type diet (8). Notably, the effect of 5°C cold exposure on liver TGs was highly TG species-dependent, with TGs containing 0 or 1 double bond not affected by cold exposure, TGs containing 2-4 double bonds being modestly increased at 5°C, and TGs containing 5-10 double bonds being highly significantly increased at 5°C. Of note, a similar pattern of effects was observed for DGs. As was suggested previously (10), it is likely that enhanced fatty acid uptake contributes to the increase in TG species within the liver following acute cold exposure at 5°C However, the reason for the selective increase in more unsaturated TG species is unclear. Indeed, we noted that plasma free fatty acids, which are largely derived from WAT, increased to a similar extent regardless of acyl chain length or saturation status. As discussed above, fatty acids released from WAT following cold-exposure (~4°C) are taken up by the liver to be converted to acylcarnitines. One possibility is that very long chain polyunsaturated fatty acids may not be as efficiently converted to acylcarnitines in the liver as long chain saturated and monounsaturated fatty acids, leading to a selective increase in TGs containing these fatty acid species.

In the vast majority of pre-clinical studies mice are maintained at housing temperatures of 20-22°C. However, it is now well appreciated that these conditions impose a moderate cold stress; indeed, when housed at 22°C over one-third of energy expenditure is devoted to maintaining core body temperature (25). Because under standard living conditions humans do not expend energy to maintain core body temperature, it has been suggested that pre-clinical studies in mice might be more predictive of human disease conditions if

421 performed at temperatures that approximate murine thermoneutrality (~30°C) (7, 25).
422 Because lipid metabolism is affected by housing at 22°C, e.g. increased endogenous TG
423 hydrolysis within BAT and increased BAT uptake of lipids derived from the circulation,
424 housing mice at thermoneutrality may be particularly important in pre-clinical models of
425 disease in which altered lipid metabolism is a major contributor to disease pathology, e.g.
426 obesity, non-alcoholic fatty liver disease, atherosclerosis. In this regard, the results of this
427 study are somewhat re-assuring as we observed no significant alterations in lipid levels in
428 skeletal muscle, subcutaneous and visceral WAT, and only 2 significantly altered lipid
429 species in the liver of mice housed at 22°C relative to 30°C, suggesting that housing mice at
430 22°C may be a confounding factor that impacts on the interpretation of disease/treatment-
431 induced alterations in lipid levels. We do however see significant alterations in BAT and
432 plasma TG levels, being decreased in both instances. This could have implications for
433 metabolic conditions in which circulating TG levels contribute to disease pathogenesis such
434 as atherosclerosis. Indeed, it was recently demonstrated that housing mice at thermoneutrality
435 potentiated the development of atherosclerosis (30). It will be interesting to determine how
436 more chronic housing at 22°C alters the plasma and tissue lipidome relative to housing at
437 thermoneutrality.

438

439 **Perspectives and Significance**

440 Our work provides a comprehensive analysis of the effect of acute cold exposure on the
441 mouse tissue and circulating lipidome. Our work identifies circulating ether lipids as having a
442 potential role in thermogenic responses as well as providing independent validation to
443 previously reported effects of cold on the plasma and tissue lipidome. Our results reveal that
444 while housing mice at thermoneutrality (30°C) results in similar lipid profiles to mice housed

445 at the standard 22°C, there are some significant alterations in the plasma and BAT lipidome.
446 This is particularly true of the more complex TGs, suggesting that TG metabolism should be
447 studied with caution in respect to thermogenic influences. The data herein provide a helpful
448 and extensive resource for those in the metabolism community, the complete data set for
449 which is provided in the Supplementary Tables accessible at the following:
450 https://doi.org/10.6084/m9.figshare.12298973.

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Author Contributions

G.P. prepared samples for mass spectrometry, analysed data and constructed figures. P.K.M. analysed data. N.A.M. co-ordinated mass spectrometry analysis. K. H. provided key intellectual input into lipidomic analysis. P.J.M. co-ordinated mass spectrometry analysis and provided key intellectual input into lipidomic analysis. A.J.M. provided key intellectual input and was involved in project conception. D.C.H. and G.I.L. designed the studies, carried out the research, analysed data and had the primary role in drafting the manuscript. All other authors contributed to editing and revising the draft manuscript.

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Figure 1. Effects of acute cold exposure on body weight, lean mass, and fat mass. Mice were weighed and body composition analysed after 24 hrs of acute cold exposure (22°C or 5°C) or having remained at 30°C. **(A)** Total body weight, **(B)** Lean mass, and **(C)** Fat mass change compared to pre measurements. Data are presented as a box and whisker plot, where the whiskers denote minimum and maximum values. Individual data points are shown within each box and whisker plot. Statistical significance was determined by 1-way ANOVA followed by Tukey's honestly significant different test. *** P<0.001; **** P<0.0001.

Figure 2. Effects of acute cold exposure on the plasma lipidome. After 24 hrs of acute cold exposure (22°C or 5°C) or having remained at 30°C, mice were killed and plasma collected for lipid analysis by mass spectrometry. (**A**) Heat map of the 239 significantly different lipid species. Each column is an individual mouse, each row is an individual lipid species. (**B**) Total and specific fatty acid species. (**C**) Total and specific acylcarnitine species. (**D**) Total and specific TG species. Data in **B**-**D** are presented as a box and whisker plot, where the whiskers denote minimum and maximum values. Individual data points are shown within each box and whisker plot. Statistical significance was determined by 1-way ANOVA followed by Tukey's honestly significant different test. * P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001.

Figure 3. Effects of acute cold exposure on plasma ether lipids. After 24 hrs of acute cold exposure (22°C or 5°C) or having remained at 30°C, mice were killed and plasma collected for lipid analysis by mass spectrometry. (**A**) Heat map of the 48 significantly different ether lipid species. Each column is an individual mouse, each row is an individual lipid species. (**B**) Total PC(O), PC(P), PE(O), and PE(P) levels. (**C**) Total PE(P) levels classified by alkenyl chain composition (16:0, 18:0, or 18:1). (**D**) Specific ether lipid species. Data in **B**-**D** are presented as a box and whisker plot, where the whiskers denote minimum and maximum values. Individual data points are shown within each box and whisker plot. Statistical

significance was determined by 1-way ANOVA followed by Tukey's honestly significant different test. * P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001.

Figure 4. Effects of acute cold exposure on the BAT lipidome. After 24 hrs of acute cold exposure (22°C or 5°C) or having remained at 30°C, mice were killed and BAT collected for lipid analysis by mass spectrometry. (**A**) Heat map of the 134 significantly different lipid species. Each column is an individual mouse, each row is an individual lipid species. (**B**) Total TG levels. (**C**) Total TG levels grouped by the sum of the acyl chain length. (**D**). Total TG levels grouped by the number of double bonds present within the acyl chains. Data in **B-D** are presented as a box and whisker plot, where the whiskers denote minimum and maximum values. Individual data points are shown within each box and whisker plot. Statistical significance was determined by 1-way ANOVA followed by Tukey's honestly significant different test. * P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001.

Figure 5. Effects of acute cold exposure on the liver lipidome. After 24 hrs of acute cold exposure (22°C or 5°C) or having remained at 30°C, mice were killed and the liver collected for lipid analysis by mass spectrometry. (**A**) Heat map of the 51 significantly different lipid species. Each column is an individual mouse, each row is an individual lipid species. (**B**) Total TG levels. (**C**) Total TG levels grouped by the number of double bonds present within the acyl chains. (**D**) TG species. (**E**) Total and specific DGs. Data in **B-E** are presented as a box and whisker plot, where the whiskers denote minimum and maximum values. Individual data points are shown within each box and whisker plot. Statistical significance was determined by 1-way ANOVA followed by Tukey's honestly significant different test. * P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001.

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Figure 1

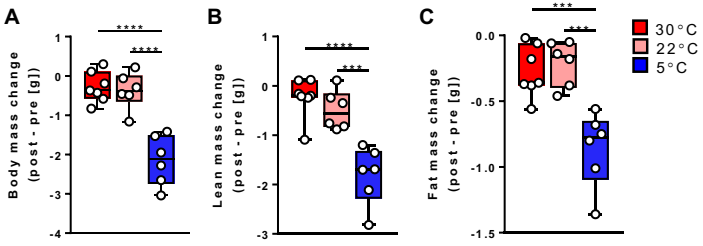

Figure 2

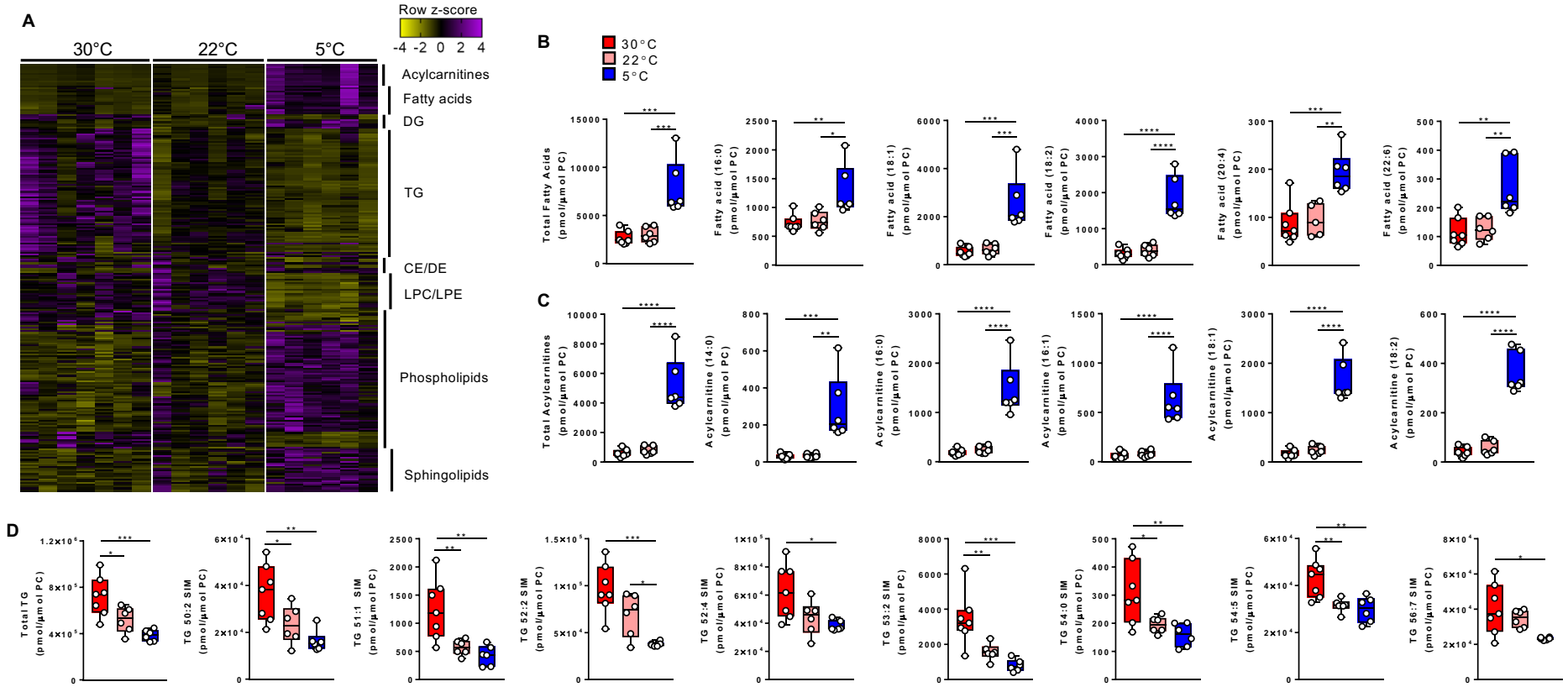

Figure 3

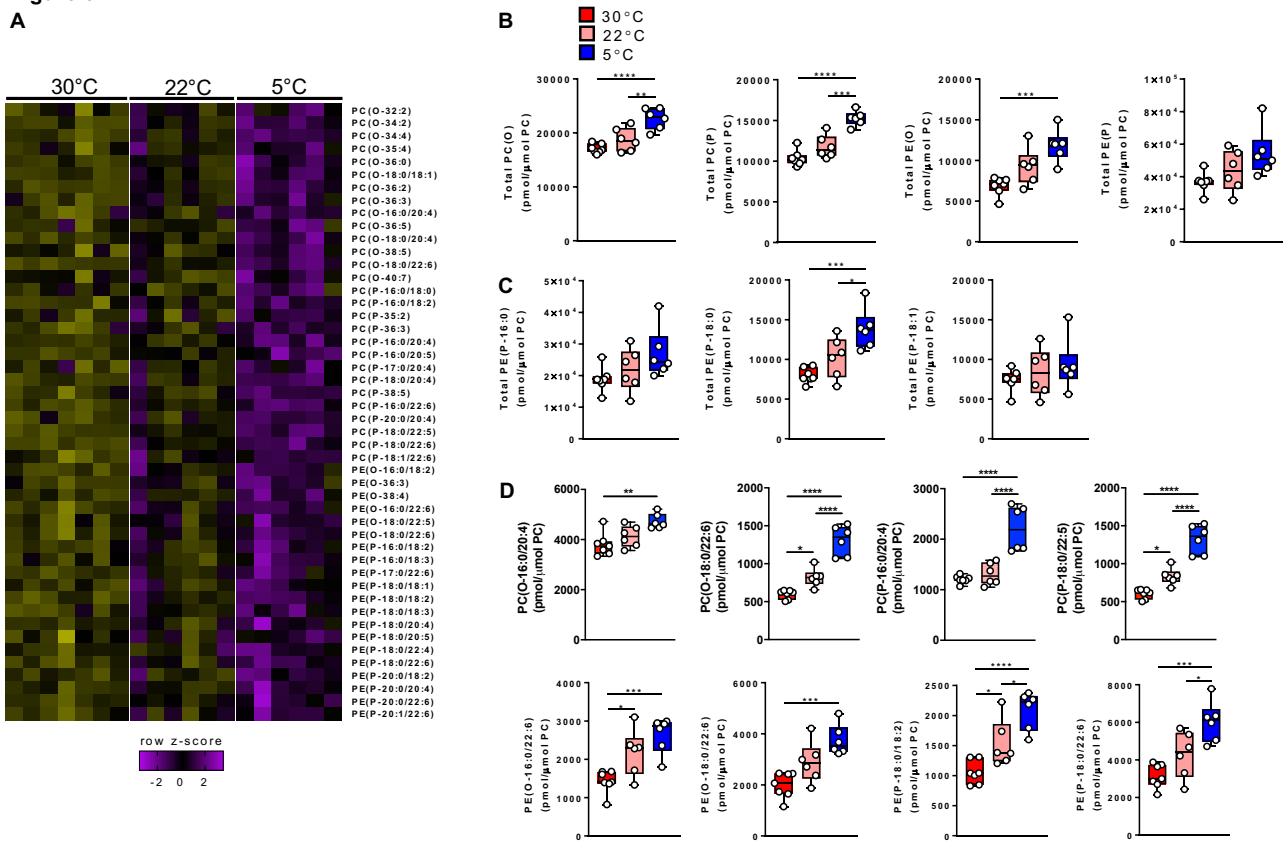

Figure 4

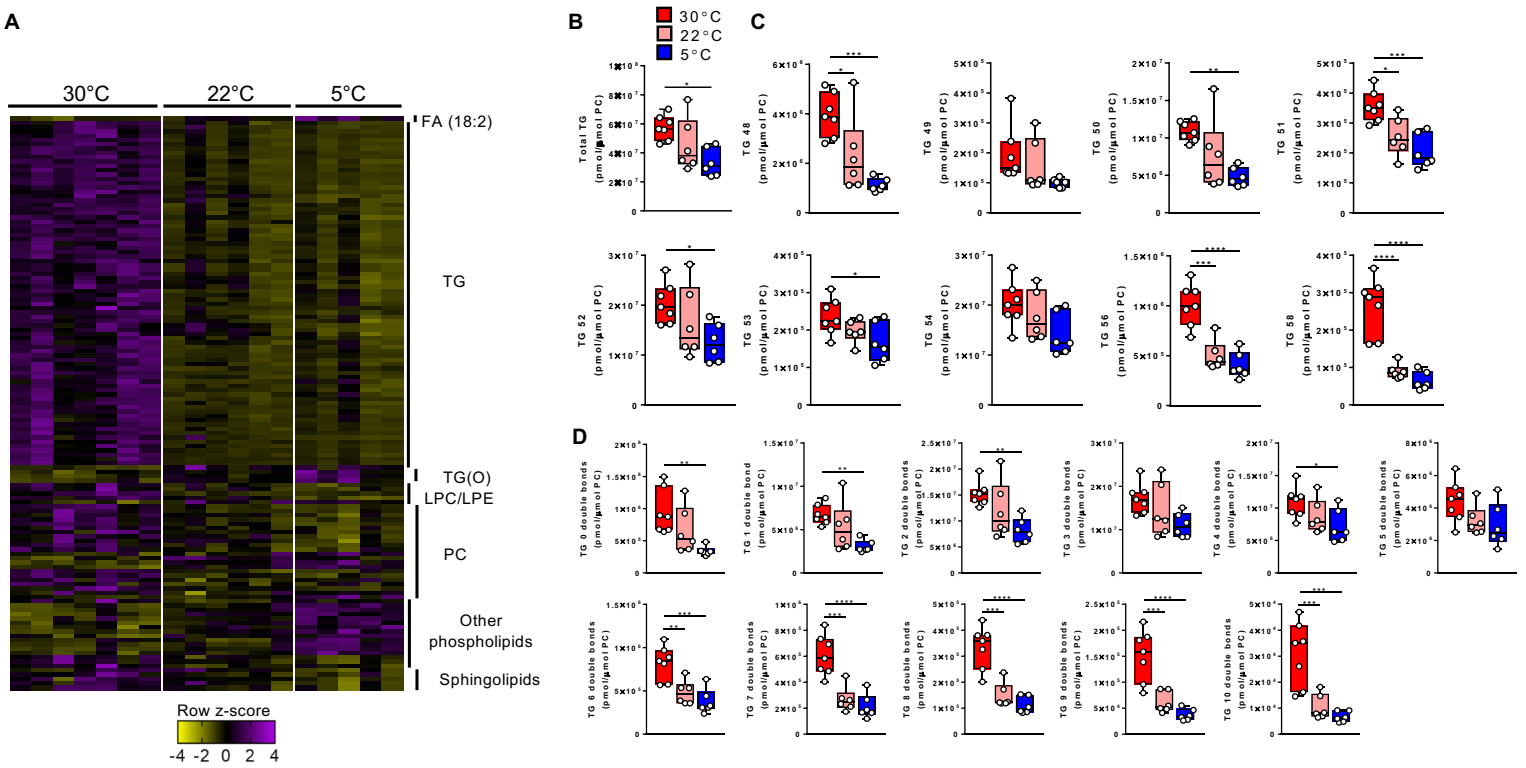

Figure 5