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A Crude Awakening: Effects of Crude Oil on Lipid Metabolism in Calanoid Copepods Terminating Diapause

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Abstract. *Calanus finmarchicus* and *Calanus glacialis* are keystone zooplankton species in North Atlantic and Arctic marine ecosystems because they form a link in the trophic transfer of nutritious lipids from phytoplankton to predators on higher trophic levels. These calanoid copepods spend several months of the year in deep waters in a dormant state called diapause, after which they emerge in surface waters to feed and reproduce during the spring phytoplankton bloom. Disruption of diapause timing could have dramatic consequences for marine ecosystems. In the present study, *Calanus* C5 copepodites were collected in a Norwegian fjord during diapause and were subsequently experimentally exposed to the water-soluble fraction of a naphthenic North Sea crude oil during diapause termination. The copepods were sampled repeatedly while progressing toward adulthood and were analyzed for utilization of lipid stores and for differential expression of genes involved in lipid metabolism. Our results indicate that water-soluble fraction exposure led to a temporary pause in lipid catabolism, suggested by (i) slower utilization of lipid stores in water-soluble fraction-exposed C5 copepodites and (ii) more genes in the β-oxidation pathway being downregulated in water-soluble fraction-exposed C5 copepodites than in the control C5 copepodites. Because lipid content and/or composition may be an important trigger for termination of diapause, our results imply that the timing of diapause termination and subsequent migration to the surface may be delayed if copepods are exposed to oil pollution during diapause or diapause termination. This delay could have detrimental effects on ecosystem dynamics.

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Abbreviations: ACAT, thiolase acetyl-CoA acetyltransferase; ALDE, aldehyde dehydrogenase; C5s, C5 copepodites; CoA, coenzyme A; CPM, counts per million; DEG, differentially expressed genes; EC, Enzyme Commission number; GCF, Genomic Core Facility; GLM, generalized linear model; GO, gene ontology; *HNF-4*, transcription factor hormone nuclear factor-4 homolog; KEGG, Kyoto Encyclopedia of Genes and Genomes; LCFAC, long-chain-fatty-acid-CoA-ligase; *NHR-E75*, nuclear hormone receptor E-75; PAH, polycyclic aromatic hydrocarbon; PPAR, peroxisome proliferator activator receptor; PW, produced water; RNA-Seq, RNA sequencing; *SREBP*, sterol regulatory element-binding protein; SVOC, semivolatile organic compounds; *TAp63*, tumor protein p73; WSF, water-soluble fraction.

Introduction

The trophic transfer of highly nutritious lipids from phytoplankton to predators through small herbivores is essential for maintaining oceanic ecosystem dynamics (Lee *et al.*, 2006). The calanoid copepods *Calanus finmarchicus* and *Calanus glacialis* are primary consumers that are widely distributed in the North Atlantic and Arctic regions (Conover, 1988; Choquet *et al.*, 2017), where they constitute a key trophic link between phytoplankton and predators (Slagstad and Tande, 1990; Sakshaug *et al.*, 1994; Reigstad *et al.*, 2006). Their life cycles include six naupliar stages that are followed by five copepodite stages and then a final molt to the reproductive adult stage. During the last copepodite stage (C5), individuals may initiate molting directly or undergo a period of dormancy called diapause.

Diapause occurs in diverse taxa, including freshwater zooplankton and insects, and is broadly considered to be an adaptation for surviving long periods with unfavorable conditions, including limited food availability, abundance of predators, and suboptimal temperatures (Koštál, 2006; Artal-Sanz and

Tavernarakis, 2009). In *Calanus* copepods, diapause involves an arrest in development, vertical migration to deep waters, and, subsequently, reduced metabolism lasting for several months (Hirche, 1996; reviewed by Baumgartner *et al.*, 2017). Prior to diapause, C5 copepodites increase their storage of neutral lipids contained in the lipid sac (Hirche, 1996). These lipids, stored as wax esters, are used as an energy reserve during the months in diapause, until the copepods "awaken" and migrate to the surface to reproduce as adults (Irigoien, 2004). Though little is known about which factors act as triggers of diapause termination in copepods, one hypothesis (the lipid accumulation window hypothesis) suggests that endogenous changes in either total lipid or lipid composition result in emergence from diapause (Irigoien, 2004; Johnson *et al.*, 2008). In a recent study (ES, unpubl. data), we investigated the rate of lipid catabolism during diapause and diapause termination. We found that the expression patterns of β-oxidation genes, and, thus, the lipid catabolism rate, seemed to vary during different phases of diapause. Gene expression patterns suggested that the copepods had a higher lipid catabolism rate at the beginning of the experimental period, that is, during early diapause, than toward the end of the study, that is, during diapause termination (ES, unpubl. data).

Despite the paramount importance of lipid transfer to higher trophic levels in marine ecosystems, knowledge on how anthropogenic pollution may influence lipid storage and metabolism within lower trophic levels is very limited. Several environmental stressors can affect lipid metabolism in marine species (Castelli *et al.*, 2014; Lee *et al.*, 2018; Toxværd *et al.*, 2018), including in calanoid copepods, which may limit the availability of lipids for secondary consumers in marine food webs. Among these stressors are petroleum-based pollutants, which can be released into the marine environment during extraction, transportation, and consumption of petrogenic oil (NRC, 2003). Zooplankton can potentially be exposed to oil components from a variety of sources, including accidental oil spills and through discharges of produced water (PW) from oil production platforms (Almeda *et al.*, 2014; Beyer *et al.*, 2016; Buskey *et al.*, 2016). Crude oil consists of a mixture of thousands of components, among which polycyclic aromatic hydrocarbons (PAHs) are generally considered the most toxic (Hylland, 2006). During an oil spill in the marine environment, oil components will be present as dispersed oil droplets or as dissolved components. Because of the higher bioavailability of the latter, the water-soluble fraction (WSF), which contains a mixture of PAHs, has the highest toxicological relevance (Murray *et al.*, 1984; Hansen *et al.*, 2009). Once present in the marine environment, these xenobiotics may cause adverse effects in organisms representing all trophic levels (Ball and Truskewycz, 2013; Fairbrother, 2013), ranging from phytoplankton (Dahl *et al.*, 2007) and macroalgae (Stepaniyan, 2008) to fish (Tuvikene, 1995) and marine mammals (Jenssen, 1996). In copepods, PAHs are known to affect survival, reproduction, feeding, and development, as well as to induce oxidative stress (Hansen *et al.*, 2008b, 2017a; Han *et al.*, 2014). Depending on their physicochemical properties (*i.e.*, hydrophobicity), PAHs can be incorporated into a specialized copepod organ called the lipid sac (Carls *et al.*, 2006; Hansen *et al.*, 2017b). Studies investigating effects of PAHs on lipid metabolism in crustaceans are scarce, and results have been ambiguous. Both increased and decreased lipid catabolism in response to exposure have been reported in studies examining lipid chemistry or enzymatic activity (Capuzzo *et al.*, 1984; Elumalai and Balasubramanian, 1999; Lavarías *et al.*, 2006, 2007). The potential influence of PAHs on the transcription of genes involved in lipid catabolic processes has yet to be assessed.

The emergence of calanoid copepods from diapause in the North Atlantic is timed in a way that is closely linked both to the phytoplankton bloom (Friedland *et al.*, 2016) and to cod (*Gadus morhua*) recruitment (Beaugrand *et al.*, 2003). If sub-lethal exposure to PAHs from petrogenic oil during diapause or diapause termination causes disruption of lipid metabolism, the timing of copepod emergence may be altered. This could in turn result in altered species interactions within the ecosystem, as was reported after the Exxon *Valdez* oil spill (Dean *et al.*, 2000). In the present study, we hypothesized that exposure to a mixture of PAHs in a WSF during diapause would create physiological stress in the copepods, resulting in increased lipid utilization, during and after termination of diapause. Thus, we expected shrinkage in the relative size of the lipid sac and increased transcription of genes encoding lipid catabolic enzymes in the WSF-exposed copepods. To test this hypothesis, we used RNA-sequencing (RNA-Seq) to investigate variations in transcription of lipid metabolism genes in *Calanus* spp. copepods collected during diapause in the Trondheimsfjord, Norway, and subsequently exposed to a WSF of a naphthenic North Sea crude oil, while being monitored during progression toward the adult stage.

Materials and Methods

Copepod collection and acclimation

Calanus C5-stage copepodites (C5s hereafter) were collected twice from sea bed depth (400 m) up to 200 m below the sea surface in the Trondheimsfjord, Norway (63°29′N, 10°18′E), using a Nansen net (Wiebe and Benfield, 2003) (mesh size: 200 μm) with a closing mechanism and a non-filtering cod end. In the Trondheimsfjord, C5s enter diapause and descend to deep waters in May; and most ascend to the surface in March the following year (Bergvik *et al.*, 2012). In the present study, C5s were collected once during early diapause (August 2017: this group constituted the reference group, REF in Fig. 1) and once later during diapause (November 2016: this group constituted the experimental group, EXP in Fig. 1). C5s in the reference group (10 individuals per sample, $n = 2$) were sorted from the bulk sample immediately after collection

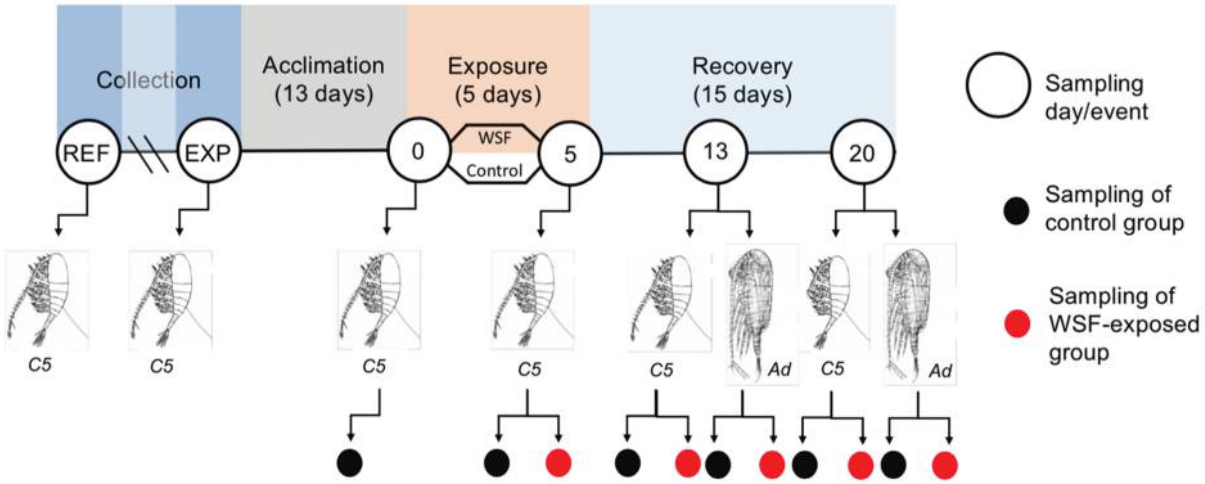

Figure 1. Timeline of the experiment. Ad, adults; EXP, sampling of experimental group; REF, sampling of reference group; WSF, water-soluble fraction.

(*in situ*) on the research vessel, were photographed for biometrical measurements as explained below (see *Sorting and experimental setup*), and then were placed in RNAlater (Thermo Fisher Scientific, Waltham, MA) for subsequent sequencing. The experimental copepods were temporarily kept in filtered seawater (salinity: ~33‰, temperature range: 4–9 °C) in 30-L buckets that were covered in 3 layers of dark plastic, until transportation to the laboratory facilities onshore (Norwegian University of Science and Technology [NTNU] Sealab, Trondheim, Norway). The animals were then acclimated in two 250-L tanks with constant flow-through of filtered seawater (8 °C, no light exposure or external stimulation) for 13 days before being divided into one control group and one group that was exposed to a WSF of a naphthenic North Sea crude oil (WSF-exposed group). The acclimation was performed to minimize stimulation prior to the experiment.

Species identification

Calanus finmarchicus (Gunnerus, 1770) has long been thought to be the only species of *Calanus* living in the Trondheimsfjord (Planque and Batten, 2000). However, after the RNA-Seq analyses in the present study were performed, a study that used molecular tools to identify *Calanus* revealed the presence of *Calanus glacialis* Jaschnov, 1955 in this fjord (Choquet *et al.*, 2017). An additional study also reported on the morphological similarity of *C. finmarchicus* and *C. glacialis* in areas of sympatry and concluded that only molecular analyses can enable reliable species identification (Choquet *et al.*, 2018). Because this information became available only after we had performed the RNA-Seq analyses, we decided to use RNA aliquots from all RNA-Seq samples (described in *RNA isolation, library preparation, and RNA-Seq*, below) to investigate genetically the potential presence of *C. glacialis* among the samples. For this purpose, the molecular-based protocol for reliable identification of *Calanus* species, described in Choquet *et al.* (2017), was adjusted to the conditions of the present study. First, RNA extracts of pooled individuals were converted into first-stranded cDNA using Invitrogen SuperScript II Reverse Transcriptase (Life Technologies, Carlsbad, CA), following the manufacturer's protocol. The produced cDNA was then used as a template for molecular species identification of each pool. In brief, a set of five nuclear molecular markers of the type InDel (Insertion or Deletion motifs) and of transcriptomic origin (T_4700, T_1338, T_1966, T_3133, T_461 in Smolina *et al.*, 2014) were amplified in a single multiplexed polymerase chain reaction (PCR). The resulting PCR amplicons were scored using a 3500xL Genetic Analyzer (Applied Biosystems, Foster City, CA), generating either a "pure" species-specific profile characteristic of *C. finmarchicus* or *C. glacialis* (Smolina *et al.*, 2014), in the case of only one species present within a pool, or a profile containing mixed signals from both species, in the case of the two species being present within a pool.

Generation of water-soluble fraction of crude oil

A naphthenic crude oil from the North Sea, which has been used previously in similar exposure studies with copepods (Jager *et al.*, 2016; Hansen *et al.*, 2017b; Øverjordet *et al.*, 2018), was chosen for the present study. Oil characterization (by gas chromatography-mass spectrometry [GC-MS]) can be found in the Appendix (Table A1). The oil was artificially weathered by simple distillation at 200 °C (Stiver and Mackay, 1984), and the +200 °C residue was collected and used for WSF generation. The system for generating continuous and constant exposures to WSF of crude oil in seawater has been described in detail previously (Nordtug *et al.*, 2011). Briefly, the oil residue was continuously pumped at a flow rate of 10 mg min^{-1} into a custom-made oil droplet generator through

a Teflon capillary from a 2.5-mL glass syringe using a syringe pump (model Aladdin AL-2000, World Precision Instruments, Sarasota, FL). Within the generator, an oil dispersion was generated by breaking up the oil into small droplets in seawater, which was pumped through Teflon tubes at a rate of 160 mL min^{-1} by a valveless metering pump (model QD with QCKC pump head, Fluid Metering, Syosset, NY). The resulting dispersion was collected continuously in a settling chamber (an inverted 5000-mL borosilicate jar with the bottom removed; DURAN, DWK Life Sciences, Mainz, Germany). The dispersion was then continuously filtered using a custom-made in-line filtration unit (250 mL) containing loosely packed fine glass wool (15 g) on top of Whatman GF/C (1.6 μm) and GF/F (0.7 μm) glass microfiber filters (Whatman, Maidstone, United Kingdom) to generate a droplet-free WSF of oil in seawater. The WSF was then led to an overflow tube with a rate of 140 mL min^{-1} and then transferred at an identical rate (~12 mL min^{-1}) to each of the exposure containers ($n = 7$) by adjusting the relative height between a floating valve at the water-receiving end and an outflow connection. The exposure containers were 5000-mL borosilicate glass jars (VWR International, Radnar, PA) with a modified spout, which created an even outflow. The outflow was collected in a plastic gutter. Clean filtered seawater was used for the control group ($n = 4$) and also used during recovery after exposure termination.

Analysis of semi-volatile organic compounds in exposure medium

Water samples taken after 48 and 96 hours of exposure were spiked with surrogate internal standards (SIS, o-terphenyl, naphthalene-d8, phenanthrene-d10, chrysene-d12, phenol-d6, and 4-methylphenol-d8) prior to extraction with dichloromethane (DCM), using the Environmental Protection Agency (EPA) method 3510C protocol (US EPA, 1996). The extracts were dried with sodium sulfate and concentrated to about 1 mL using a Zymark Turbovap 500 Concentrator (Hopkinton, MA). Recovery internal standards (RIS, 5a-androstane, fluorene-d10, and acenaphthene-d10) were added prior to analysis of semi-volatile organic compounds (SVOCs) by GC-MS and quantified by modifications of EPA method 8270D (US EPA, 1998; Faksness *et al.*, 2012).

Sorting and experimental setup

After the acclimatization period, the copepods were sorted from the 250-L tanks by using ladles and a 64-μm sieve and were transferred into the experimental containers of either clean filtered seawater (control group, $n = 4$) or WSF (exposed group, $n = 7$). Sampling of non-exposed C5s for RNA-Seq was performed simultaneously during this sorting (day 0, control group, three individuals per replicate, Fig. 1). The WSF exposure was maintained for five days, after which C5s were sampled from both the control and WSF-exposed groups (day 5, three individuals per replicate). Recovery (clean filtered seawater in all experimental containers) was initiated on day 5 and lasted until day 20. Sampling of C5s was again performed during recovery on days 13 and 20 (3 individuals per replicate), which corresponded to 1 and 2 weeks after termination of WSF exposure. On day 13, adults started to appear in sufficient numbers for sampling. Thus, 3 adults per replicate were sampled for RNA-Seq on days 13 and 20, in addition to C5s.

All sampled individuals were irreversibly euthanized with tricaine methane-sulfonate (MS-222, Finquel, 1.5 g L^{-1} seawater, Argent Laboratories, Redmond, WA). Exposure to MS-222 and total handling time was less than five minutes, approximately the same for all copepods in all treatments. All individuals were photographed using a CCD still-video camera (Sony DWF-sx900) operated by Fire-i software (Unibrain, San Ramon, CA) connected to a dissecting microscope (Meica M80, Leica Microsystems). The images were stored on a PC to enable biometrical measurements (described below, in *Biometry*). Each sampling day, copepods (C5s, adults, or both) were sampled and stored in RNAlater, first at 4 °C for 24 hours, then at −20 °C until subsequent sequencing analyses were performed.

All handling, except the photographing, was performed in darkness at 8 °C, using a halogen flashlight with a red glass filter for illumination. Red light was used because it penetrates minimally through water compared to other visible wavelengths (Clarke, 1936). The experimental containers were kept behind light-blocking curtains throughout the experiment, and the copepods were not fed. These efforts were implemented to mimic the conditions of diapausing *C. finmarchicus* in the Trondheimsfjord. Also, darkness was maintained to avoid potential interactive effects of light and oil exposure (Duesterloh *et al.*, 2002; Miljeteig *et al.*, 2013).

Biometry

Biometrical measurements were performed on stored images using previously described methods (Hansen *et al.*, 2008a). Lipid sac volume and prosome volume were estimated using methods previously reported (Miller *et al.*, 1998). Relative lipid content was calculated as the ratio of lipid sac volume to prosome volume.

Statistical analyses of biometrical data were performed in R (ver. 3.3.1; R Foundation for Statistical Computing, Vienna). Outliers (three) were removed using Tukey's method where data points above or below 1.5(interquartile range) were defined as outliers. Differences in relative lipid content (%) as a function of time from the initiation of the experiment between control and exposed copepods were investigated in C5s and adults separately by using ANCOVAs with time (number of days) and group as explanatory variables. An interaction term between time and group was included in the models to investigate differences in the rate of lipid utilization

between the two groups. If this interaction was not significant ($P > 0.25$; see Underwood, 1987 for choice of this P-value), the interaction term was removed from the models. Visual inspection of the residuals confirmed normal distribution.

RNA isolation, library preparation, and RNA-Seq

Samples for high-throughput RNA-Seq were selected from all available samples to most closely represent the median relative lipid content for each group at each time point. This selection consisted of two or three replicates (3–10 individuals in each replicate) from each group, that is, combination of treatments (control or WSF exposed), stages (C5 or adult), and sampling time (reference, days 0, 5, 13, and 20; see Fig. 1). Pooling was conducted in order to incorporate biological material from a larger number of individuals into a limited number of RNA-Seq libraries (Todd *et al.*, 2016), as is common in gene expression studies with copepods (reviewed by Tarrant *et al.*, 2019).

The copepods from each sample were first removed from RNAlater, gently dried on a paper towel, and weighed. Total RNA extraction was then performed using the Qiagen RNeasy Plus Universal Mini Kit (Qiagen, Valencia, CA) with additional use of a QiaShredder column, following the manufacturer's protocol. The RNeasy kit includes a genomic DNA removal step. The final eluation volume of RNA was 30 μL. RNA quality was assessed using a model 2100 Bioanalyzer instrument (Agilent, Santa Clara, CA). RNA Integrity Number (RIN) values could not be obtained because the hydrogen bonds in arthropod 28S rRNA are disrupted in the denaturation heating step prior to Bioanalyzer analysis, resulting in fragments that migrate closely with 18S rRNA (McCarthy *et al.*, 2015). RNA quality was therefore assessed using electropherogram summary graphs and gel images. All analyzed samples were of high quality, containing a strong 18S band and little or no evidence of genomic DNA contamination (large bands) or degradation (a smear of smaller bands).

For RNA sequencing, cDNA libraries were synthesized from total RNA (40 ng μL^{-1} input), using the Illumina TruSeq Stranded mRNA sample preparation kit (Illumina, San Diego, CA), with minor modifications adjusted for smaller volumes than in the manufacturer's protocol. The final volume of cDNA libraries was 22 μL. Prior to RNA sequencing, the cDNA libraries were pooled and normalized, and a quality control was performed on a Bioanalyzer instrument by the sequencing facility.

Samples were sequenced at the Genomic Core Facility (GCF) at NTNU, Trondheim, Norway, with 75-bp (base pair) paired-end reads on an Illumina HiSeq 500 HO flow cell. The 23 libraries were multiplexed across four lanes on the Illumina flow cell. Adapter sequences were trimmed at the GCF. Demultiplexed and adapter-trimmed FASTQ (Illumina) files were generated using bcl2fastq (ver. 2.18), using default settings; and the quality of trimmed reads was assessed using FastQC (ver. 0.10). All libraries passed standard fastqc checks for quality metrics, including per-sequence quality score, per-base N content (the percentage of base calls at each position for which an N was called instead of a known base), per-sequence GC (guanine-cytosine) content, overrepresented sequences, sequence length distribution, and adapter content. We retained 400 million reads in total and an average of ~15 million reads per sample (Table A2).

Bioinformatic analyses

A reference transcriptome (PRNJA231164) previously assembled from *C. finmarchicus* C5s collected from the Trondheimsfjord contains 241,140 transcripts and includes 96.7% of Benchmarking Universal Single-Copy Ortholog (BUSCO) genes (Tarrant *et al.*, 2014, 2019). To take advantage of new annotation tools and to incorporate recent additions to the Swiss-Prot database, the entire reference transcriptome was re-annotated (Skottene *et al.*, 2019) using Trinotate (ver. 3.0; Bryant *et al.*, 2017). Within Trinotate, transcripts were compared against Swiss-Prot by using BLASTX (ver. 2.2.30; National Center for Biotechnology Information, Bethesda, MD). This information was then used by Trinotate to assign gene ontology (GO) terms and KEGG (Kyoto Encyclopedia of Genes and Genomes) orthology (KO) groups.

Read mapping and estimation of abundance were performed using scripts bundled within Trinity (ver. 2.0.6; Haas *et al.*, 2013). Reads were mapped to the transcriptome using Bowtie (ver. 1.0.0; Langmead *et al.*, 2009), and transcript abundances were estimated using the RSEM package (ver. 1.2.12; Li and Dewey, 2011). Differentially expressed genes (DEGs) between the control group and the reference group, and between the WSF-exposed group and the reference group, were identified using a generalized linear model (GLM) with a negative binomial distribution, using packages edgeR (ver. 3.6.8) and limma (ver. 3.20.9) within Bioconductor in R (ver. 3.2.3; Robinson *et al.*, 2010). The implications of comparing both control and WSF-exposed groups to the reference group are explored in the *Discussion*. Group (control and WSF exposed), stage (adult and C5), and sampling time (reference and days 0, 5, 13, and 20) were used in the design matrix in the GLM. Expected gene counts from RSEM were used as input, and genes with very low counts per million (CPM < 1) were filtered out. Trimmed mean of M-value normalization (TMM) was performed, and tagwise dispersion was calculated using the Cox-Reid profile-adjusted likelihood method, which allows for multiple factors (group, stage, and time) in the GLM (Robinson *et al.*, 2010; McCarthy *et al.*, 2012).

Because the overall goal of the present study was to examine effects of WSF exposure on lipid metabolism, the gene expression analysis focused on transcripts associated with the fatty acid β-oxidation pathway and potential master regulators of lipid metabolism. Transcriptome-wide responses to the WSF exposure, such as GO enrichment analysis and principal

Table 1

Direction of regulation of transcripts associated with enzymes in the β-oxidation pathway in control and water-soluble fraction (WSF)-exposed Calanus *spp. C5 copepodites compared to the reference group*

Fig. 3	EC	Annotation	Transcript ID	Day 5	Day 13	Day 20
A	6.2.1.3	Long-chain-fatty-acid-CoA ligase (LCFAC) 1	comp274130	⇓	⇓	⇓
A	6.2.1.3	Long-chain-fatty-acid-CoA ligase (LCFAC) ACSBG2	comp273007_c0		⇓	↓
A	6.2.1.3	Long-chain-fatty-acid-CoA ligase (LCFAC) ACSBG2	comp271341_c0			⇑
B	4.2.1.17	Enoyl-CoA hydratase, mitochondrial	comp263616		↓	
B	1.1.1.35	Aldehyde dehydrogenase, mitochondrial (ALDE)	comp262846		⇓	↓
B	2.3.1.16	Acetyl-CoA acetyltransferase, mitochondrial (ACAT)	comp266860_c1	⇓	⇓	
B	2.3.1.16	3-ketoacyl-CoA thiolase, mitochondrial	comp262743_c0			↓
D	1.1.1.284 (1.1.1.1)	S-(hydroxymethyl)glutathione dehydrogenase	comp257585_c0			⇓
D	1.2.1.31 (1.2.1.8 1.2.1.3)	Alpha-aminoadipic semialdehyde dehydrogenase	comp269490_c0			↓
D	1.2.1.3	Aldehyde dehydrogenase family 3 member B1	comp268106_c0			⇓

Exposure period: days 0–5; recovery period: days 6–20. ↓, control; ⇓ or ⇑, WSF-exposed group. CoA, coenzyme A; EC, Enzyme Commission number.

component analyses, will be reported elsewhere. To identify genes coding for enzymes in the fatty acid β-oxidation pathway, amino acid sequences for each enzyme were obtained for *Daphnia pulex* from the KEGG database. *Daphnia pulex* was the only crustacean with an available fatty acid β-oxidation pathway in the KEGG database as of December 2017 (dpx00071), and it was the species most commonly retrieved as a top result in BLASTX queries with *C. finmarchicus* transcripts in a study by Lenz *et al.* (2014). A nucleotide database was made from the *C. finmarchicus* reference transcriptome, and the amino acid sequence corresponding to each gene in the *D. pulex* β-oxidation pathway was used in a tblastx (BLAST+, ver. 2.7.1) query (translated nucleotide sequence toward amino acid sequence). The e-value cutoff was set at 10^{-7}. The same method as described above was used (with tblastn) when identifying master regulators. The amino acid sequences used as queries are detailed in Table A3. The *SREBP* sequence was obtained from *Daphnia magna*. Two partial sequences encoding *TAp63* were obtained from *Mytilus galloprovincialis*, and both sequences had the same top hit in *C. finmarchicus*. The amino acid sequence for *HNF-4* was obtained from *Tigriopus japonicus*. Because of the importance of the peroxisome proliferator activator receptor (PPAR) as a master regulator of energy and lipid homeostasis in many other species (Varga *et al.*, 2011), we investigated the presence of this receptor in *C. finmarchicus* by using the human sequence as a query. The top hit had a 35% identity with a sequence that was annotated as *NHR-E75*. We investigated the expression of this sequence further.

Presenting gene expression patterns

In order to outline the general expression pattern, only the transcripts and associated β-oxidation enzymes that are "uniquely" expressed differently in either the control group or the WSF-exposed group compared to the reference group are presented (Tables 1, 2). For example, on day 5 in the control group, one transcript was upregulated and one was downregulated in C5s (Fig. A1; Table A4, transcript ID marked with superscript 1). These were also up- and downregulated, respectively, in the

Table 2

Direction of regulation of transcripts associated with enzymes in the β-oxidation pathway in control and water-soluble fraction (WSF)-exposed Calanus *spp. adults compared to the reference group*

Fig. 3	EC	Annotation	Transcript ID	Day 5	Day 13	Day 20
A	6.2.1.3	Long-chain-fatty-acid-CoA ligase 1 (LCFAC)	comp271267_c0		↓	⇓
B	4.2.1.17, 1.1.1.211	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	comp261049_c0		↓	
C	1.3.8.8	Long-chain specific acyl-CoA dehydrogenase, mitochondrial	comp264590_c2			⇓
D	5.3.3.8	Delta3-Delta2-enoyl-CoA isomerase	comp273172_c0		↓	
D	1.1.1.284, 1.1.1.1	Alcohol dehydrogenase class-3	comp262350_c0			↓
D	1.1.1.284, 1.1.1.1	Alcohol dehydrogenase class-3	comp273523_c0		⇑	
D	1.2.1.31, 1.2.1.8, 1.2.1.3	Alpha-aminoadipic semialdehyde dehydrogenase	comp269490_c0		⇓	
D	1.2.1.3	Fatty aldehyde dehydrogenase	comp274839_c0			⇓
D	1.2.1.3	Aldehyde dehydrogenase family 1 member A3	comp272276_c0			⇓

Exposure period: days 0–5; recovery period: days 6–20. ↓, control; ⇓ or ⇑, WSF-exposed group. CoA, coenzyme A; EC, Enzyme Commission number.

WSF-exposed group on day 5 (Fig. A1; Table A4, transcript ID marked with superscript 1). These two transcripts are thus removed in order to show the general pattern. The two remaining transcripts (comp274130 and comp266860_c1) that were differentially expressed in C5s on day 5 were only downregulated in the WSF-exposed group and are presented along with the annotation of the associated β-oxidation enzyme (Table 1).

Results

Species determination

Of the 22 RNA samples, each consisting of 3–10 individuals, that were analyzed for species identification, 43.4% contained only *Calanus finmarchicus*, 43.4% contained a mix of *C. finmarchicus* and *Calanus glacialis*, and 8.6% contained only *C. glacialis* (Table 3). Within the mixed samples it was not possible to determine the number of individuals belonging to each of the two species because the analysis was performed on pooled RNA aliquots. Mixed samples accounted for 33% and 44% in the control and the WSF-exposed groups, respectively.

Table 3

Distribution of the two species Calanus finmarchicus *and* Calanus glacialis *in all RNA-sequencing samples*

Stage	Group	Day	Replicate	*C. finmarchicus*	*C. glacialis*	Mix
C5	Ref	—	1			✓
C5	Ref	—	2			✓
C5	C	0	1	✓		
C5	C	0	2			✓
C5	C	0	3			✓
C5	C	5	1	✓		
C5	C	5	2	✓		
C5	E	5	1			✓
C5	E	5	2			✓
C5	C	13	1	✓		
C5	C	13	2	✓		
C5	E	13	1			✓
C5	E	13	2			✓
C5	C	20	1		✓	
C5	E	20	1		✓	
C5	E	20	2	✓		
Ad	C	13	1	✓		
Ad	C	13	2			✓
Ad	E	13	1	✓		
Ad	C	20	1	✓		
Ad	C	20	2			✓
Ad	E	20	1	✓		
Ad	E	20	2	NA	NA	NA
% of total				43.4	8.6	43.4

Sample determination analyses using InDel markers was performed on RNA aliquots containing pooled RNA from 3 or 10 (reference group only) individuals. Ad, adults; C, control; E, WSF-exposed; NA, no amplification (unknown species ID); ref, reference group.

Table 4

Concentrations of semi-volatile organic compound groups in the water-soluble fraction (WSF) exposure medium at 48 and 96 hours after day 0, measured by gas chromatography-mass spectrometry in selection ion monitoring (SIM) mode

	48 hours (mg L^{-1})	96 hours (mg L^{-1})	Mean ± SD	% of total
Σ all compounds	97.68	102.58	100.13 ± 3.47	100
Σ decalins	1.46	1.68	1.57 ± 0.16	1.57
Naphthalenes	81.51	85.96	83.74 ± 3.15	83.63
2–3-ring PAHs	14.22	14.53	14.38 ± 0.22	14.36
4–6-ring PAHs	0.33	0.35	0.34 ± 0.01	0.34
C0-C5-phenols	0.17	0.07	0.12 ± 0.07	0.12

Mean and standard deviation (SD) were calculated from concentrations measured at 48 and 96 hours. PAH, polycyclic aromatic hydrocarbon.

Semi-volatile organic compound analysis of exposure medium

Of the SVOCs in the exposure medium, naphthalenes were the most prevalent (Table 4, 83.63%). Two- or three-ring PAHs were the second most prevalent group (14.36%), followed by total decalins (1.57%), four- to six-ring PAHs (0.34%), and C0–C5 phenols (0.12%). This PAH composition is comparable to previous studies using WSFs prepared from this oil (*e.g.*, Hansen *et al.*, 2017b).

Lipid content and expression of lipid metabolism genes

Relative lipid content. In C5s, relative lipid content differed with time between the WSF-exposed group ($n = 306$) and the control group ($n = 185$) (P for interaction $= 0.022$, Fig. 2A). Control C5s had a more pronounced reduction in lipid content as a function of time (estimate ± SE $= -0.21\% \pm 0.07\%$ per day) than the exposed group (estimate ± SE $= -0.06\% \pm 0.04\%$ per day). In the adults, change in relative lipid content over time did not differ between the control group ($n = 136$) and the WSF-exposed group ($n = 264$) (P for interaction $= 0.576$, Fig. 2B). Also, there was no effect of time on relative lipid content in adults ($P = 0.596$). Removing the interaction term from the model showed that the exposed adults had a higher relative lipid content than the control adults (control adults, 12.14% ± 0.36%; exposed adults, 13.21% ± 0.61%; $P = 0.003$).

Expression of β-oxidation genes. Relative to the reference group (early diapause C5s, sampled for RNA-Seq *in situ*), there was a general pattern of a higher number of downregulated β-oxidation genes in the WSF-exposed C5s than in the control C5s, particularly on days 5 and 13 (Table 1). Magnitude of regulation (fold change, CPM), F-values, P-values, and false discovery rates for all differentially expressed transcripts ($P < 0.05$) are given in the Appendix (Table A4; Fig. A1).

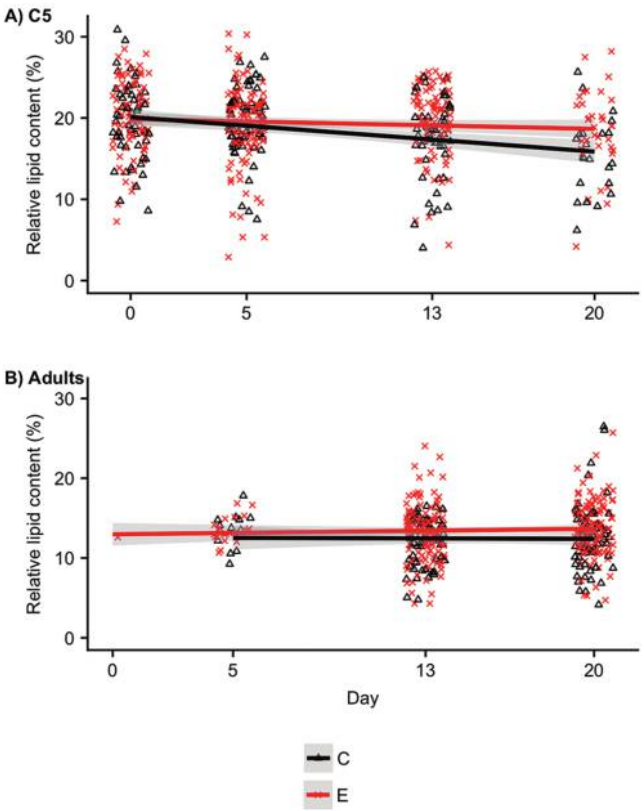

Figure 2. Regression of relative lipid content with time (days since water-soluble fraction [WSF] exposure start) in control and WSF-exposed (A) C5 copepodites (C5s) and (B) adults of *Calanus* spp. WSF exposure period: days 0–5. Recovery period: days 6–20. (A) WSF-exposed C5s, $n = 306$. Control C5s, $n = 185$. ANCOVA, $F_{3,478} = 7.78$, $R^2 = 0.04$. The WSF-exposed C5s (E) had a less steep decline in lipid content with time than the control C5s (C) ($P < 0.05$). (B) WSF-exposed adults, $n = 306$. Control adults, $n = 136$. ANCOVA, $F_{2,395} = 4.48$, $R^2 = 0.02$. There was no change in lipid content in control or exposed adults with time, but exposed adults had a significantly higher mean lipid content than control adults ($P < 0.05$).

On day 5, two unique transcripts were downregulated in the WSF-exposed C5s compared to the reference group (Table 1). These transcripts were associated with two enzymes in the β-oxidation pathway (where CoA is coenzyme A): long-chain-fatty-acid-CoA-ligase (LCFAC, Enzyme Commission number [EC] 6.2.1.3, Fig. 3A) and the thiolase acetyl-CoA acetyltransferase (ACAT, EC 2.3.1.16, Fig. 3B). LCFAC is the first enzyme in the β-oxidation pathway and is involved in the attachment of CoA to fatty acids in preparation for the transportation from cytosol into the mitochondrial matrix. It is a rate-limiting step in β-oxidation (Soupene and Kuypers, 2008; Shriver and Manchester, 2011). In C5s on day 13, four unique transcripts were downregulated in the WSF-exposed group (Table 1). These encoded three different enzymes in the β-oxidation pathway, including LCFAC and ACAT (Fig. 3A, B). One unique transcript encoding one enzyme was downregulated in control C5s on day 13 (Table 1). On day 20, the observed pattern of more downregulated genes in the exposed C5s was reversed, because four unique transcripts encoding four enzymes in the β-oxidation pathway were downregulated in the control C5s (Table 1), among them LCFAC (Fig. 3A). In contrast, within the WSF-exposed C5s, only three unique transcripts encoding two enzymes were downregulated; and one transcript (encoding LCFAC, Fig. 3A) was upregulated (Table 1).

No adults were sampled on day 5 because of low abundance. However, in adults on day 13, there were more downregulated β-oxidation genes in the control group than in the WSF-exposed group (Table 2), which was opposite the observed pattern in the C5s. Specifically, three unique transcripts encoding three β-oxidation enzymes were downregulated (*e.g.*, LCFAC), and one transcript encoding one enzyme was upregulated in the control adults (Table 2; Fig. 3A). One unique transcript encoding one enzyme was downregulated in the WSF-exposed adults on day 13 (Table 2). On day 20, however, there were more downregulated genes in the WSF adults than in the control adults. Only one unique transcript encoding one enzyme was downregulated in the control adults (Table 2), while four transcripts encoding four enzymes were downregulated in the WSF-exposed adults, including LCFAC (Table 2; Fig. 3A). Several of the genes regulated in adults were different types of alcohol and aldehyde dehydrogenases (ALDEs; EC 1.1.1.284 and EC 1.2.1.3, Fig. 3D).

Master regulators. Genes encoding four potential master regulators of lipid metabolism were differentially expressed in the WSF-exposed and the control C5 and/or adults when compared to the reference group (Fig. 4). The pattern of differential expression differed between the control group and the WSF-exposed group, particularly in the adults. Compared to the reference group, hepatocyte nuclear factor 4 (*HNF-4*) was significantly downregulated in WSF-exposed C5s, but not in the control C5s, on day 13. Nuclear hormone receptor E-75 (*NHR-E75*) was significantly downregulated in control adults, but not in WSF-exposed adults, on day 13. On day 20 however, *NHR-E75* was only downregulated in the WSF-exposed adults, not in control adults. Sterol regulatory element-binding protein cleavage protein (*SREBP*) and tumor protein p73 (*TAp63*) were both significantly downregulated in the control adults, but not in the exposed adults, on day 13.

Discussion

Water-soluble fraction exposure disrupts lipid metabolism in Calanus *spp. C5 copepodites terminating diapause*

The results of the present study indicate that exposure to the WSF of a naphthenic North Sea crude oil during diapause termination altered lipid metabolism in *Calanus* spp. C5s. While we expected that exposure to PAHs in a WSF would cause physiological stress in the diapausing copepods and result in an increased lipid catabolism, our results indicate a decreased utilization of lipids in WSF-exposed *Calanus* C5s. This is indicated both by significantly slower reduction of

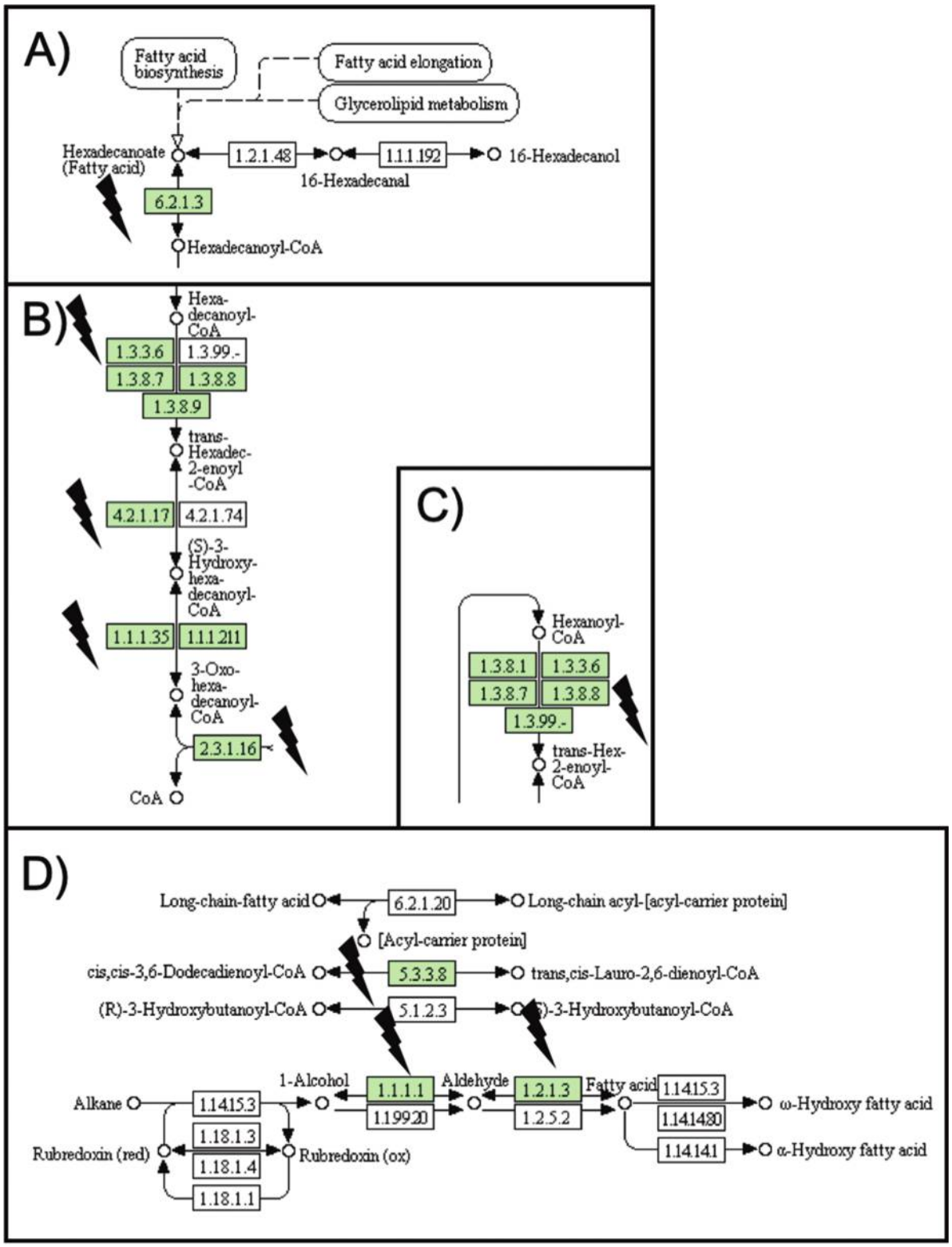

Figure 3. Enzymes (Enzyme Commission number [EC] in green or white boxes) in the β-oxidation pathway associated with differentially expressed transcripts in *Calanus* spp. C5 copepodites (C5s) and adults. Water-soluble fraction (WSF) exposure period: days 0–5. Recovery period: days 6–20. Lightning symbol indicates effect (up- or downregulation [$P < 0.05$] compared to the reference group [early diapause group]) in either WSF-exposed or control *Calanus* spp. C5s or adults. Figure obtained from the Kyoto Encyclopedia of Genes and Genomes, based on *Daphnia pulex* (Kanehisa and Goto, 2000; Kanehisa *et al.*, 2016, 2017). Only enzymes in the pathway with significant effects are shown. Subplots are denoted with letters (A–D), referred to in the text: (A) effects on EC 6.2.1.3; (B) effects on EC 1.3.3.6, EC 4.2.1.17, EC 1.1.1.35, and EC 2.3.1.16; (C) effects on EC 1.3.8.8; (D) effects on EC 5.3.3.8, EC 1.1.1.1, and EC 1.2.1.3.

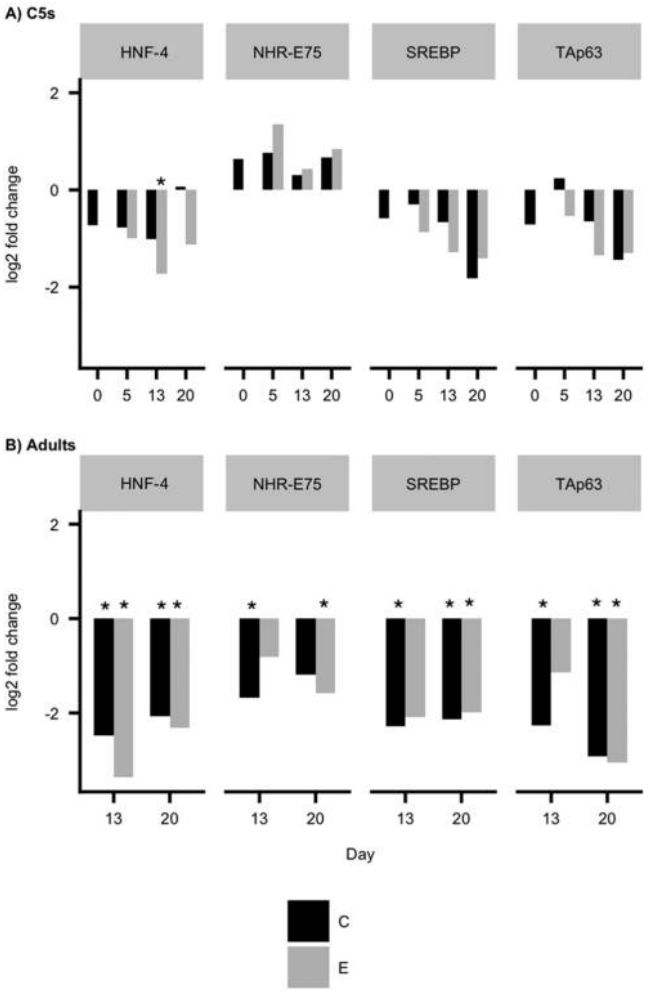

Figure 4. Differentially expressed (log_2 fold change) master regulator genes (*HNF-4*, hepatocyte nuclear factor 4; *NHR-E75*, nuclear hormone receptor E-75; *SREBP*, sterol regulatory element-binding protein cleavage-activating protein; and *TAp63*, tumor protein p73) in control (C, black bars) and water-soluble fraction (WSF)-exposed (E, gray bars) C5 copepodite (C5s; A) and adult (B) *Calanus* spp. compared to the reference group. WSF exposure period: days 0–5. Recovery period: days 6–20. A positive fold change indicates upregulation compared to the reference group; a negative fold change indicates downregulation. Asterisks above bars indicate a statistically significant ($P < 0.05$) difference from the reference group.

lipids in the lipid sac over time in the WSF-exposed than in control copepods and by the observed differential expression patterns of lipid metabolism genes. Our initial hypothesis is therefore rejected. The observed downregulation of β-oxidation genes in the WSF-exposed copepods suggests a lower maximum capacity for production of β-oxidation enzymes, which may indicate a lower lipid catabolic rate.

The species identification analyses performed on the RNA aliquots of the samples revealed that most samples contained at least a few individuals of *Calanus glacialis*. Whether the presence of *C. glacialis*, along with *Calanus finmarchicus*, in some RNA-Seq samples influenced the gene expression patterns is difficult to discern with certainty. However, we may assume that the heterogeneity of the samples is somewhat balanced by the similarity in diapause behavior between the two species (reviewed by Baumgartner and Tarrant, 2017) and by the high conservation of the investigated lipid metabolic genes (*e.g.*, Tsai *et al.*, 2014). Indeed, the β-oxidation pathway is considered to be an evolutionarily well-conserved metabolic process (Schulz, 1991). In addition, the overall proportions of mixed samples were relatively similar in the control and the WSF-exposed groups. Therefore, cautious conclusions can still be made based on these data. Nevertheless, we highly recommend performing molecular species identification analyses prior to pooling *in situ* collected *Calanus* copepods in future studies to avoid such complications.

*Rationale behind comparing gene expression with the reference group (*in situ*, early diapause C5 copepodites)*

When examining effects of exposure to a stressor, comparisons are usually made between the exposed group and a control group that are sampled at the same time points. Underlying such comparisons is the assumption that the two groups remain comparable throughout the study with respect to aspects other than the endpoints of interest. In the present study where the copepods progressed developmentally throughout the experiment, this assumption is not met because the exposed and control groups are likely to differ with regard to development, activity level, and diapause state as a result of the WSF exposure itself, as has been previously suggested (Billiard *et al.*, 2008; Grenvald *et al.*, 2013). In addition, *Calanus* C5s are notorious for asynchronous development within a population (Tarrant *et al.*, 2016). This complicates comparisons of gene expression between different groups sampled periodically over a relatively long developmental period. Therefore, the exposed and control groups were compared with the reference group, which was collected *in situ* in the Trondheimsfjord and assumed to be in an early state of diapause and, thus, development. Gene expression comparisons between the control and WSF-exposed copepods sampled on the same days were analyzed and are reported in the Appendix (Tables A5, A6). However, for the reasons stated above, these will not be discussed because they are not considered to properly reflect the biological effects of WSF exposure on lipid metabolism.

Downregulation of more β-oxidation genes in the water-soluble fraction-exposed C5 copepodites

On day 13 (one week after WSF exposure was terminated), four transcripts encoding four enzymes in the β-oxidation pathway were downregulated in the WSF-exposed C5s, while only one was downregulated in the control C5s. Transcripts associated with LCFAC appeared to be particularly responsive to WSF exposure (Fig. 3A). Several different transcripts associated with this enzyme were downregulated in the exposed C5s on days 5 and 13 (both up- and downregulation were observed on day 20). The attachment of CoA to fatty acids prior to transport into mitochondria, a reaction catalyzed by LCFAC, is a rate-limiting step of β-oxidation (Soupene

and Kuypers, 2008; Shriver and Manchester, 2011). Because effects of the WSF exposure were observed on several transcripts encoding this enzyme throughout the experimental period, we suggest that this may be a sensitive step for disruption by WSF exposure within the β-oxidation pathway. Because this is a rate-limiting step of β-oxidation, expression of the corresponding transcripts may serve as a proxy for the rate of lipid catabolism.

Day 20: back to normal?

The marked pattern of downregulation of lipid catabolism genes in the WSF-exposed C5s appeared to change on the last day of the experiment, day 20, which was 15 days after the WSF exposure was terminated. This is illustrated by transcripts associated with ACAT (Fig. 3B) and an ALDE (EC 1.1.1.35, Fig. 3B): genes encoding these enzymes were downregulated in the WSF-exposed C5s on day 13 but not on day 20. Rather, these transcripts were downregulated in the control C5s on day 20. Also, two different transcripts associated with the rate-limiting LCFAC (Fig. 3A) were regulated dissimilarly (one up, one down) on day 20 (Table 1), which illustrates that the previously clear pattern of downregulation of lipid catabolism in the WSF-exposed group became less obvious on day 20. Fewer downregulated β-oxidation genes suggest a higher capacity to produce enzymes involved in lipid catabolism in the WSF-exposed C5s on day 20. Together, this may indicate that the suggested pause in lipid catabolism due to the PAH exposure was temporary, because it increased 15 days after termination of WSF exposure. These results are complicated somewhat by the developmental asynchrony, which might have been enhanced by WSF exposure. On day 20 of the experiment, the number of C5s in both groups was greatly outnumbered by the number of adults, and the C5s sampled at day 20 may represent animals that developed particularly slowly. The consequences of this for lipid metabolism are unclear.

Overall, these results make it possible to derive hypothetical patterns of lipid catabolism in C5s over the course of the experiment (Fig. 5). A higher number of downregulated β-oxidation genes in WSF-exposed C5s on days 5 and 13 suggests a lower maximum capacity of production of enzymes associated with lipid catabolism. On day 20, this pattern was no longer evident. Together, this suggests that the lipid catabolic rate in the WSF-exposed C5s was lower than in the control copepods from day 5 to at least day 13, before it increased (gray line, Fig. 5). In the control copepods, there was more downregulation of lipid catabolic genes on day 20, suggesting a reduction in lipid catabolism rate (black line, Fig. 5).

Lipid utilization and gene expression patterns differed in adults

Lipid content variations during the course of the experiment and the expression pattern of lipid metabolism genes differed between the C5s and the adult copepods. Although there was no time-dependent change in the relative lipid content in either control adults or WSF-exposed adults, the relative lipid content was significantly higher in the exposed adults than in the control group (Fig. 2B). This may be linked to the suggested pause in lipid catabolism in the WSF-exposed C5s, resulting in excess lipid stores in the WSF-exposed adults compared to the control group.

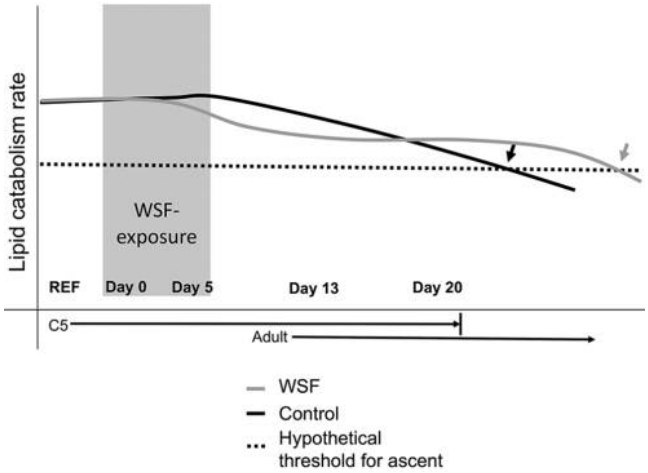

Figure 5. Hypothetical lipid catabolism rate in *Calanus* spp. C5 copepodites (C5s) exposed to a water-soluble fraction (WSF) from day 0 to day 5, with recovery from day 6 to day 20. WSF-exposed C5s had a less steep decline in relative lipid content in the lipid sac than control C5s, and on days 5 and 13 there were more β-oxidation genes that were downregulated in WSF-exposed C5s than in the control C5s. Downregulation of these genes suggests a lower maximum capacity of production of enzymes associated with lipid catabolism. On day 20, this pattern was no longer evident. Together, this suggests that the lipid catabolic rate in the WSF-exposed copepods was lower than in the control copepods from day 5 to at least day 13, before it increased (gray line). In the control C5s, there was a slow reduction in lipid catabolism rate, as more β-oxidation genes were downregulated on day 20 than on days 5 and 13. Black and gray arrows indicate potential timing of migration to the surface for control and WSF-exposed copepods, respectively. The exposure period is highlighted in light gray. Note that the copepods were not fed in the present study.

Differential expression of β-oxidation genes suggests that exposure to the WSF affected lipid catabolism in the adults, as well as in the C5s. While the control adults had several downregulated β-oxidation genes on day 13, including the rate-limiting LCFAC (Fig. 3A), the WSF-exposed adults had only one downregulated β-oxidation transcript. This suggests a lower lipid catabolism on day 13 in the control adults and a comparably higher lipid catabolism in the WSF-exposed adults. This contrasts with the pattern in the C5s, where the expression of lipid catabolic genes on day 13 was high in the control group and lower in the WSF-exposed group. On day 20, however, there was a higher number of downregulated β-oxidation genes in the WSF-exposed adults, including the rate-limiting LCFAC (Fig. 3A), while only one transcript was downregulated in the control group. This suggests a downregulation of the lipid catabolism in the WSF-exposed adults on day 20, which was not observed in the control adults. Downregulation of genes involved in energy metabolism was reported

in adult *C. finmarchicus* fed a diet of a saxitoxin-producing dinoflagellate, after an initial upregulation (Roncalli *et al.*, 2016), consistent with metabolic depression as a response to general physiological stress.

As mentioned, the expression pattern in adults contrasts with the pattern in the C5s, where we observed fewer downregulated β-oxidation genes in WSF-exposed C5s on day 20, suggesting a higher rate of β-oxidation. The causes of the opposite patterns observed in the C5s and in the adults are not clear, although it is tempting to suggest that differences in the physiology between these two life stages most likely played a major role. Adult and C5 copepods have differing energetic demands, and WSF exposure may thus cause different responses in the control and exposed copepods. The consequence of the downregulation of lipid catabolism in WSF-exposed adults on day 20 may be that less energy from lipid catabolism is available for reproduction and migration to surface waters.

Master regulators of lipid metabolism also indicate disruption of lipid metabolism

NHR-E75 is a member of the same nuclear receptor family as the ecdysone receptor (EcR) and is involved in molting and developmental progression in arthropods (Mané-Padrós *et al.*, 2008; Nakagawa and Henrich, 2009). NHR-E75 may be a functional equivalent of the vertebrate subtype PPARγ, which is a master regulator of lipid metabolism (Smith, 2002; Hong and Park, 2010), with potential for disruption by environmental contaminants (*e.g.*, Routti *et al.*, 2016). This master regulator was significantly downregulated in control adults on day 13, but not 20, while in the WSF-exposed adults, *NHR-E75* was not downregulated on day 13, but it was downregulated on day 20. SREBP is a membrane-bound transcription factor that can stimulate lipid biosynthesis, as shown in insects, where it has an essential role in membrane production (Dobrosotskaya *et al.*, 2002). TAp63 is closely linked to lipid metabolism in mice (Su *et al.*, 2012). *SREBP* and *TAp63* were significantly downregulated in control adults on days 13 and 20 but significantly downregulated in the exposed adults only on day 20. Depending on the roles these master regulators play in copepods, the fact that downregulation occurred later in the exposed copepods than in control copepods may support our theory of a PAH-induced pause in lipid catabolism. However, because the specific roles of each master regulator in *C. finmarchicus* are unknown, it is difficult to evaluate potential effects of the observed differences in expression of these genes.

Reduced utilization of lipids in the lipid sac in water-soluble fraction-exposed C5 copepodites

The relative lipid content declined during the time of the experiment in the control C5s, while the lipid content remained stable in the WSF-exposed C5s (Fig. 2A). This suggests reduced utilization of the energy stores from the lipid sac in WSF-exposed C5s terminating diapause. Decreased utilization and mobilization of lipid reserves caused by exposure to petroleum hydrocarbons have previously been reported in other crustaceans, for example, lobster larvae (*Homarus americanus*) (Capuzzo *et al.*, 1984) and giant mud crab (*Scylla serrata*) (Elumalai and Balasubramanian, 1999). PAH exposure and lipid catabolism in copepods were investigated in a recent study where overwintering *C. glacialis* were exposed to the PAH pyrene (Toxværd *et al.*, 2018). The exposure resulted in a reduction in both the mobilization of lipid stores from the lipid sac and the capacity to rebuild lipid stores after exposure (Toxværd *et al.*, 2018). The molecular basis for this observation was not investigated in that study. The gene expression results of our study show a downregulation of several β-oxidation genes in WSF-exposed copepods (see *Discussion*). Downregulation of these genes indicates a lower capacity to produce lipid catabolic enzymes. Thus, we can suggest that exposure to PAHs in a WSF of a North Sea crude oil affects this biochemical catabolic pathway in calanoid copepods.

Ecological consequences of water-soluble fraction-induced disruption of lipid metabolism

The lipid accumulation window hypothesis argues that accumulating lipids above some threshold triggers the entry into diapause (Pepin and Head, 2009). When lipids are depleted below some threshold during diapause, termination might be triggered (Johnson *et al.*, 2008). A PAH-induced reduction in maximum capacity to produce enzymes involved in lipid catabolism may result in a delayed termination of diapause and, thus, a delayed migration to surface waters. This postponement in resurfacing could result in the copepods missing the spring phytoplankton bloom, potentially resulting in starvation, reduced reproductive output, low survival of offspring, and a subsequent collapse of the population. This hypothetical scenario is illustrated in Figure 5. Ultimately, oil exposure in deep waters can shift marine ecosystem dynamics. Whether the observed effects of WSF exposure on lipid catabolism also influence the ability of the C5s to molt into reproductive adults, that is, the C5 : adult ratio, should be investigated in future studies. For now, no method exists to induce diapause in the lab, making it impossible to expose copepods to oil during active feeding stages and then investigate effects during diapause. This is currently a key experimental limitation for assessing effects of exposure to oil pollution in more detail in these species.

Conclusions

Our results indicate that exposure to PAHs in a WSF of crude oil alters lipid metabolism in *Calanus* spp. C5s, terminating diapause by causing temporary reduction (*i.e.*, a pause) in lipid catabolism. We showed that WSF-exposed *Calanus*

spp. C5s had a less steep decline in relative lipid content than the control C5s and that on days 5 and 13 more genes involved in lipid catabolism were downregulated in WSF-exposed copepods than in the control copepods. Downregulation of these genes suggests a lower capacity to produce enzymes associated with lipid catabolism. Protein abundance and enzymatic activities were not investigated here and should be subjects of future studies. Because endogenous lipid content can be an important trigger for termination of diapause, our results imply that the timing of termination and subsequent migration to the surface may be shifted if copepods are exposed to oil pollution during diapause or diapause termination. Because of the importance of *Calanus* spp. in the marine ecosystems of the North Atlantic, changes to these dynamics can have dire ecological consequences.

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Data Availability

Sequence data have been submitted to the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov) under BioProject PRJNA420690. The annotation of the *Calanus finmarchicus* reference transcriptome is available at https://figshare.com/articles/Annotation_of_Calanus_finmarchicus_transcriptome_NCBI_Bioproject_PRJNA231164_/8199416.

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Appendix

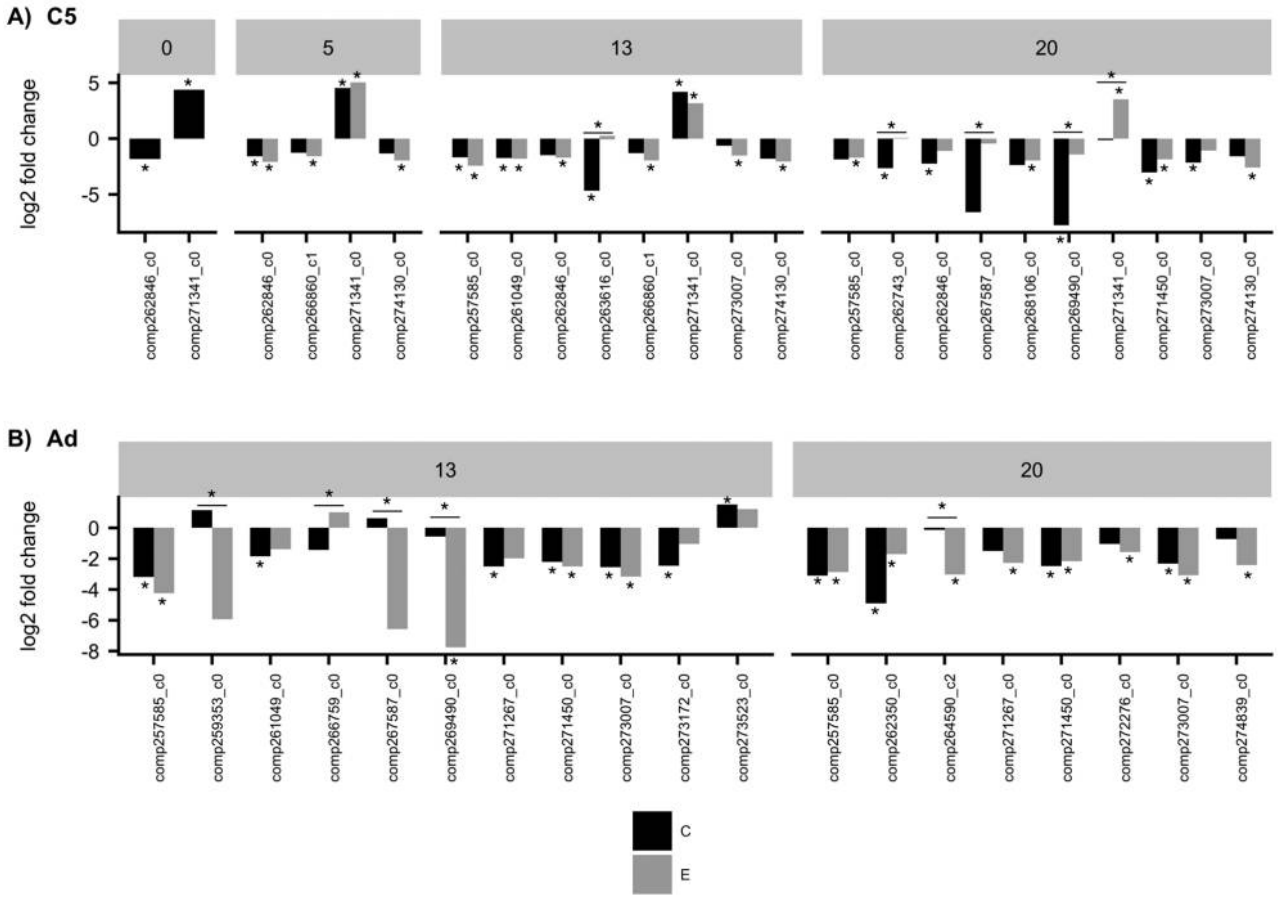

Figure A1. Differentially expressed ($\log_2$ fold change) β-oxidation genes in control (C; black bars) and water-soluble fraction (WSF)-exposed (E; gray bars) *Calanus* spp. C5 copepodites (C5; A) and adults (Ad; B) exposed to a WSF from day 0 to day 5, compared to the reference group. E, WSF-exposed group; C, control group. Recovery from day 6 to day 20. All bars show $\log_2$ fold change compared to the reference group. Asterisks signify statistical significant difference ($P < 0.05$) between comparisons.

Table A1

Chemical characterization of crude oil and water samples taken during the experiment

Sample name	Crude oil profile (g kg^{-1})	WS (48 h; $\mu g\ L^{-1}$)	WS (48 h; $\mu g\ L^{-1}$)	WS (96 h; $\mu g\ L^{-1}$)
Decalin	0.734	0.838	0.958	1.017
C1-decalins	1.404	0.433	0.483	0.532
C2-decalins	1.125	0.102	0.113	0.130
C3-decalins	0.790	ND	ND	ND
C4-decalins	0.662	ND	ND	ND
Benzo(b)thiophene	ND	ND	ND	ND
Naphthalene	0.872	18.870	18.552	18.976
C1-naphthalenes	1.831	32.689	32.195	34.600
C2-naphthalenes	2.297	19.780	19.920	21.824
C3-naphthalenes	1.662	8.767	8.710	8.786
C4-naphthalenes	0.921	1.769	1.762	1.771
Biphenyl	0.309	3.790	3.894	4.139
Acenaphthylene	0.025	0.051	0.060	0.062
Acenaphthene	0.034	0.340	0.342	0.382
Dibenzofuran	0.039	0.422	0.423	0.468
Fluorene	0.137	1.453	1.433	1.622
C1-fluorenes	0.329	1.917	1.822	1.837
C2-fluorenes	0.445	1.229	1.228	1.184
C3-fluorenes	0.366	0.411	0.360	0.345
Phenanthrene	0.205	1.810	1.751	1.743
Anthracene	ND	ND	ND	ND
C1-phenanthrenes/anthracenes	0.448	1.468	1.465	1.401
C2-phenanthrenes/anthracenes	0.507	0.569	0.539	0.541
C3-phenanthrenes/anthracenes	0.470	0.149	0.131	0.140
C4-phenanthrenes/anthracenes	0.349	0.058	0.059	0.047
Dibenzothiophene	0.025	0.236	0.228	0.230
C1-dibenzothiophenes	0.086	0.277	0.266	0.260
C2-dibenzothiophenes	0.123	0.128	0.124	0.126
C3-dibenzothiophenes	0.114	ND	ND	ND
C4-dibenzothiophenes	0.066	ND	ND	ND
Fluoranthene	0.016	0.071	0.056	0.055
Pyrene	0.021	0.037	0.034	0.036
C1-fluoranthrenes/pyrenes	0.180	0.117	0.111	0.112
C2-fluoranthenes/pyrenes	0.197	0.079	0.073	0.075
C3-fluoranthenes/pyrenes	0.202	ND	0.042	0.055
Benz(a)anthracene	ND	ND	ND	ND
Chrysene	0.013	0.007	0.004	0.005
C1-chrysenes	0.095	0.017	0.000	0.012
C2-chrysenes	0.121	ND	ND	ND
C3-chrysenes	0.066	ND	ND	ND
C4-chrysenes	ND	ND	ND	ND
Benzo(b)fluoranthene	0.008	ND	ND	ND
Benzo(k)fluoranthene	ND	ND	ND	ND
Benzo(e)pyrene	0.009	ND	ND	ND
Benzo(a)pyrene	0.003	ND	ND	ND
Perylene	0.006	ND	ND	ND
Indeno(1,2,3-c,d)pyrene	0.002	ND	ND	ND
Dibenz(a,h)anthracene	ND	ND	ND	ND
Benzo(g,h,i)perylene	0.004	ND	ND	ND
Phenol	ND	0.069	0.074	0.066
C1-phenols (o- and p-cresol)	ND	0.106	0.074	ND
C2-phenols	ND	ND	ND	ND
C3-phenols	ND	ND	ND	ND
C4-phenols	ND	ND	ND	ND
C5-phenols	ND	ND	ND	ND
30 ab hopane	0.149	0.000	0.000	0.000

Data shown are from 48 hours ($n = 2$) and 96 hours ($n = 1$). One replicate from 96 hours was contaminated and thus not analyzed. ND, not detected; WS, water sample.

Table A2

Number of reads per sample

Accession no.	Stage	Group	Day	Replicate	M seqs
SRX3459593	C5	Ref	—	1	7.7
SRX3459594	C5	Ref	—	2	11
SRX3459596	C5	C	0	1	10.3
SRX3459589	C5	C	0	2	9.4
SRX3459595	C5	C	0	3	7.1
SRX3459591	C5	C	5	1	12
SRX3459590	C5	C	5	2	8.3
SRX3459606	C5	E	5	1	10.9
SRX3459605	C5	E	5	2	11.3
SRX3459592	C5	C	13	1	12
SRX3459587	C5	C	13	2	9.4
SRX3459604	C5	E	13	1	14.5
SRX3459603	C5	E	13	2	8
SRX3459588	C5	C	20	1	5.6
SRX3459602	C5	E	20	1	29.9
SRX3459601	C5	E	20	2	24.1
SRX3459600	Ad	C	13	1	8.6
SRX3459599	Ad	C	13	2	14.9
SRX3459585	Ad	E	13	1	4.7
SRX3459598	Ad	C	20	1	7.6
SRX3459597	Ad	C	20	2	5.1
SRX3459584	Ad	E	20	1	5.3
SRX3459583	Ad	E	20	2	7.6

Shown are National Center for Biotechnology Information accession numbers assigned to each sample library. Ad, adults; C, control; E, water-soluble fraction exposed; M seqs, number of reads per sample library in millions; ref, reference group.

Table A3

Top hit sequences of master regulator genes identified in Calanus finmarchicus

Gene	Transcript ID	Annotation in *C. finmarchicus* transcriptome	Sequence obtained from	% identity	E-value	Accession no.
SREBP	comp273734_c0	Sterol regulatory element-binding protein	*Daphnia magna*	36.32	0.00E+00	KZS19898.1
HNF-4	comp272555_c0	Transcription factor hormone nuclear factor-4 homolog	*Tigriopus japonicus*	64.2	0.00E+00	AID52844.1
TAp63	comp270344_c0	Tumor protein p73	*Mytilus galloprovincialis*	39.42	2.41E-21	ACD76065.2
PPARa	comp271239_c0	Nuclear hormone receptor E-75	*Homo sapiens*	35.4	6.24E-57	NP_001001928.1

Table A4

ß-oxidation gene expression results of Calanus *spp. C5 copepodites and adults compared to the reference group*

Group	Stage	Day	Transcript ID	Annotation	EC	log FC	log CPM	*F*	*P*	FDR
Control	C5	0	comp271341_c0	Long-chain-fatty-acid-CoA ligase ACSBG2	EC:6.2.1.3	4.379	5.475	17.183	0.001	0.052
		0	comp262846_c0[1]	Aldehyde dehydrogenase, mitochondrial	EC:1.1.1.35	−1.834	7.696	21.330	0.008	0.158
		5	comp271341_c0[1]	Long-chain-fatty-acid-CoA ligase ACSBG2	EC:6.2.1.3	4.527	5.475	17.191	0.001	0.107
		5	comp262846_c0	Aldehyde dehydrogenase, mitochondrial	EC:1.1.1.35	−1.570	7.696	12.203	0.043	0.415
		13	comp271341_c0	Long-chain-fatty-acid-CoA ligase ACSBG2	EC:6.2.1.3	4.186	5.475	15.027	0.001	0.066
		13	comp261049_c0	Hydroxyacyl-CoA dehydrogenase, mitochondrial	EC:4.2.1.17 1.1.1.211	−1.732	6.887	13.081	0.026	0.271
		13	comp257585_c0	S-(hydroxymethyl)glutathione dehydrogenase	EC:1.1.1.284 1.1.1.1	−1.679	5.144	9.076	0.033	0.293
		13	comp263616_c0	Enoyl-CoA hydratase, mitochondrial	EC:4.2.1.17	−4.668	0.604	5.440	0.045	0.325
		20	comp271450_c0	Glutaryl-CoA dehydrogenase, mitochondrial	EC:1.3.8.6	−3.045	5.849	18.971	0.006	0.309
		20	comp269490_c0	Alpha-aminoadipic semialdehyde dehydrogenase	EC:1.2.1.31 1.2.1.8 1.2.1.3	−7.761	3.149	8.785	0.009	0.343
		20	comp262743_c0	3-ketoacyl-CoA thiolase, mitochondrial	EC:2.3.1.16	−2.656	7.694	14.475	0.015	0.383
		0	comp262846_c0	Aldehyde dehydrogenase, mitochondrial	EC:1.1.1.35	−2.230	7.696	13.521	0.033	0.463
		20	comp273007_c0	Long-chain-fatty-acid-CoA ligase ACSBG2	EC:6.2.1.3	−2.146	5.490	12.685	0.042	0.493
	Ad	13	comp257585_c0	S-(hydroxymethyl)glutathione dehydrogenase	EC:1.1.1.284 1.1.1.1	−3.185	5.144	29.483	0.000	0.007
		13	comp273007_c0	Long-chain-fatty-acid-CoA ligase ACSBG2	EC:6.2.1.3	−2.545	5.490	31.320	0.001	0.025
		13	comp271267_c0	Long-chain-fatty-acid-CoA ligase 4	EC:6.2.1.3	−2.507	5.837	27.320	0.002	0.028
		13	comp271450_c0	Glutaryl-CoA dehydrogenase, mitochondrial	EC:1.3.8.6	−2.195	5.849	19.990	0.005	0.051
		13	comp261049_c0	Hydroxyacyl-CoA dehydrogenase, mitochondrial	EC:4.2.1.17 1.1.1.211	−1.846	6.887	14.998	0.017	0.097
		13	comp273172_c0	Delta3-Delta2-enoyl-CoA isomerase	EC:5.3.3.8	−2.448	3.736	5.433	0.033	0.138
		13	comp273523_c0	Alcohol dehydrogenase class-3	EC:1.1.1.284 1.1.1.1	1.529	7.565	13.962	0.049	0.171
		20	comp257585_c0	S-(hydroxymethyl)glutathione dehydrogenase	EC:1.1.1.284 1.1.1.1	−3.108	5.144	27.740	0.000	0.009
		20	comp271450_c0	Glutaryl-CoA dehydrogenase, mitochondrial	EC:1.3.8.6	−2.481	5.849	24.587	0.002	0.032
		20	comp273007_c0	Long-chain-fatty-acid-CoA ligase ACSBG2	EC:6.2.1.3	−2.330	5.490	26.275	0.003	0.046
		20	comp262350_c0	Alcohol dehydrogenase class-3	EC:1.1.1.284 1.1.1.1	−4.913	1.909	8.267	0.011	0.087

Table A4 (*Continued*)

Group	Stage	Day	Transcript ID	Annotation	EC	log FC	log CPM	*F*	*P*	FDR
WSF-exposed	C5	5	comp271341_c0[1]	Long-chain-fatty-acid-CoA ligase ACSBG2	EC:6.2.1.3	5.050	5.475	20.521	0.000	0.028
		5	comp262846_c0[1]	Aldehyde dehydrogenase, mitochondrial	EC:1.1.1.35	−2.093	7.696	20.987	0.008	0.120
		5	comp274130_c0	Long-chain-fatty-acid-CoA ligase 1	EC:6.2.1.3	−1.970	3.371	7.860	0.029	0.212
		5	comp266860_c1	Acetyl-CoA acetyltransferase, mitochondrial	EC:2.3.1.16	−1.577	5.881	10.594	0.039	0.240
		13	comp257585_c0	S-(hydroxymethyl)glutathione dehydrogenase	EC:1.1.1.284 1.1.1.1	−2.434	5.144	18.439	0.002	0.061
		13	comp271341_c0	Long-chain-fatty-acid-CoA ligase ACSBG2	EC:6.2.1.3	3.188	5.475	9.351	0.008	0.107
		13	comp266860_c1	Acetyl-CoA acetyltransferase, mitochondrial	EC:2.3.1.16	−1.967	5.881	15.949	0.011	0.130
		13	comp261049_c0	Hydroxyacyl-CoA dehydrogenase, mitochondrial	EC:4.2.1.17 1.1.1.211	−1.813	6.887	14.486	0.019	0.165
		13	comp274130_c0	Long-chain-fatty-acid-CoA ligase 1	EC:6.2.1.3	−2.042	3.371	8.232	0.026	0.187
		13	comp262846_c0	Aldehyde dehydrogenase, mitochondrial	EC:1.1.1.35	−1.698	7.696	14.162	0.030	0.200
		13	comp273007_c0	Long-chain-fatty-acid-CoA ligase ACSBG2	EC:6.2.1.3	−1.531	5.490	12.253	0.046	0.246
		20	comp271341_c0	Long-chain-fatty-acid-CoA ligase ACSBG2	EC:6.2.1.3	3.534	5.475	11.301	0.004	0.087
		20	comp274130_c0	Long-chain-fatty-acid-CoA ligase 1	EC:6.2.1.3	−2.595	3.371	12.941	0.005	0.097
		20	comp271450_c0	Glutaryl-CoA dehydrogenase, mitochondrial	EC:1.3.8.6	−1.872	5.849	14.974	0.015	0.155
		20	comp268106_c0	Aldehyde dehydrogenase family 3 member B1	EC:1.2.1.3	−1.969	3.711	9.139	0.026	0.196
		20	comp257585_c0	S-(hydroxymethyl)glutathione dehydrogenase	EC:1.1.1.284 1.1.1.1	−1.693	5.144	9.515	0.029	0.205
	Ad	13	comp257585_c0	S-(hydroxymethyl)glutathione dehydrogenase	EC:1.1.1.284 1.1.1.1	−4.235	5.144	22.950	0.001	0.073
		13	comp273007_c0	Long-chain-fatty-acid-CoA ligase ACSBG2	EC:6.2.1.3	−3.173	5.490	23.773	0.005	0.184
		13	comp269490_c0	Alpha-aminoadipic semialdehyde dehydrogenase	EC:1.2.1.31 1.2.1.8 1.2.1.3	−7.761	3.149	9.164	0.008	0.211
		13	comp271450_c0	Glutaryl-CoA dehydrogenase, mitochondrial	EC:1.3.8.6	−2.506	5.849	13.981	0.019	0.299
		20	comp273007_c0	Long-chain-fatty-acid-CoA ligase ACSBG2	EC:6.2.1.3	−3.075	5.490	42.434	0.000	0.009
		20	comp257585_c0	S-(hydroxymethyl)glutathione dehydrogenase	EC:1.1.1.284 1.1.1.1	−2.867	5.144	24.208	0.000	0.014
		20	comp271267_c0	Long-chain-fatty-acid-CoA ligase 4	EC:6.2.1.3	−2.267	5.837	22.447	0.004	0.047
		20	comp264590_c2	Long-chain specific acyl-CoA dehydrogenase, mitochondrial	EC:1.3.8.8	−3.033	2.996	20.336	0.006	0.054
		20	comp271450_c0	Glutaryl-CoA dehydrogenase, mitochondrial	EC:1.3.8.6	−2.153	5.849	19.107	0.006	0.056
		20	comp274839_c0	Fatty aldehyde dehydrogenase	EC:1.2.1.3	−2.419	3.201	6.538	0.029	0.132
		20	comp272276_c0	Aldehyde dehydrogenase family 1 member A3	EC:1.2.1.3	−1.573	5.338	13.658	0.043	0.162

Transcripts that were commonly expressed in control and WSF-exposed C5s on day 5, and thus removed when presenting gene expression patterns, are indicated with a superscript 1. Ad, adults; CoA, coenzyme A; EC, Enzyme Commission number; FDR, false discovery rate; log CPM, $\log_2$ counts per million; log FC, $\log_2$ fold change; WSF, water-soluble fraction.

Table A5

ß-oxidation gene expression results in control Calanus *spp. C5 copepodites and adults compared to the WSF-exposed copepods sampled on the same day*

Stage	Day	Transcript ID	Annotation	EC	log FC	log CPM	*F*	*P*	FDR
C5	5	None							
	13	comp263616_c0	Enoyl-CoA hydratase, mitochondrial	EC:4.2.1.17	4.902	0.604	6.054	0.034	1
	20	comp262743_c0	3-ketoacyl-CoA thiolase, mitochondrial	EC:2.3.1.16	2.701	7.694	14.902	0.014	0.976
		comp269490_c0	Alpha-aminoadipic semialdehyde dehydrogenase	EC:1.2.1.3	6.964	3.149	6.327	0.027	0.976
		comp271341_c0	Long-chain-fatty-acid-CoA ligase ACSBG2	EC:6.2.1.3	3.681	5.475	5.605	0.031	0.976
		comp267587_c0	Short-chain specific acyl-CoA dehydrogenase, mitochondrial	EC:1.3.8.1	7.045	2.455	5.118	0.038	0.976
Ad	13	comp259353_c0	Acetyl-CoA acetyltransferase A, mitochondrial	EC:2.3.1.9	−7.087	2.101	5.055	0.039	1
		comp266759_c0	Probable 3-hydroxyacyl-CoA dehydrogenase B0272.3	EC:1.1.1.35	2.436	2.494	4.798	0.044	1
		comp267587_c0	Short-chain specific acyl-CoA dehydrogenase, mitochondrial	EC:1.3.8.1	−7.196	2.455	5.665	0.030	1
		comp269490_c0	Alpha-aminoadipic semialdehyde dehydrogenase	EC:1.2.1.3	−7.210	3.149	7.349	0.017	1
	20	comp264590_c2	Long-chain specific acyl-CoA dehydrogenase, mitochondrial	EC:1.3.8.8	−2.910	2.996	18.448	0.008	1

Ad, adults; CoA, coenzyme A; EC, Enzyme Commission number; FDR, false discovery rate; log CPM, $\log_2$ counts per million; log FC, $\log_2$ fold change; WSF, water-soluble fraction.

Table A6

Gene expression results of master regulator genes in control Calanus *spp. C5 copepodites and adults compared to WSF-exposed copepods sampled on the same day*

Stage	Day	Transcript ID	Annotation	log FC	log CPM	*F*	*P*	FDR
C5	5	comp271239_c0	*NHR-E75*	0.585	7.902	2.091	0.443	1
C5	13	comp271239_c0	*NHR-E75*	0.122	7.902	0.092	0.872	1
C5	20	comp271239_c0	*NHR-E75*	0.179	7.902	0.128	0.849	1
Ad	13	comp271239_c0	*NHR-E75*	0.864	7.902	3.138	0.348	1
Ad	20	comp271239_c0	*NHR-E75*	−0.391	7.902	0.917	0.612	1
C5	5	comp273734_c0	*SREBP*	−0.586	4.318	1.973	0.461	1
C5	13	comp273734_c0	*SREBP*	−0.624	4.318	2.106	0.447	1
C5	20	comp273734_c0	*SREBP*	0.411	4.318	0.532	0.702	0.978
Ad	13	comp273734_c0	*SREBP*	0.195	4.318	0.119	0.857	1
Ad	20	comp273734_c0	*SREBP*	0.142	4.318	0.100	0.868	1
C5	5	comp270344_c0	*TAp63*	−0.774	2.479	1.537	0.419	1
C5	13	comp270344_c0	*TAp63*	−0.702	2.479	1.095	0.495	1
C5	20	comp270344_c0	*TAp63*	0.149	2.479	0.026	0.916	1
Ad	13	comp270344_c0	*TAp63*	1.135	2.479	1.608	0.408	1
Ad	20	comp270344_c0	*TAp63*	−0.117	2.479	0.020	0.927	1
C5	5	comp272555_c0	*HNF-4*	−0.226	4.333	0.282	0.775	1
C5	13	comp272555_c0	*HNF-4*	−0.718	4.333	2.611	0.385	1
C5	20	comp272555_c0	*HNF-4*	−1.187	4.333	5.299	0.216	0.976
Ad	13	comp272555_c0	*HNF-4*	−0.877	4.333	1.809	0.470	1
Ad	20	comp272555_c0	*HNF-4*	−0.241	4.333	0.278	0.777	1

Ad, adults; FDR, false discovery rate; *HNF-4*, transcription factor hormone nuclear factor-4 homolog; log CPM, $\log_2$ counts per million; log FC, $\log_2$ fold change; *NHR-E75*, nuclear hormone receptor E-75; *SREBP*, sterol regulatory element-binding protein; *TAp63*, tumor protein p73; WSF, water-soluble fraction.