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Dietary chlorpyrifos-methyl exposure impair transcription of immune-, detoxification- and redox signaling genes in leukocytes isolated from cod (*Gadus morhua*)

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A B S T R A C T

Inclusion of new environmental toxicants increase with the amount of plant ingredients substituting marine proteins and oils in feed for farmed Atlantic salmon (*Salma salar*). Agricultural pesticides like chlorpyrifos-methyl, present in commercial salmon feeds, may affect salmon immune and detoxification responses. Atlantic cod (*Gadus morhua*), surrounding the net pens, grazing on feces and uneaten pellets may be affected accordingly. The aim of this study was to analyze transcription responses in Atlantic cod head kidney tissue and isolated leukocytes following dietary chlorpyrifos-methyl inclusions and possible interactions with proinflammatory signals. Head kidney tissues and leukocytes were isolated from cod fed diets contaminated with chlorpyrifos-methyl (0.5 mg/kg, 2.4 mg/kg, 23.2 mg/kg) for 30 days. The isolated leukocytes were further challenged with bacteria (lipopolysaccharide (LPS), virus (polyinosinic acid:polycytidylic acid (PIC) mimic and L-arginine, an immuno-modulating amino acid, *in vitro*.

The LPS-induced transcription of the interleukin genes *il-1β, il-6, il-8* increased in leukocytes isolated from cod fed chlorpyrifos-methyl 23.2 mg/kg, compared to cod fed the control diet, indicating increased inflammation. Transcriptional levels of carnitine palmitoyl transferase (*cpt1a),* aryl hydrogen receptor (*ahr*) and *catalase (cat)* were all reduced by dietary inclusions of chlorpyrifos-methyl in the leukocytes. The findings suggests that dietary chlorpyrifos-methyl exposure impair inflammation, detoxification and redox signaling in cod leukocytes.

1. Introduction

Limited marine resources require increasing use of plant ingredients in the feed of farmed carnivorous fish species. Along with plant proteins and oils, unwanted substances like pesticides from agricultural activity may end up in commercial fish feed. Organophosphorus pesticides such as chlorpyrifos-methyl are often found in trace amounts in today's aquafeeds. According to a Norwegian survey (2017), chlorpyrifos-methyl levels in commercial Atlantic salmon (*Salmo salar*) feed ranged from 11 to 26 μg/kg [1]. Salmon production in Norway is based on housing in open net pens in the sea. To produce 1 kg of salmon around 0.5 kg of feces and uneaten pellets are generated [2]. Uneaten feed pellets and feces may slip through the net pens and introduce dietary contaminants to marine organisms feeding on the nutritious leftovers [3,4].

Chlorpyrifos-methyl is widely used to control unwanted insects in agriculture and is a moderately persistent contaminant with a half -life ranging from days to months [5]. Agricultural chlorpyrifos-methyl can impact many non-target terrestrial species like the developing stage of worker honeybees (*Apis mellifera)* [6], freshwater species like the three-spined stickleback (*Gasterosteus aculeatus)* [7] and amphibians [8,9]. Mammalian species could also be affected due to the runoff into rivers and ground water [10,11]. In fish, several toxic effects of chlorpyrifos-methyl have been reported. In salmon liver, chlorpyrifos-methyl exposure affects lipid metabolisms as well as induce oxidative stress [12–14]. Studies on common carp (*Cypriunus carpio* L.) suggested that chlorpyrifos-methyl may cause immunotoxicity [15,16]. Yang et al. [17] showed that chlorpyrifos-methyl induced apoptosis and autophagy in common carp lymphocytes. In Atlantic salmon and cod (*Gadus morhua*), the effects of chlorpyrifos-methyl inclusion in diets have been studied in liver and brain respectively [13,18].

To cope with immunotoxic chemical stress, farmed fish needs an optimized diet. Arginine is an essential amino acid in salmon [19–21] and other carnivorous fish species. Arginine requirements in fish are not stable and varies with fish species, fish size, fish health and environmental conditions [22,23]. In fish, the influence of arginine supplementation on the pattern recognition receptor (PPR) induced inflammation responses is largely unknown, but over the years it is

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reported that appropriate arginine supplementation will benefit fish immunity to fight against environmental stress and infections [24–28].

Arginine metabolisms produce nitric oxide (NO) and citrulline via nitric oxide synthase and polyamines via arginase 1 and 2, of which NO and polyamines directly can modulate gene expression and regulate nutrient availability of immune cells, both in mammals and fish [20,21,26,29,30]. In mammals, the arginine metabolic pathways cross inhibit each other on the level of arginine breakdown products, respectively. In the mammalian immune system, macrophage biology is driven by the phenotype of macrophage arginine metabolism that is prevalent in the ongoing immune response, meaning that an evolving immune response could be either pro- or anti-inflammatory [29]. Arginine was reported to exert immune-modulatory functions, regulating innate and adaptive immune responses, inhibit leukocyte apoptosis and regulate bacterial infections in fish [31]. Martins et al. [26] showed that both LPS and PIC could modulate genes involved in polyamine synthesis in salmon liver cells and head kidney leukocytes. Also reported is that increasing amounts of arginine in the diet may impair fish disease resistance and the amount of arginine supplemented to the diet should be evaluated in feeding practices. [22,24,32,33].

Besides inducing oxidative stress, LPS and PIC also induce specific and common immune responses in head kidney leukocytes isolated from salmon and cod [26,34]. Holen et al. [35] and Iliev et al. [36] have previously shown that LPS partly signals through p38 MAPK. LPS signaling in fish differ from mammals as mammalian TLR4 binding to LPS requires MD-2 and CD14 costimulatory molecules [37] not found in fish genomes to date [38,39]. Although TLR4 has been cloned and characterized in many fish species it seems that LPS is not recognized by fish TLR4 [40]. PIC is structurally similar to double stranded RNA which is present in some viruses and stimulates TLR3 and virus induced gene markers like *isg15* [34].

The aim of this study was to monitor dietary chlorpyrifos-methyl induced effects on immune tissue/leukocyte gene responses in Atlantic cod. Chlorpyrifos-methyl interactions with inflammatory responses was further monitored *in vitro*. In this trial, diets to cod were supplemented with chlorpyrifos-methyl in three concentrations (0.5 mg/kg, 2.4 mg/kg, 23.2 mg/kg), and the fish were fed for 30 days. A control diet without chlorpyrifos-methyl addition was included. From the main trial, a total of 24 fish, 6 fish per 4 diets were examined. The head kidney tissues were harvested and besides keeping small pieces of tissues for later transcriptional analysis, the remaining tissues were used to isolate the head kidney leukocytes. Selected leukocyte cultures were exposed to inflammatory inducers like LPS and PIC, and also to the immunomodulatory amino acid L-arginine. The applied *in vitro* head kidney cell model has been utilized in several earlier fish experiments, in which fish were fed specific diets and isolated cells were challenged with inflammatory inducers *in vitro* [35,41–43].

2. Materials and methods

2.1. Dietary chlorpyrifos-methyl exposure experiment

Locally bred Atlantic cod were obtained from an ongoing exposure experiment (total fish = 276) conducted at Tromsø Aquaculture Research Station, Kårvika, Tromsø, Norway. The experimental design is described in detail by Olsvik et al. [18]. In short, the dietary exposure experiment lasted for 30 days (May 22nd-June 21st, 2018). The experimental diet was Amber Neptum (Skretting, Norway) for marine fish 3 mm. During this period, the fish (150 g) used in this experiment received either experimental diet without chlorpyrifos-methyl, 0.5 mg/kg chlorpyrifos-methyl inclusion in the diet; 4.2 mg/kg chlorpyrifos-methyl inclusion in the diet or 23.2 mg/kg chlorpyrifos-methyl inclusion in the diet. Exposure concentrations were based on prior investigations [13,14]. Fiberglass tanks (500 L), 23 fish per tank, were supplied with filtered seawater and there were 3 tanks per treatment. The feed intake per tank was assessed and recorded daily. To determine the average dose of chlorpyrifos consumed (nanogram per kilogram fish) per day, feed intake per tank was quantified by collecting feed waste once a day post feeding more closely described under Material and Methods, Table 1, [18]. The fish were reared in seawater with salinity 34 g/L, at 7 °C, 77% oxygen saturation, using 24 h light regime which is a natural photoperiod at 69°N. Before the *in vitro* experiments, the fish were euthanized by an overdose of tricaine methane sulfonate (0.07 g/L) followed by a quick blow to the head.

2.2. Atlantic cod head kidney tissue

Head kidneys were isolated from Atlantic cod at the end of 30 days exposure period. Cells were obtained from six fish per diet (the total number of fish used was 24). For each fish, small pieces of the harvested head kidney tissues were immersed in 1 mL RNA*later* (R0901, Merck). The remaining tissue were used to isolate head kidney leukocytes for *in vitro* exposure.

2.3. Atlantic cod head kidney leukocyte isolation

For each fish, the remaining head kidney was added to a sterile isolation buffer containing 9 g NaCl/L and 7 g EDTA/L, pH 7.2. The cells were aspirated with a syringe and then squeezed through a 40 μM Falcon cell strainer. The cells were transferred to 50 mL tubes and washed by centrifugation in a Hettich Zentrifugen, 320R, at 600 g, 5 min, and 4 °C. Cell pellets were resuspended in the isolation buffer and layered carefully on top of equal amounts of diluted Percoll with density 1.08 g/mL. To remove the red blood cells from the cell supernatant, the tubes were centrifuged at 800 g, 5 min, at room temperature. The remaining immune cells in the supernatant and Percoll layer were collected and pelleted by centrifugation, 800*g*, 4 °C, for 5 min. Two additional washing steps were performed before resuspending the cells in cL-15. The viability of the isolated cells was assessed by trypan blue counting (T10282, Thermo Fischer Scientific. The viability of the leukocytes was high (>90%).

2.4. Cell medium and reagents

L-15 medium (L5520, Sigma Aldrich, Buchs, Switzerland)) was supplemented with 10% fetal bovine serum (FBS)(BioWhittaker, cat#14–801F), pen/strep (50U/mL, BioWhittaker, cat#17-602E), 2% 2 mM glutamax™ 100 × (Gibco, cat#35056) and was designated complete medium (cL-15). Lipopolysaccharide was derived from *Pseudomonas aeruginosa* (LPS, cat# L-7018). Polyinosinic acid:polycytidylic acid, (PIC, cat# P9582) and L-arginine (cat#A8094) were purchased from Sigma Aldrich.

2.5. In vitro challenging study

Leukocytes (n = 24), 1×10^7, were seeded into 6 well culture plates (Costar, cat#3335) and medium was added to a final volume of 2 mL. The cells were plated in either standard cL-15 medium (measured concentration of 1.82 mM arginine, control) or L-15 medium supplemented with L-arginine to concentrations of 3.63 mM (x2Arg, surplus arginine) on the day of cell isolation. The cultures were incubated for 24 h in the dark in a normal atmosphere incubator (Sanyo Incubator) at 9 °C. The second day of culturing, some of the wells received 100 μg/mL LPS or 50 μg/mL PIC. Untreated cultures were included as controls. All cultures were incubated for 24 h post LPS or PIC addition before being sampled.

2.6. RNA extraction

Total RNA was extracted from the head kidney cells, using RNeasy®Plus kit (Qiagen) and from head kidney tissue using EZ1RNA universal tissue kit (Qiagen, Crawly, UK), according to the manufacturer's instructions, and frozen at −80 °C. The quantity and quality of RNA

Table 1
Primers for qPCR.

Primer	Forward	Reverse	Accession number
cyp1a1	*CCTTGACCTCTCGGAGAAAGAC*	*CGCCCCGCTAGCTATAGACA*	*EX721847*
casp3	*CAGGCTGTGGAGAGCACCTT*	*GTGGTCCTCCTGGGACACAGT*	*EX726570*
cat	*GCCAAGTTGTTGAGCAGGTT*	*CTGGGATCACGCACCGTATC*	*DQ270487*
il-1β	*GGAGAACACGGACGACCTGA*	*CGCACCATGTCACTGTCCTT*	*EU007443*
cox2(ptgs2)	*CCGGACTGGATTCAATGGAG*	*GGACCGACATGTTGTTGACG*	*XM_030372675*
isg15	*CACCCGGTGCCAATATCTTC*	*CCCAGGTCGGACGGTGTAG*	*EX725117*
il-8	*GTTTGTTCAATGATGGGCTGTT*	*GACCTTGCCTCCTCATGGTAATACT*	*EUOO7442*
il-6	*TGAAGAAGGAGTACCCCGACAAT*	*GGTGCCTCATCTTTTCCTCAATG*	*JF309111*
nqo2	*ACAGCCAGGGCATCTTCAAG*	*GGCCACAGGGTGACATTCAT*	*XM_030344330*
fasl	*TCCCCGACACACCACTTTGT*	*AGGTCCACACGCTGTCCTTT*	*XM_030372933*
cpt1a	*CCTGACCAGCTACGCCAAGT*	*GACCCATGGTGCCGTTCTT*	*XM_030365625*
ahr	*CAACCGGCGGTCCACAT*	*GCACCATGCAGTTGCCAGTA*	*EX728781*
alox5	*ACGACTGGCTGATCGCAAG*	*CATGGCCACTCCGAACATCT*	*XM_030378694.1*
cd36	*CATGAACCTGTCGCCTCTGA*	*CTATCAAATCCTTCCGACTGGC*	*EX722461*
usp40[a]	*TCCTTTCCTGCGTTGTTCCT*	*GCCGTTCCACCTCAAAAGATG*	*XM_030356001*
actb[a]	*CACGCCGAGCGTGGATTA*	*ACGAGCTAGAAGCGGTTTGC*	*XM_030351415*
ef1a[a]	*CGGTATCCTCAAGCCCAACA*	*GTCAGAGACTCGTGGTGCATC*	*EX722124*

[a] Reference genes.

were assessed using the NanoDrop ND-1000 UV Spectrophotometer (NanoDropTechnologies, Wilmington, DE, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). RNA integrity was assessed using the RNA 6000 Nano LabChip® kit (Agilent Technologies, Palo Alto, CA, USA). The samples used in this experiment had 260/280 nm absorbance ratios of 2.0 ± 0.1 and 260/230 nm ratios of

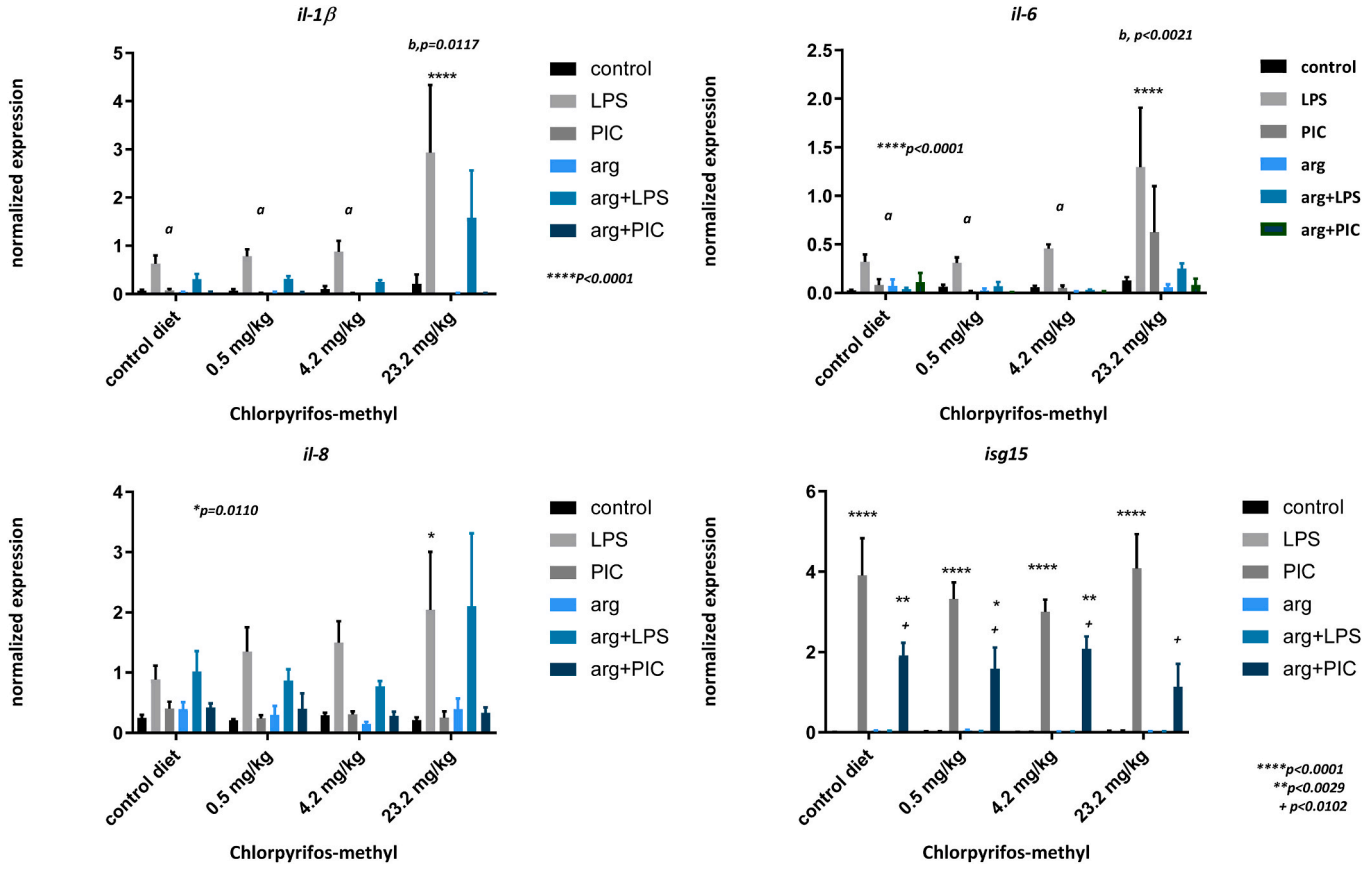

Fig. 1. Chlorpyrifos-methyl 23.2 mg/kg inclusions in diets to cod increased LPS-induced gene transcription of *il-1β, il-6 and il-8* in isolated leukocytes. Chlorpyrifos-methyl inclusions in diets to cod had no effect on PIC-induced *isg15* transcription. Diet a ± b, n = 6.
a) LPS induced significant *il-1β* transcription (****$p < 0.0001$) in leukocytes isolated from cod fed on 23,2 mg/kg chlorpyrifos-methyl inclusions in the diet. Difference between diets (b, $p = 0.0117$)
b) LPS triggered significant *il-6* transcription (****$p < 0.0001$) in leukocytes isolated from cod fed on 23,2 mg/kg chlorpyrifos-methyl inclusion in the diet. Difference between diets (b, $p < 0.0021$)
c) LPS induced significant *il-8* transcription (*$p = 0.0110$) in leukocytes isolated from cod fed on 23,2 mg/kg chlorpyrifos-methyl inclusions in the diet. No difference between the diets.
d) PIC induced transcription of *isg15* in leukocytes isolated from cod, all diets (****$p < 0.0001$). Surplus arginine reduced the PIC induced transcription of *isg15* in all diets (+ $p < 0.0344$).

2.4 ± 0.1 (mean ± STDEV, n = 24) and RIN-values between 8 and 10 indicating RNA samples suitable for RT-qPCR.

2.7. Quantitative real time RT- PCR (qPCR)

A twostep RT-qPCR amplification protocol was used to quantify the transcription levels of the target genes in head kidney tissue as well as in head kidney leukocytes, using the CFX Maestro™software version 1.1 (BioRad lab Inc). The qbase^{plus}software analyzed quantification cycle (Cq) values. The Cq values obtained were normalized using three reference genes *ef1a*, *actb* and *usp40*. Table 1 shows the q PCR primers used in this experiment.

2.8. Statistics

Gene expression between diets and treatments were analyzed using two-way ANOVA with Tukey's multiple comparison test, while the diet differences in head kidney tissues were analyzed with one-way ANOVA using GraphPad Prism 8. Letters (***a*** ± ***b***) describe diet differences while the sign (*) describes significant differences within a treatment compared to its respective control.

The sign (+) describes significant differences between PIC alone and PIC + surplus arginine.

3. Results

3.1. Immune gene transcription in leukocytes were modulated by dietary chlorpyrifos-methyl

LPS-induced *il-1β* transcription (****$p < 0.0001$), was high in leukocytes isolated from cod fed 23.2 mg/kg chlorpyrifos-methyl giving a significant difference between the diets (*b*, $p = 0.0117$) (Fig. 1a). The same pattern was observed for *il-6* as leukocytes isolated from cod fed the 23.2 mg/kg chlorpyrifos-methyl diet, expressed significantly higher levels of *il-6* (****$p < 0.0001$) than leukocytes from the other dietary groups, resulting in a significant difference between diets (*b*, $p = 0.0021$) (Fig. 1b). LPS-induced *il-8* transcription (*$p = 0.0110$) was different from control culture only in leukocytes isolated from cod fed the 23.2 mg/kg chlorpyrifos-methyl diet. There were no significant differences between the diets (Fig. 1c). High PIC-induced *isg15* transcription (****$p < 0.0001$) was observed in leukocytes isolated from head kidneys from all dietary groups when compared to respective control cultures. Adding surplus arginine to the cultures significantly reduced the PIC-induced transcription of *isg15* in the diets (control diet $^{+}p < 0.0012$, 0.5 mg/kg chlorpyrifos-methyl diet $^{+}p = 0.0102$ and, 23.2 mg/kg methyl diet $^{+}p < 0.0001$) (Fig. 1d).

3.2. Eicosanoid pathways in leukocytes may be modulated by dietary chlorpyrifos-methyl

Cox2 transcription was significantly induced by LPS in leukocytes isolated from cod fed the control diet (**$p = 0.0016$) and the 23.2 mg/kg chlorpyrifos-methyl diet (*$p = 0.035$) when compared to respective control cultures. Dietary inclusions of chlorpyrifos-methyl of 0.5 mg/kg (*b*, $p = 0.0199$) and 4.2 mg/kg (*b*, $p = 0.008$) resulted in a general inhibition of *cox2* transcription (Fig. 2a). Dietary 23.2 mg/kg chlorpyrifos-methyl reduced leukocytic *alox5* transcription compared to the other diets (*b*, $p = 0.0192$). No leukocyte culture treatment affected *alox5* transcription (Fig. 2b).

3.3. Dietary chlorpyrifos-methyl triggered leukocyte transcription of cd36, nqo2 and fasl, when leukocytes were cultured together with surplus arginine and PIC

Cd36 transcription was increased when surplus arginine was cultured together with PIC in leukocytes isolated from cod fed on 4.2 mg/kg (****$p < 0.0001$, *b*, $p = 0.0163$) and 23.2 mg/kg (***$p = 0.0004$, *b*, $p = 0.0154$) chlorpyrifos-methyl diet. Interactions between diets and cell treatments were observed ($p < 0.0001$) (Fig. 3a). Leukocytic *nqo2* transcription was increased when surplus arginine was cultured together with PIC, in leukocytes obtained from cod fed 4.2 mg/kg (****$p < 0.0001$, *b*, $p = 0.0386$) and 23.4 mg/kg (****$p < 0.0001$, *b*, $p = 0.0086$) chlorpyrifos-methyl diets. Interactions between diets and cell treatments were observed ($p = 0.0386$) (Fig. 3b). Leukocytic *fasl* transcription was increased when surplus arginine was cultured with PIC, in leukocytes obtained from cod fed 0.5 mg/kg (*$p = 0.0332$), 4.2 mg/kg (****$p < 0.0001$) and 23.2 mg/kg chlorpyrifos-methyl (***$p = 0.0009$) compared to control diet (*b*, $p < 0.0001$). Interactions between diets and treatments were measured ($p = 0.0148$) (Fig. 3c).

3.4. Transcription of detoxification genes in leukocytes was inhibited by dietary chlorpyrifos-methyl

Compared to the other diets, transcription of *ahr* was downregulated

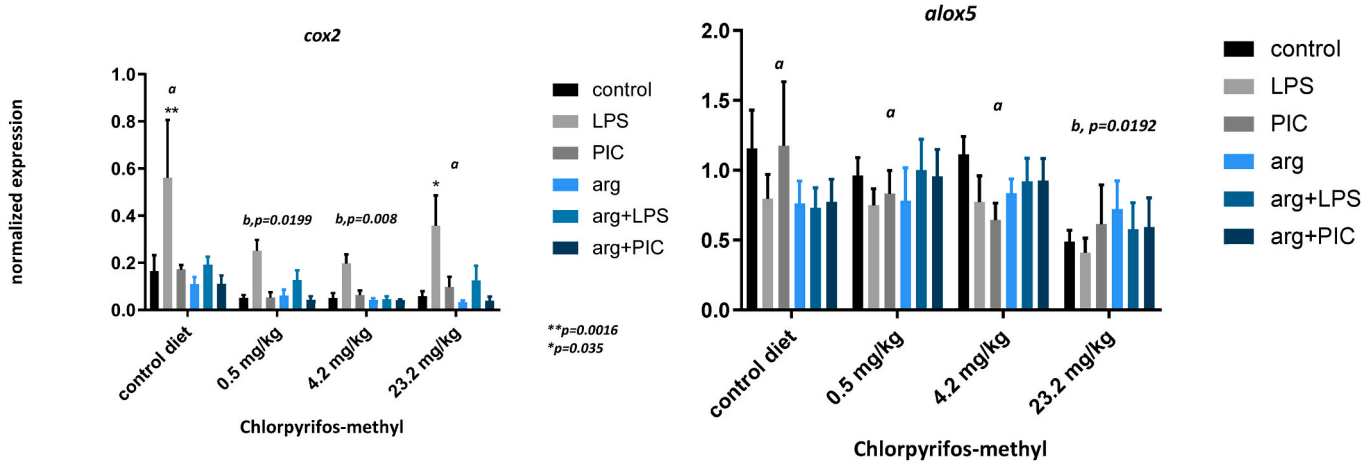

Fig. 2. Downregulation of genes involved in eicosanoid metabolism in isolated leukocytes when including chlorpyrifos-methyl in diets to cod. Diet a±b, n = 6
a) *Cox2* transcription was generally depressed in leukocytes isolated from cod fed 0.5 and 4.2 mg/kg chlorpyrifos-methyl inclusions in the diets (b, $p = 0.0199$ and b, $p = 0.008$, respectively). LPS induced significant *cox2* transcription particularly in leukocytes isolated from cod fed the control diet (**$p = 0.0016$) but also from cod fed the 23.3 mg/kg chlorpyrifos-methyl diet (*$p = 0.035$), when compared to respective control cultures.
b) alox5 transcription was reduced in leukocytes isolated from cod fed 23.2 mg/kg chlorpyrifos-methyl inclusion in diet (b, $p = 0.0192$).

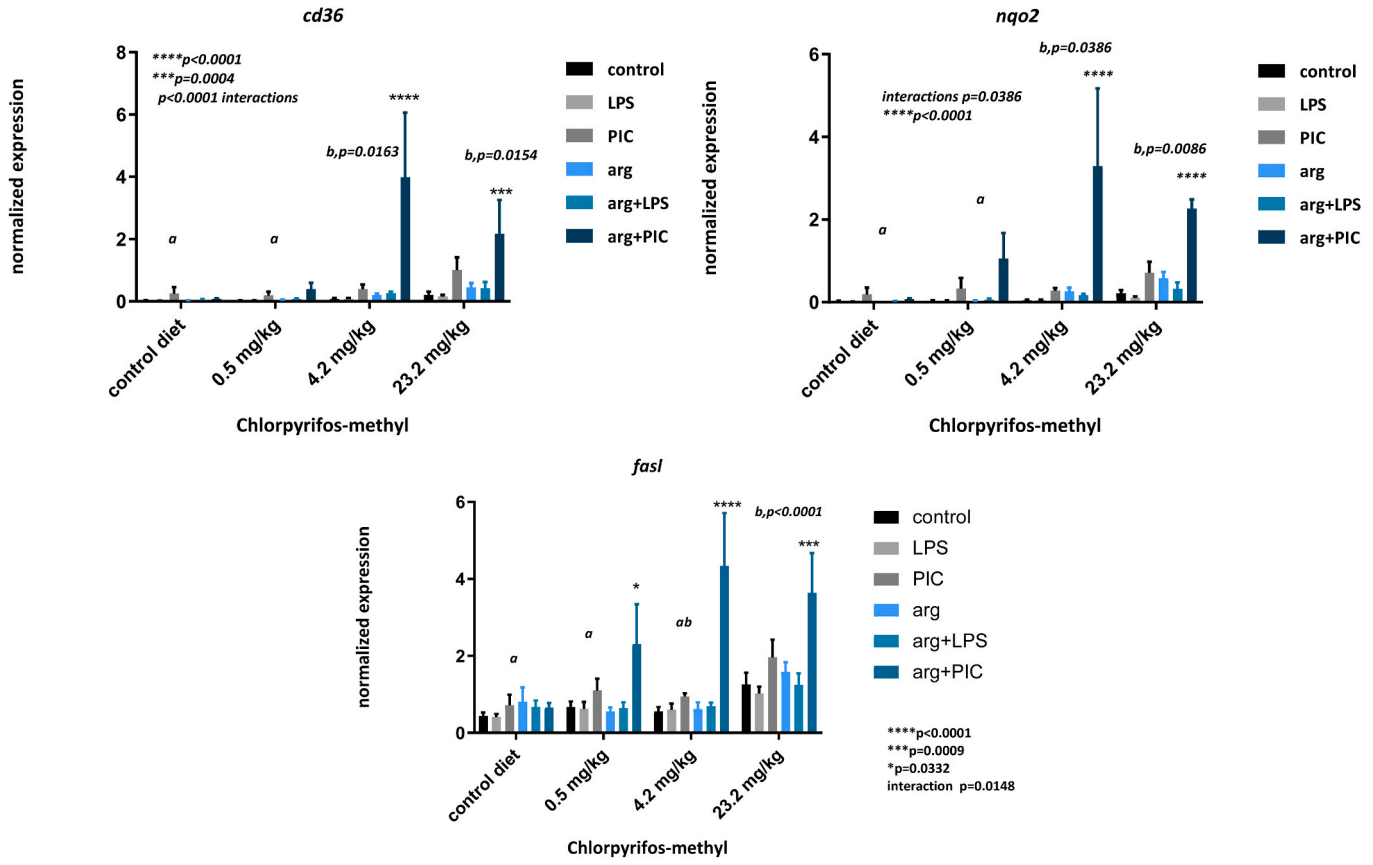

Fig. 3. Surplus arginine cultured together with PIC significantly increased transcription of the genes involved in fatty acid transport *cd36*, phase II detoxification gene *nqo2* and fatty acyl synthetase ligand gene, *fasl,* in leukocytes isolated from cod fed on chlorpyrifos-methyl inclusions in the diets. Diet a ±b, n = 6
a) Gene transcription of *cd36* was significantly increased when leukocytes isolated from cod fed on 4.2 and 23.2 mg/kg chlorpyrifos-methyl inclusions in diet were cultured together with PIC and surplus arginine (****$p < 0.0001$ and ***$p = 0.0004$, respectively) compared to respective control cultures. Diet differences (b, $p = 0.0163$ and b, $p = 0.0154$). Interactions between diets and treatments were observed (p < 0.0001).
b) Transcription of *nqo2* was significant upregulated in leukocytes isolated from cod fed on 4.2 and 23.2 mg/kg chlorpyrifos-methyl inclusions in the diet when cultured together with surplus arginine and PIC (****$p < 0.0001$), compared to respective control cultures. Diet differences (b, $p = 0.00386$ and b, $p = 0.0086$, respectively). Interaction between diets and treatments was observed ($p = 0.0386$).
c) Gene transcription of *fasl* increased significantly when isolated leukocytes from cod fed on 0.5, 4.2 and 23.2 mg/kg chlorpyrifos-methyl were cultured with surplus arginine and PIC (*$p = 0.0332$, ****$p < 0.0001$ and ***$p = 0.0009$), compared to control cultures, respectively. Diet inclusion of 23.2 mg/kg chlorpyrifos-methyl differed from the other diets (b, $p < 0.0001$). Interaction between diets and treatments was observed, ($p = 0.0148$).

in leukocytes isolated from cod dieting on 4.2 mg/kg (*b*, $p < 0.0001$) and 23.2 mg/kg (*b*, $p < 0.0001$) chlorpyrifos-methyl. PIC alone downregulated *ahr* transcription in the control diet (*$p < 0.0001$) and in the 0.5 mg/kg chlorpyrifos-methyl diet (*$p < 0.0001$) compared to respective control cultures. Surplus arginine supplementation had no effect on *ahr* transcription in these leukocytes. (Fig. 4a). The transcription profile of *cyp1a1* in leukocytes from cod dieting on 0.5 mg/kg (*b, p = 0.0015*) and 4.2 mg/kg (*b*, $p = 0.0082$) chlorpyrifos-methyl differed from the transcription profile of *cyp1a1* in the control diet and 23.2 mg/kg chlorpyrifos-methyl diet (Fig. 4b). Compared to control diet, *cat* transcription was downregulated in leukocytes isolated from cod fed the chlorpyrifos-methyl diets (0.5 mg/kg, *b*, $p = 0.0007$; 4.2 mg/kg, *b*, $p < 0.0001$; 23.2 mg/kg, *b*, $p < 0.0001$). In the control diet, when leukocytes were treated with surplus arginine together with PIC, the transcription of *cat* was significantly lower than the respective control culture (*$p = 0,021$) (Fig. 4c). *Cpt1a* transcription was reduced in leukocytes isolated from cod fed 0.5 mg/kg- (*b*, $p = 0.006$), 4.2 mg/kg (*b*, $p < 0.0001$) and 23.2 mg/kg chlorpyrifos methyl (*b*, $p < 0.0001$). Compared to respective control cultures, PIC downregulated *cpt1a* transcription significantly in leukocytes isolated from the control diet (*$p < 0.0001$) and 0.5 mg/kg chlorpyrifos-methyl diet (*$p < 0.0001$) (Fig. 4d).

3.5. Leukocytic transcription of gstp1, but not caspase-3 (casp3), was increased by chlorpyrifos-methyl inclusion in diet

Gstp1 transcription in isolated leukocytes differed significantly in cells obtained from cod fed 0.5 mg/kg, (*b*, $p = 0.0013$) and 23.2 mg/kg (*b*, $p = 0.0018$) chlorpyrifos-methyl, compared to control diet and the 4.2 mg/kg chlorpyrifos-methyl diet. Fig. 5a). Leukocytic *casp3* transcription was not affected by any of the diets or treatments (Fig. 5b).

3.6. Dietary chlorpyrifos-methyl and effects on head kidney tissue gene transcription

Head kidney tissues (n = 24) harvested from the same cod individuals of which leukocytes were isolated from and cultured *in vitro*, were analyzed for transcriptional responses with a panel of selected genes. The head kidneys were analyzed for *il-1β, il-8, cox2, fasl, cpt1a, gstp1, cyp1a1, ahr, cat, casp3* and *alox5*. Fig. 6 a-j) and Fig. 7. The expression of *cd36* and *nqo2* were extremely low in this tissue regardless of diet, and results are not shown. Compared to the control diet, none of these genes were differentially expressed in the head kidney tissue isolated from the chlorpyrifos-methyl exposed groups. One exception was *alox5* (Fig. 7) which was significantly higher expressed in head kidney obtained from cod fed the 4.2 mg/kg (*b*, $p = 0.0218$) and 23.2 mg/kg (*b*,

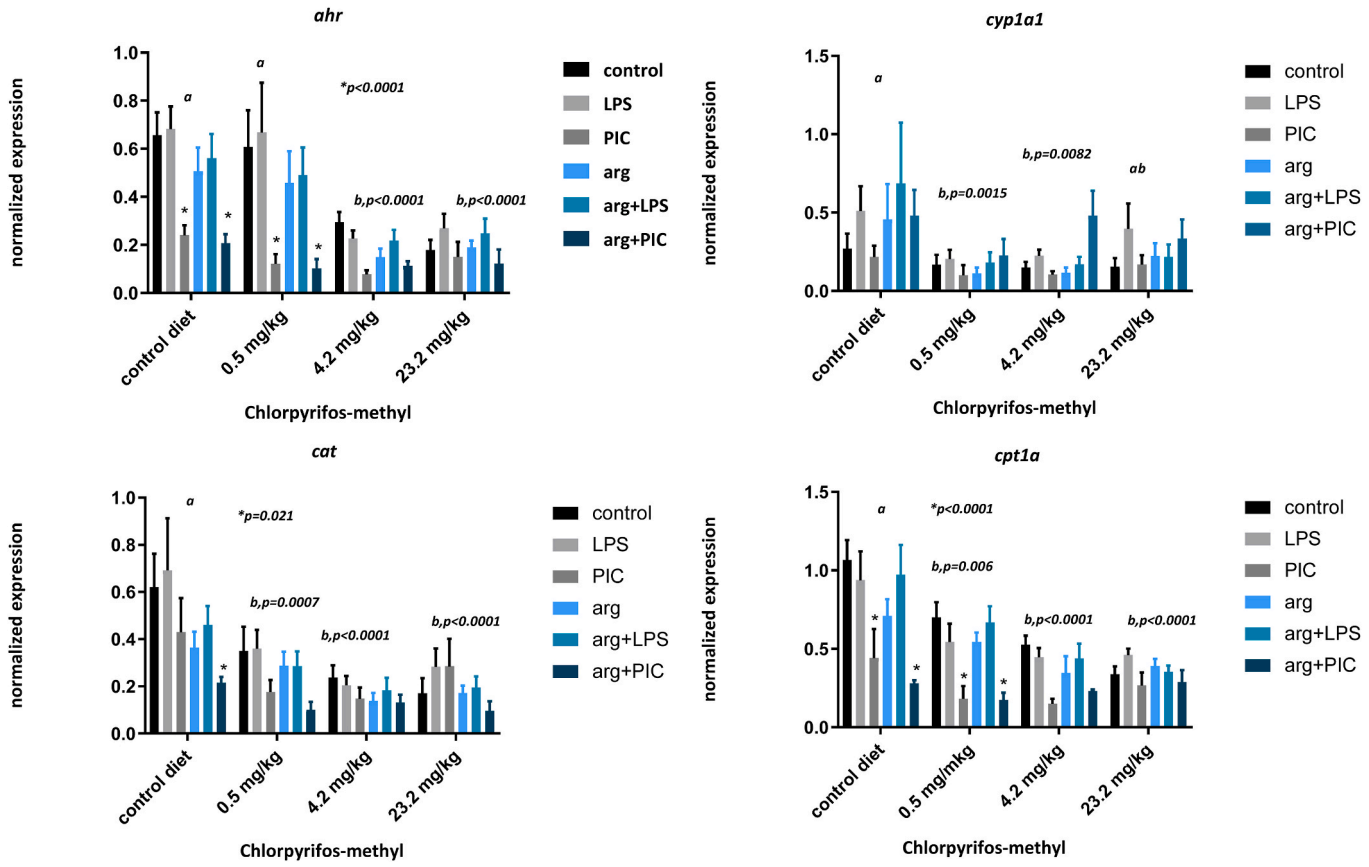

Fig. 4. Downregulation of detoxification genes *ahr* and *cyp1a1*, redox gene *cat* and fatty acid oxidation enzyme gene *cpt1a* in isolated leukocytes when including chlorpyrifos-methyl in diets to cod. Diet a±b, n = 6

a) Isolated leukocytes from cod fed on 4.2 and 23.2 mg/kg chlorpyrifos-methyl inclusions in the diet depressed transcription of *ahr* (b, $p < 0.0001$). PIC alone downregulated *ahr* gene responses in the control diet and in diet with 0.5 mg/mg chlorpyrifos-methyl inclusion (*$p < 0.0001$) compared to respective control cultures.

b) Isolated leukocytes from cod fed on 0.5, and 4.2 mg/kg chlorpyrifos-methyl inclusions in the diet reduced transcription of *cyp1a1* (b, $p = 0.0015$ and b, $p = 0.0082$, respectively)

c) Isolated leukocytes from cod fed on 0.5, 4.2 and 23.2 mg/kg chlorpyrifos-methyl inclusions in the diet reduced transcription of *cat* compared to control diet (b, $p = 0.0007$, b, $p < 0.0001$ and b, $p < 0.0001$, respectively). In control diet, when the leukocytes were treated with surplus arginine and PIC, transcription of *cat* was significantly lower than the respective control culture (*$p = 0.021$).

d) Isolated leukocytes from cod fed on 0.5, 4.2 and 23.2 mg/kg chlorpyrifos-methyl inclusions in the diet reduced transcription of *cpt1a* compared to control diet (b, $p = 0.006$, b, $p < 0.0001$ and b, $p < 0.0001$, respectively). PIC downregulated *cpt1a* in control diet and 0.5 mg/kg chlorpyrifos-methyl diet compared to respective control cultures (*$p < 0.0001$).

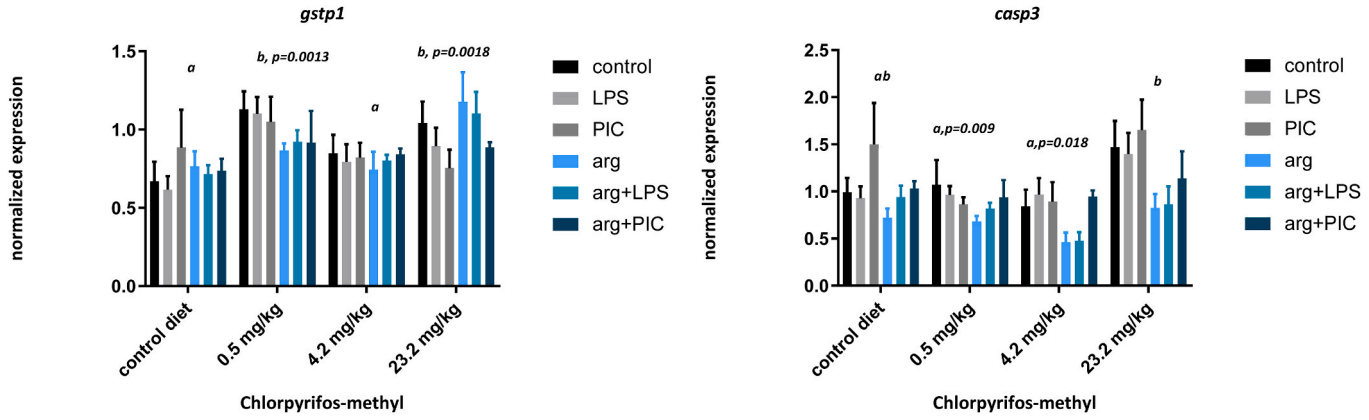

Fig. 5. Transcriptional upregulation of class II detoxification gene *gstp1* in isolated leukocytes when including chlorpyrifos-methyl in diets to cod. Apoptotic *casp3* gene transcription was not affected by the chlorpyrifos-methyl diets. Diet a±b, n = 6

a) Isolated leukocytes from cod fed on 0.5 and 23.2 mg/kg chlorpyrifos-methyl inclusions in the diet upregulated transcription of *gstp1* (b, $p = 0.0013$ and b, $p = 0.0018$) compared to the other diets.

b) Isolated leukocytes from cod fed any of the chlorpyrifos-methyl diets did not affect *casp3* transcription when compared to control diet.

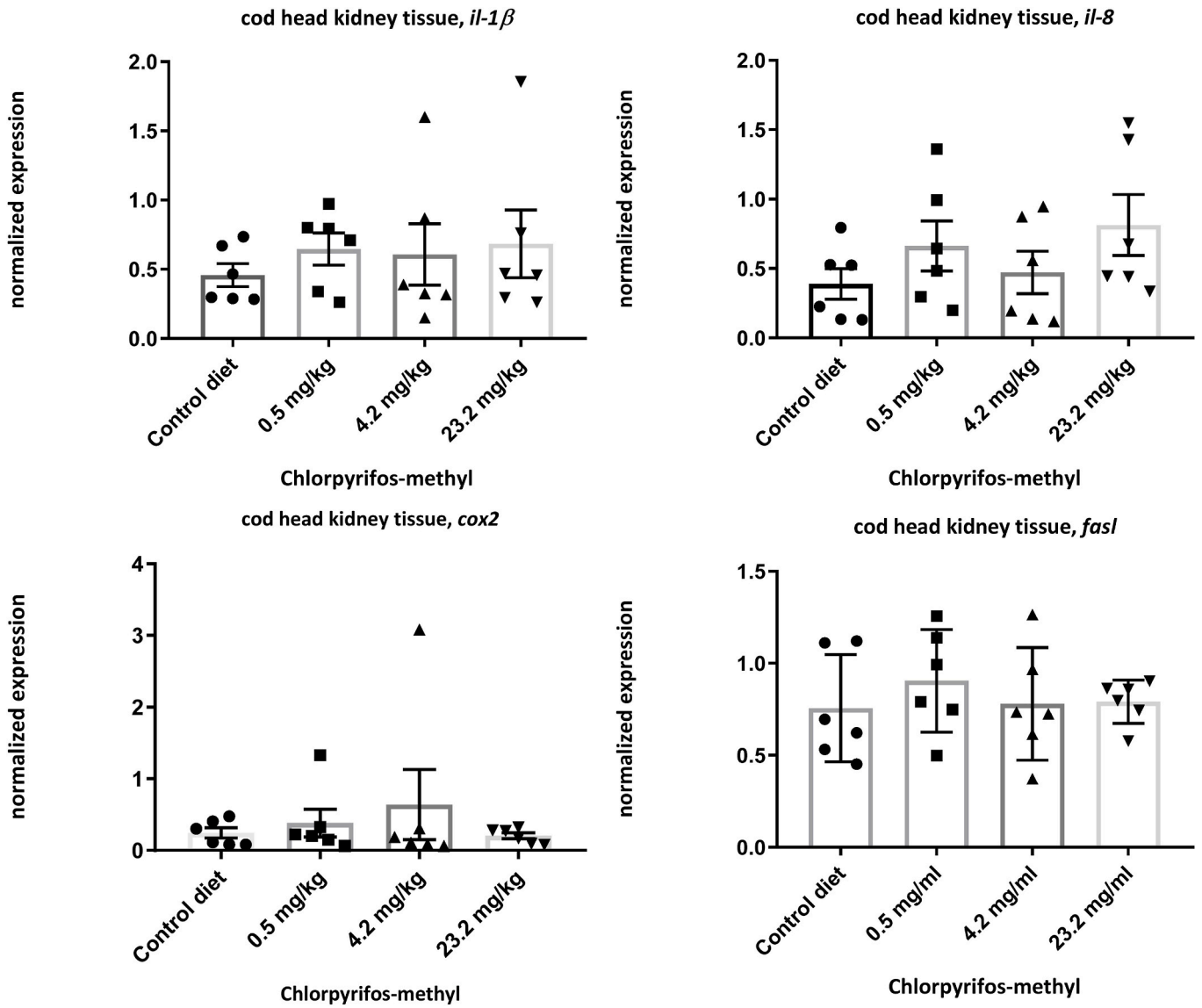

Fig. 6. Head kidney tissue (n = 6 per diet, 24 in total) harvested from cod fed on chlorpyrifos-methyl inclusions in the diet showed no significant transcription responses, compared to control diet, when analyzing:
a) il-1β
b) il-8
c) cox2
d) fasl
e) cpt1a
f) gstp1
g) cyp1a1
h) ahr
i) cat
j) casp3.

p = 0.0256) chlorpyrifos-methyl.

4. Discussion

Chlorpyrifos-methyl inclusions in diets to Atlantic cod had insignificant impact on transcription of the selected genes in head kidney whole tissue. One exception was the transcription of *alox5* which was significantly higher expressed in the 4.2 mg/kg and 23.1 mg/kg chlorpyrifos exposure groups. Head kidney tissue is composed of a wide variety of cells including leukocyte and nucleated red blood cells in various stages of maturation as well as groups of melano-macrophages. Oxidative responses may also be associated with post translational modifications of proteins rather than transcriptional variation of genes [44]. Accumulation of chlorpyrifos-methyl was not assessed in the tissue of head kidneys.

Our earlier transcription studies with salmon and cod head kidneys obtained from fish fed on specific diets or exposed to toxicants without adding a costimulatory signal, show that head kidney tissue is relatively unresponsive to external stimuli [41]. Fish liver is the primary tissue counteracting stressful challenges caused by feed or chemicals [18,45,46]. Olsvik et al. [18] showed that chlorpyrifos-methyl accumulates in cod liver, and affects cholesterol and steroid biosynthesis, detoxification

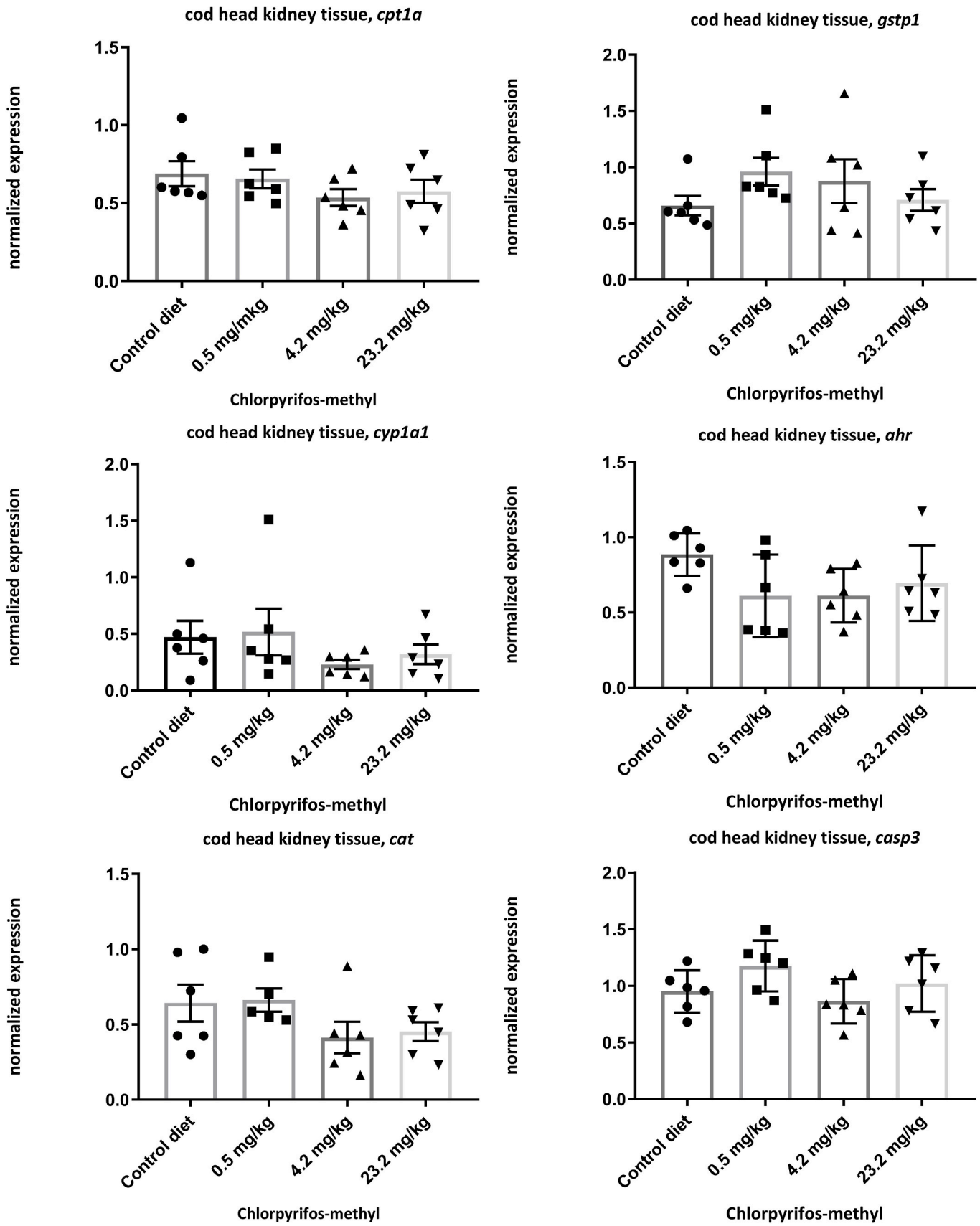

Fig. 6. (*continued*).

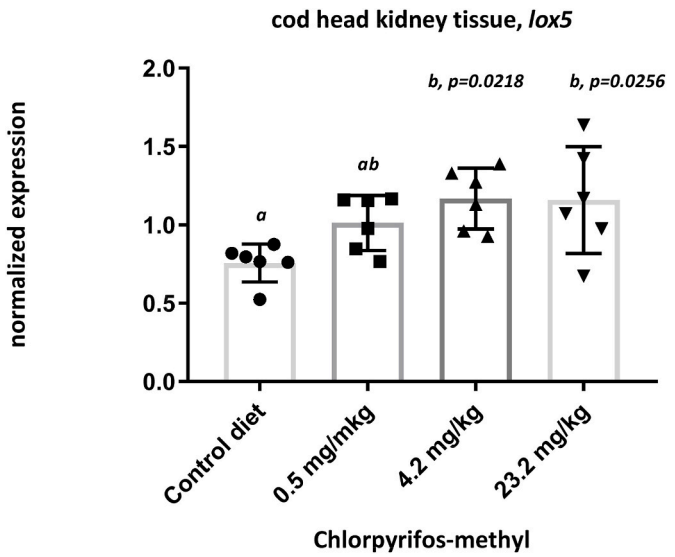

Fig. 7. Head kidney tissue harvested from cod fed on 4.2 and 23.2 mg/kg chlorpyrifos-methyl inclusions in the diets increased transcription of *alox5* (b, $p = 0.0218$ and b, $p = 0.0256$, respectively), compared to control diet. Diet a ± b (n = 6).

by glutathione-S-transferase and energy demand.

Dietary inclusion of chlorpyrifos-methyl alone did not change the expression of the selected immune genes in the isolated leukocytes as compared to leukocyte culture controls and control feed. This situation changed when the leukocytes were challenged with proinflammatory inducers like LPS, PIC and/or surplus concentrations of the immune-modulator L-arginine. These findings indicate that the proliferative capacity of cod leukocytes was not decreased by chlorpyrifos-methyl exposure. However, LPS-induced transcription of the immune genes *il-1β, il-6* and *il-8* was significantly increased in leukocytes isolated from cod fed 23.2 mg/kg chlorpyrifos-methyl, suggesting exaggerated inflammation in the leukocytes. A possible explanation for this could be that the high concentration of chlorpyrifos-methyl interferes with LPS-signaling mechanisms in the leukocytes.

In line with this, Holen & Olsvik [47] showed that LPS cultured together with phenanthrene in cod leukocytes interfered with the aryl hydrocarbon receptor (AhR) and *cyp1a1* transcription. Furthermore, the expression of *il-1β* was significantly increased in head kidney of common carp [48] and Chinook salmon (*Onchorhynchus tshawytscha*) [49] exposed to chlorpyrifos-methyl in freshwater. Zahran et al. [50] found that *il-8, il-1β* and *tnfα* were upregulated in head kidney and spleen of Nile tilapia (*Oreochromis niloticus*) when exposed to chlorpyrifos-methyl. Taken together, these studies show that chlorpyrifos-methyl exposure interfere with the immune response in kidney and spleen of fish.

Eder et al. [49] observed that the antiviral transcripts *inf-γ* and *mx* were unaffected by chlorpyrifos-methyl, when examining head kidney tissues from chinook salmon and common carp after freshwater exposure [48]. In the present experiment, there were no difference in the PIC-induced transcription of the antiviral gene *isg15* in head kidney leukocytes. However noticeable, surplus arginine cultured together with PIC reduced *isg15* transcription in leukocytes from all dietary groups. Surplus arginine may thus modulate virus induced immune gene responses regardless of dietary inclusions of chlorpyrifos-methyl.

The *cox2* gene is rapidly inducible and tightly regulated under basal conditions and *cox 2* transcription is upregulated during LPS challenge both in salmon liver and head kidney leukocytes [26,30,41], in cod leukocytes [34] and by IL-1β protein in mammals [51]. Soybean oil (n-6/n-3 ratio = 24) diet can also trigger *cox2* transcription in salmon indicating a link to fatty acid metabolism [41]. In the present study, chlorpyrifos-methyl exposure seemed to inhibit transcription of *cox2* in cod leukocytes. This observation probably indicates impaired prostaglandin regulation by chlorpyrifos-methyl exposure. As reported earlier, there seemed to be a stable endogenous transcription of *alox5* in salmon leukocytes [30]. This was also observed in cod leukocytes obtained from all dietary groups in this study except from fish fed the diet with 23.2 mg/kg chlorpyrifos-methyl, in which a*lox5* was downregulated. This expression pattern was the opposite of what was observed in head kidney whole tissue. The presence of other cell types in tissue, like nucleated red blood cells, may impact the overall expression of *alox5* and other immune genes, after chlorpyrifos-methyl exposure. This needs to be further assessed.

Another effect of surplus arginine was observed when analyzing the phase II detoxification gene NAD(P)H-dependent quinine oxidoreductases (*nqo2)*, fatty acyl synthetase ligand (*fasl*) and a multitarget gene encoding a protein also involved in fatty acid transport, *cd36*. Leukocytes isolated from cod fed on 4.2- and 23.2 mg/kg chlorpyrifos methyl did not respond to chlorpyrifos-methyl in the diet except when surplus arginine was added together with PIC, inducing high transcription of *nqo2*, *fasl* and *cd36,* compared to the control diet. The effect of surplus arginine seemed to be dose dependent reflecting the concentration of chlorpyrifos-methyl inclusions in the diet. In mammals, leukocytes play a significant role of responsive balance through the activities of arginine hydrolyzing enzymes nitric oxide synthase 2 (iNOS) and arginase 1. Nitric oxide (NO) production of iNOS is mostly proinflammatory but arginase 1 expressing macrophages contributes to resolution of inflammation and wound repair. NOS enzymes metabolize L-arginine to citrulline, and nitric oxide. NO can be antiviral or immunopathologic depending on virus type and tissue. The expression of NO and arginase enzymes during a viral infection might therefore result in different outcomes depending on virus and tissue involved [52]. In this experimental set up, dietary chlorpyrifos-methyl, surplus arginine, and PIC triggered gene expression of *nqo2*, *fasl* and *cd36*. Several pathways are probably interacting involving the arginine-hydrolyzing enzymes and NO production.

The genes, carnitine palmitoyl transferase (*cpt1a*) which is essential in fatty acid oxidation, aryl hydrogen receptor (*ahr*), which upon xenobiotic binding activates the expression of multiple phase I and II xenobiotic chemical metabolizing enzyme genes like *cyp1a1 (p450)*, and the redox gene *cat,* were all reduced by nearly all diet inclusions of chlorpyrifos-methyl when measured in the isolated leukocytes. These results suggest that chlorpyrifos-methyl generally affects mechanisms associated with detoxification, cytolytic capacity and redox signaling in cod leukocytes. It has been reported that organophosphorus pesticides like chlorpyrifos-methyl diminish natural killer cells and cytotoxic activities in aquatic and human models through different and indirect mechanisms [53–55]. Also reported, organophosphorus pesticides induced apoptosis in cultured human lymphocytes of peripheral blood [56] and chlorpyrifos-methyl induced apoptosis and increased apoptotic gene *casp3* in the human monocyte cellular line U937 [57]. In contrast, in the present study, *casp3* transcription was not affected by dietary chlorpyrifos-methyl when compared to control diet.

Zahran et al. [50] reported decreased lysozyme activity in Nile tilapia due to chlorpyrifos-methyl exposure. Overproduction of reactive oxygen species (ROS) correlated with reduced levels of antioxidant enzymes in exposed fish. Mechanisms of detoxification of ROS include several enzymes, among others, CAT, and glutathione-S-transferase 1 (GSTP1). In the present experiment, gene transcription of *cat* was downregulated, while transcription of the phase II detoxification enzyme, *gstp1* [58], was increased by chlorpyrifos-methyl in the diet. The different effects of chlorpyrifos-methyl may be due to different ROS inducing pathways. One of these pathways is affecting transcription factors like nuclear factor erythroid factor 2 (nfr2) and nuclear transcription factor c-jun, inducing activation of ARE (antioxidant response element) in the nucleus which promote the expression of *gstp1* (and also *nqo2* discussed above) [58]. The expression of phase II detoxification genes, *gstp1* and *nqo2*, (*nqo2* with the help of PIC signaling and surplus

arginine) may contribute to detoxification of chlorpyrifos-methyl in these specific cultures of cod leukocytes.

The main toxic effect of chlorpyrifos-methyl is the irreversible inhibition of acetylcholinesterase (AchE) activity in nerve cells [59]. The target molecule of organophosphorus pesticides is the AchE enzyme, blocking its activity resulting in increased levels of neurotransmitter acetylcholine (Ach) in the nervous system. According to Kawashima et al. [60] and Toleda-Ibarra [61], this will affect lymphocytes as they express muscarinic acetylcholine receptor (mAChR). It has been suggested that antigenic stimuli may activate the lymphoid cholinergic system resulting in increased production of acetylcholine and upregulating c-fos expression and inducing cytokine synthesis [60,62]. Recently, the effect of the organophosphorus pesticide, diazinon, was evaluated on the cholinergic system of immune cells of Nile tilapia as possible targets of organophosphorus pesticide immunotoxicity [61,63,64]. The authors suggested that the non-neural lymphocytic cholinergic system may be a target in the mechanism of organophosphorus pesticides either indirectly by interaction with the ACh receptors or directly by inhibiting AChE. Possibly, the cholinergic system may also be affected by chlorpyrifos-methyl in cod leukocytes proposing multitarget effects of chlorpyrifos-methyl. This needs to be further evaluated.

5. Summary

This study was conducted to gain insight into whether fish feed contaminated by agricultural pesticides like chlorpyrifos-methyl might interfere with the transcription of genes in cod immune cells *in vitro*. To simulate infection, LPS or PIC and/or the immunomodulator L-arginine was added as costimulatory signals. Despite the insignificant transcriptional response of chlorpyrifos-methyl exposure on genes in head kidney tissue, gene transcription in isolated leukocytes was affected by dietary chlorpyrifos-methyl, with or without costimulatory signals.

- Dietary chlorpyrifos-methyl, 23.2 mg/kg, may increase inflammation by triggering transcription of LPS-induced immune genes (*il-1β, il-6, il-8*) in the leukocytes.
- Dietary chlorpyrifos-methyl (0.5-, 4.2- and 23.2 mg/kg) did not affect PIC-induced immune gene *isg15* transcription in the leukocytes.
- Surplus arginine reduced PIC-induced *isg15* transcription in leukocytes suggesting that arginine supplementation may be beneficial for modulating immune responses during viral diseases.
- Dietary chlorpyrifos-methyl may affect eicosanoid metabolism by downregulating *cox2* and *alox5* (23.2 mg/kg), thereby affecting inflammation in the leukocytes.
- Dietary chlorpyrifos-methyl suppressed the transcription of *cpt1a*, *ahr*, *cyp1a1* and *cat* in the leukocytes, suggesting dysregulation of mechanisms associated with detoxification, cytolytic capacity and redox signaling.
- Dietary chlorpyrifos-methyl increased the transcription of *gstp1* in the leukocytes which probably is indicative of enhanced detoxification capacity.
- Isolated leukocytes from cod fed 23.2 mg/kg chlorpyrifos-methyl, added surplus arginine and PIC, showed increased *cd36*, *nqo2* and *fasl* transcription when compared to control diet. Differential expression of these genes could signal an impact on fatty acid metabolism and detoxification mechanisms.

Ethics statement

The experiment complied with the guidelines of the Norwegian Regulation on Animal Experimentation and EC Directive 86/609/EEC (FOTS approval ID 15057).

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Author contribution

Elisabeth Holen: planned the experiment, executed and wrote the manuscript. Marit Espe: planned and executed the trial, reviewed the manuscript. Anett K. Larsen: executed the trial and reviewed the manuscript while. Pål A. Olsvik: planned the experiment and reviewed the manuscript.

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