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Morphological alterations in the liver of yellow perch (*Perca flavescens*) from a biological mercury hotspot

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Abstract Mercury (Hg) contamination is a global issue due to its anthropogenic release, long-range transport, and deposition in remote areas. In Kejimkujik National Park and National Historic Site, Nova Scotia, Canada, high concentrations of total mercury (THg) were found in tissues of yellow perch (*Perca flavescens*). The aim of this study was to evaluate a possible relationship between THg concentrations and the morphology of perch liver as a main site of metal storage and toxicity. Yellow perch were sampled from five lakes known to contain fish representing a wide range in Hg concentrations in fall 2013. The ultrastructure of hepatocytes and the distribution of Hg within the liver parenchyma were analyzed by transmission electron microscopy (TEM) and electron energy loss spectrometry (EELS). The relative area of macrophage aggregates (MAs) in the liver was determined using image analysis software and fluorescence microscopy. No relation between general health indicators (Fulton's condition index) and THg was observed. In line with this, TEM examination of the liver ultrastructure revealed no prominent pathologies related to THg accumulation. However, a morphological parameter that appeared to increase with muscle THg was the relative area of MAs in the liver. The hepatic lysosomes appeared to be enlarged in samples with the highest THg concentrations. Interestingly, EELS analysis revealed that the MAs and hepatic lysosomes contained Hg.

Keywords Methyl mercury · Yellow perch · Macrophage aggregates · Ultrastructure

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Introduction

Increasing mercury (Hg) concentrations in aquatic and terrestrial ecosystems is of major concern, since Hg is distributed through long-distance transport on a global scale and deposited even in remote areas (Durnford et al. 2010; Selin 2009; Streets et al. 2011). Due to bacterial methylation in aquatic ecosystems, methyl-mercury (MeHg) is produced from inorganic Hg. MeHg bioaccumulates in biota and biomagnifies up the food web, leading to potentially toxic Hg concentration in fish and fish-eating wildlife (Evers et al. 1998; Lescord et al. 2014; Wyn et al. 2010). Total Hg (THg) concentrations found

in muscle tissue of fish are composed of approximately 95 % MeHg (Bloom 1992). Various effects of chronic dietary MeHg exposure are documented in laboratory and field studies of fish from highly contaminated areas. These include decreased growth (Friedmann et al. 1996), impaired gonadal development (Friedmann et al. 1996; Kidd and Batchelar 2011), oxidative damage through the production of reactive oxygen species (Zaman and Pardini 1996), and histological alterations in the liver and kidney such as an increase in macrophage aggregates (MAs) (Giari et al. 2008; Mela et al. 2007; Raldúa et al. 2007). From such studies, a whole-body Hg threshold effect level of 0.2 μg g^{-1} wet weight (wet wt) has been proposed which is presumed to be protective of fish health (Beckvar et al. 2005). Higher Hg levels, therefore, may lead to adverse effects.

Kejimkujik National Park and National Historic Site (KNPNHS) is located in southwestern Nova Scotia, Canada, and has no direct anthropogenic point sources of pollution (O'Driscoll et al. 2005). However, due to high concentrations of MeHg found in yellow perch (*Perca flavescens*) and its predator, the common loon (*Gavia immer*), within and outside the park, the region is known as a biological mercury "hotspot" (Evers et al. 2007). Moreover, between the years 1996–1997 and 2006–2007, THg concentrations in yellow perch within KNPNHS increased, on average, by 29 % in 10 out of 16 lakes (Wyn et al. 2010). Muscle THg concentrations (range 0.09 to 2.13 μg g^{-1} wet wt) of KNPNHS perch determined in 2009–2010 exceeded the 0.2 μg g^{-1} wet wt threshold in 57 % of the sampled fish (Batchelar 2011). Further, the overall mean muscle THg concentration (0.43±0.014 μg g^{-1} wet wt) (Batchelar et al. 2013) exceeded that reported for yellow perch in other parts of northeastern North America (0.351±0.198 μg g^{-1} wet wt) (Dittman and Driscoll 2009; Kamman et al. 2005). General health endpoints such as Fulton's condition index (*K*) or liver somatic index (LSI) of KNPNHS perch with high Hg concentrations were not decreased compared to conspecifics with low Hg (Batchelar et al. 2013). However, a conspicuous change found in individuals with higher Hg concentrations was the increased frequency of MAs in the liver, spleen, and kidney (Batchelar et al. 2013).

MAs are complex, discrete groups of pigmented phagocytic cells (Agius and Roberts 2003). They are primarily associated with the reticuloendothelial tissue of the hematopoietic organs of fish, kidney and spleen, but also occur in the liver (Agius and Roberts 2003; Wolke 1992). Evidence suggests that MAs sequester products of cellular degradation, trap and retrain antigens, and detoxify endogenous and exogenous materials (Agius and Roberts 2003; Meseguer et al. 1994; Vigliano et al. 2006; Wolke 1992). Moreover, MAs increase in size and frequency under conditions of environmental stress including exposure to anthropogenic pollutants (Agius and Roberts 2003; Blazer et al. 1987; Passantino et al. 2014; Schwindt et al. 2008). Batchelar et al. (2013) concluded that the enhanced presence of MAs in tissues of yellow perch from KNPNHS is suggestive of a negative impact of Hg at the cellular level. The positive relationship between the frequency of MAs and tissue Hg concentrations has also been reported for other wild fish species (Barst et al. 2011; Meinelt et al. 1997; Schwindt et al. 2008). Furthermore, laser ablation and inductively coupled plasma mass spectrometry showed that Hg and MAs were co-localized in hepatic tissue and that Hg accumulated in higher amounts in MAs than in the surrounding liver parenchyma (Barst et al. 2011; Batchelar et al. 2013).

The objectives of the present study were (1) to re-evaluate the relationship between MAs in the liver and muscle THg levels in yellow perch of KNPNHS, as reported by Batchelar et al. (2013), by means of a novel quantitative approach, and (2) to determine whether an increase in MA area in the liver is associated with alterations in the ultrastructure of the hepatocytes. Given the relationship between structure and function, an alteration of hepatic structure may reveal a change in liver function and indicate how Hg might be detoxified in this organ. Characteristics of MAs in the liver, e.g., area, number, and distribution across different liver sections, were quantified by the use of image analysis software to establish a high-throughput screening method. Liver ultrastructure was analyzed using transmission electron microscopy, and electron energy loss spectrometry (EELS) was used to locate Hg in the liver tissue.

Materials and methods

Study sites and sampling

KNPNHS is located in southwestern Nova Scotia, Canada, and is remote from urban or industrial input and mainly influenced by tourism and outdoor recreation. Lakes within KNPNHS are shallow, oligotrophic, and acidic (pH ≤6) (O'Driscoll et al. 2005; Wyn et al. 2010). Five lakes known to contain fish representing a wide range in Hg concentrations (Batchelar et al. 2013; Wyn et al. 2010) (Fig. 1) were chosen, and yellow perch were captured in September of 2013 using trap nets, set overnight in shallow littoral zones of the lakes. In total, four perch from each lake ranging in size from 11 to 15 cm were selected for dissection. The fork length (±0.1 cm) and body weight (±0.1 g) were recorded before euthanizing the fish by spinal severance. Fish were dissected according to procedures approved by the University of New Brunswick's Animal Care Committee (Canada). For optimal fixation, the fish liver was perfused in situ with 1.5 % glutardialdehyde, 1.5 % paraformaldehyde, and 2.5 % polyvinylpyrrolidone (Fisher Scientific, Ottawa, Canada) in 0.1 M phosphate buffer (Sigma-Aldrich, Oakville, Canada; pH 7.6) as described by Grund et al. (2010). After the ventricle was incised, the liver was perfused via the hepatic portal vein and terminated after a color change. Afterward, the liver was excised and washed

Fig. 1 Study lakes within Kejimkujik National Park and National Historic Site, Nova Scotia, Canada. 1: NorthCranberry, 2: Puzzle, 3: Cobrielle, 4: Hilchemakaar, 5: Upper Silver

several times with perfusion solution. The liver was cut into two approximately 1-cm slices that were transferred into 2-ml microcentrifuge tubes (Fisher Scientific, Ottawa, Canada), filled with perfusion fixative, and stored at 4 °C for histology and electron microscopy. A dorsal muscle fillet was collected above the lateral line of each perch, flash frozen in liquid nitrogen, and afterward stored at −40 °C for THg analysis. Sex was recorded and Fulton's condition index (K) was determined as $K=100\times$(total weight {g} / fork length^3 {cm}).

Mercury analysis

THg was analyzed as described by Batchelar et al. (2013). Muscle tissue samples were lyophilized using a FreeZone 12 (Labconco, Kansas City, USA) and ground using a glass rod. Percent moisture in muscle tissue was calculated for each sample. THg concentrations in the muscle samples were measured using 10 mg of freeze-dried, homogenized tissue and atomic absorption spectrophotometry (Milestone Direct Mercury Analyzer, DMA-80, Milestone, Sorisole, Italy) at the University of New Brunswick, Saint John, Canada (based on EPA Method 7473). For quality assurance, method blanks, a 10 ng liquid Hg standard (Stock solution from Ultra Scientific, North Kingstown, USA), an intralaboratory standard (yellow perch muscle sample), standard reference material DORM-2 (dogfish muscle) from the National Research Council of Canada, and sample duplicates were analyzed every ten samples. The mean percent recoveries of the liquid Hg standard and DORM-2 were 107.37±2.6 and 82.02±4.84 % (n=3), respectively. The mean THg in method blanks was 3.5±2.8 ng g^{-1} dry wt. The limit of detection (LOD) was determined as follows: $\mathrm{LOD} = \bar{x}_{\mathrm{blank}} + (3 \times \sigma_{\mathrm{blank}})$ and the LOD was found to be 9.5 ng g^{-1} dry wt. The coefficient of variation for the yellow perch intralab standard was 1.8 % (n=3), and the overall precision of the three duplicates was within 3.9 %. All chemicals used to make the calibration standards were analytical grade (Fisher Scientific, Ottawa, Canada). All THg concentrations are presented on a wet wt basis.

Macrophage aggregate analysis

To quantify the MAs, liver samples were dehydrated and embedded in paraffin (Histosec, Merck, Darmstadt, Germany) according to standard protocols. Briefly, 2-μm stepwise paraffin sections (five total at 20-μm steps) were produced per fish using a microtome (Microm HM 340 E, Microm GmbH, Walldorf, Germany). The paraffin sections were mounted on 3-aminopropyltriethoxysilane-coated microscope slides (Sigma-Aldrich, Buchs, Switzerland) and dewaxed. For the specific staining of the MAs, biotinylated *Hippeastrum* hybrid (Amaryllis) lectin (HHL, Vector Laboratories, Reactolab, Servion, Switzerland) was used, which was afterward detected with a streptavidin Alexa Fluor 647 (Jackson ImmunoResearch Laboratories, Milan Analytica, Rheinfelden, Switzerland) conjugate (Fig. 2). Stained liver sections were viewed with fluorescence microscopy (IN Cell Analyzer, GE Healthcare Life Sciences GmbH, Glattbrugg, Switzerland, excitation filter Cy5 647 nm, ×60 magnification), and a representative 5.14-mm^2 field per section was photographed. We used image analysis (IN Cell Investigator, GE Healthcare Live Sciences GmbH, Glattbrugg, Switzerland) to automate MA quantification. Thereby, the MA fluorescent signal was selected by composing a mask based on color intensity and MA shape. Thus, MAs were discriminated from nonspecific cellular fluorescent signals (background signal), which were minimized with size exclusion parameters. The software quantified the area of each individual MA fluorescent signal in square micrometers per image, and data were exported to a spreadsheet. For quality assurance, each measured MA area was controlled for specificity of the applied selection mask. The relative tissue area occupied by MAs was calculated as the sum of MA area (μm^2) / total section area (μm^2). In addition, the total number of MAs per section was counted. All five replicates were averaged and compared against muscle THg concentrations of each individual. In four of the 20 fish, only three to four sections were analyzed and one sample was completely excluded from analysis due to an undetectable MA fluorescence signal (n=19).

Ultrastructure analysis

Liver samples were cut into 1–2-mm pieces and rinsed three times in cacodylate buffer (0.1 M, pH 7.4) before postfixation with osmium tetroxide (1 % in cacodylate buffer, 4 °C; Chemie Brunschwig, Basel, Switzerland) for 2 h. After three washes with cacodylate buffer, the specimens were dehydrated in a graded series of ethanol, transferred to absolute acetone, and then embedded in Epon (Merck, Zug, Switzerland; Grogg, Stettlen, Switzerland; Sigma-Aldrich, Buchs, Switzerland). The resin was polymerized for 5 days at 60 °C. Nine fish were selected according to their THg muscle concentrations (three each of high, medium, and low THg). Blocks were then trimmed, and regions of interest were identified as based on 1-μm semithin sections that were stained with toluidine blue (Sigma-Aldrich, Buchs, Switzerland). Ultrathin sections of 70 nm in thickness were obtained with diamond knives (Diatome, Biel, Switzerland) on an ultramicrotome (Reichert-Jung Ultracut E, Leica, Heerbrugg, Switzerland). Sections were double-stained with 0.5 % uranyl acetate for 30 min at 40 °C (Sigma-Aldrich, Steinheim, Germany) and 3 % lead citrate for 10 min at 20 °C (Laurylab, Saint Fons, France) using an automated tissue stainer (Ultrastain®, Leica, Vienna, Austria). Afterward, sections were examined using a transmission electron microscope (Philips CM12, FEI, Eindhoven, The Netherlands). Micrographs were captured with a Mega View III camera using the iTEM software (version 5.2; Olympus Soft Imaging Solutions GmbH, Münster, Germany). Between 10 and 20 micrographs varying in magnification from×2650 to×11,500 were taken from every sample. To assess ultrastructural changes of the hepatocytes, heterogeneity and disturbance of intracellular compartmentation of the hepatocytes; morphology of nucleus, mitochondria, rough endoplasmic reticulum (RER), and

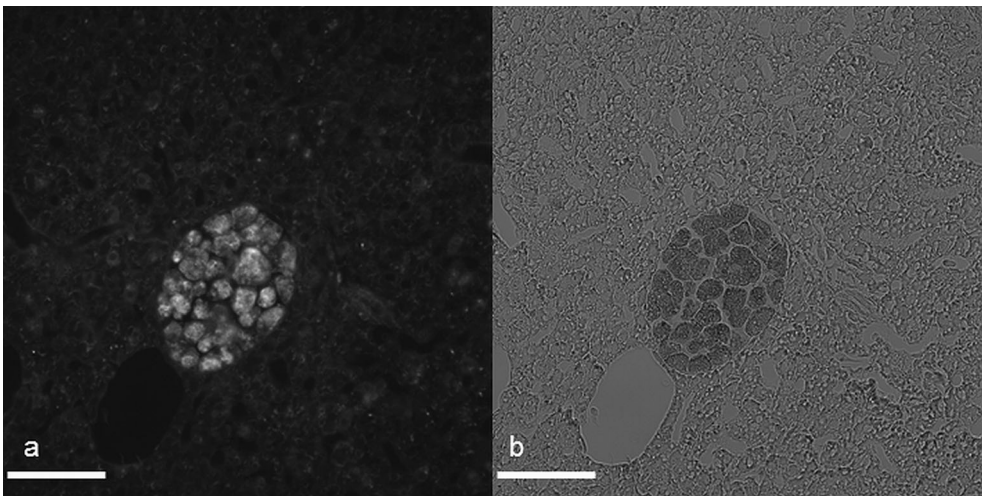

Fig. 2 Macrophage aggregate in yellow perch (*Perca flavescens*) liver labeled with biotinylated *Hippeastrum* hybrid (Amaryllis) lectin and detected with a streptavidin Alexa Fluor 647 conjugate. **a** Viewed with fluorescence microscopy (excitation filter Cy5 647 nm, ×60 magnification) or **b** bright-field microscopy (×60 magnification). *Scale bar*=50 μm

lysosomes (size and frequency); and lipid and glycogen contents were analyzed. These parameters were classified using the following semiquantitative categories: − no observed alterations, + mild alterations, ++ moderate alterations, and +++ strong alterations. In addition, the composition of the MAs was viewed at the ultrastructural level. Another additional seven fish were selected on the basis of their THg muscle concentrations to represent a range of Hg concentrations. We analyzed these samples for the size of lysosomal elements using the toluidine-stained 1-μm semithin sections and light microscopical examination (×100 magnification, oil immersion, Olympus BX51, Olympus optical co. (Europe) GmbH, Hamburg, Germany). The same categories as described above were measured. Both analyses were done blind and repeated twice. Fish data were ordered by THg muscle concentration to investigate its relation to the observed categories for each parameter and whether it depicts a pattern.

Electron energy loss spectroscopy

EELS was used to determine the presence and distribution of Hg in liver parenchyma of yellow perch. Upon interacting with the ultrathin tissue section, electrons of the primary beam loose energy. The amount of energy loss of selected, inelastically scattered electrons is then measured with a spectrometer which allows for elemental microanalysis of the sample (Kapp et al. 2007). The distribution of Hg in liver ultrathin sections from fish with the highest THg muscle concentration was analyzed using electron microscopy (Tecnai F20 equipped with a postcolumn energy filter yielding element-specific contrast, FEI, Eindhoven, Netherlands). Zero-loss images recorded at 0-eV energy loss and element-specific contrast for Hg were obtained by digital acquisition. The acceleration voltage was 200 kV, and Hg was detected at its M peak (energy loss 2295 eV) with the three-window method by using a slit with an energy window of about 60 eV.

Statistics

Normality assumptions (Kolmogorov-Smirnov test, Shapiro-Wilk test) were tested prior to parametric analysis. Relative MA area and number in the liver, fork length, weight, Fulton's condition index, and THg muscle concentration were compared among lakes. One-way analysis of variance (ANOVA) followed by a Tukey's multiple comparison test was used to determine significant differences between lakes in muscle THg concentrations, Fulton's condition index, and relative MA area and number in the liver. Fish length and weight were analyzed using the Kruskal-Wallis test (KW) followed by Dunn's multiple comparison test, because these data did not pass the tests for Gaussian normal distribution. Sex differences were not tested due to the small (n=1) sample size for males. Analysis of covariance (ANCOVA) found that the slopes of relationships between relative area of the liver occupied with MAs and muscle THg concentrations were not different among lakes (interaction term; p=0.485). The same was found for the slopes of relationships between fish length, weight, condition, and muscle THg concentrations among lakes (interaction term; length: p=0.08, weight: p=0.745, condition: p=0.09). Fulton's condition index ($F_{4,15}$=0.94, p=0.470, r^2=0.200), fish length (KW: p=0.227), and weight (KW: p=0.097) did not significantly differ among lakes. ANCOVA found no differences for the relationship of MA area and muscle THg concentrations among the lakes. Therefore, fish data from all lakes were pooled and least squares linear regression was used to evaluate the relationship between relative MA area and number, fish length, weight, condition, and muscle THg. Analysis was done with the open source program R (Dinno 2014; Komsta 2011; R Core Team 2014), and figures were created with GraphPad Prism 5 software (GraphPad Software, San Diego, USA). Potential outliers were identified with Grubb's outlier test (Grubbs 1950, 1969). The statistical significance was determined with a type I error (α) of 0.05.

Results

Muscle mercury concentrations and fish condition

Total Hg muscle concentrations in yellow perch ranged from 0.25 to 0.70 μg g^{-1} wet wt and were significantly different among lakes ($F_{4,15}$=10.04, p=0.0004, r^2=0.73). Highest THg muscle concentrations were found in fish from North Cranberry, which differed significantly from all other lakes, and were 2-fold higher than mean THg in fish from Upper Silver (Table 1). Muscle THg did not relate with length nor weight of the fish across all lakes (length: $F_{1,18}$=4.27, p=0.053, r^2=0.19 and weight: $F_{1,18}$=1.84, p=0.191, r^2=0.09). Yellow perch were found to have Fulton condition indices ranging from 0.94 to 1.53 among individuals, but there were no significant differences between lakes ($F_{4,15}$=0.94, p=0.470, r^2=0.200). Likewise, no relationship existed between THg levels and fish condition index ($F_{1,18}$=0.16, p=0.697, r^2=0.008) when data were pooled across lakes.

Quantification of macrophage aggregates using fluorescence microscopy

The liver of yellow perch showed neither necrotic foci nor parasitic infestations. However, MAs were observed in the livers of all fish. The distribution of MAs in the liver was homogenous. This was indicated from the fairly low variation of relative MA area between the five replicates (25.5 %). The relative area occupied by MAs in the liver varied significantly between lakes ($F_{4,14}$=9.77, p=0.0005, r^2=0.74), with highest

Table 1 Mean (±SD) length (cm), weight (g), condition (*K*), THg (μg g^{-1} wet wt) in muscle, relative MA area, and number of MAs in liver tissues of yellow perch (*Perca flavescens*) from Kejimkujik National Park and National Historic Site, Nova Scotia, Canada

Lake	Length (cm)	Weight (g)	Fulton's condition index (*K*)	Muscle THg (μg g^{-1}) wet wt	Relative MA area	Number of MAs
North Cranberry	13.73±1.27	29.5±10.28	1.12±0.07	0.61±0.09**	0.0094±0.0045**	55.5±31.7*
Upper Silver	12.2±1.54	19.36±8.31	1.02±0.07	0.28±0.04	0.00067±0.0003	10.2±1.9
Hilchemakaar	14.25±0.5	34.85±5.12	1.2±0.01	0.39±0.05	0.0015±0.0006	10.6±1.9
Cobrielle	14.43±0.41	33.71±5.1	1.14±0.27	0.40±0.1	0.00028±0.0008	20.7±3.0
Puzzle	14.15±0.47	30.45±4.33	1.07±0.05	0.41±0.07	0.0045±0.0002	28.4±9.7

ANOVA or Kruskal-Wallis and Tukey's or Dunn's multiple comparison test were used to analyze differences among lakes. Relative MA area: relative area occupied with macrophage aggregates in the liver

*$p<0.01$; **$p<0.001$

values in fish from North Cranberry Lake when compared to all other lakes. Mean relative MA area in the liver from North Cranberry fish was 14 times higher than in Upper Silver, the lake with the lowest Hg fish. The slopes of the relationship between relative MAs area and muscle THg concentration did not differ significantly among lakes (interaction term; $p=0.485$). Neither length nor weight of all fish was related to the relative area of MAs (length: $F_{1,16}=1.77$, $p=0.20$, $r^2=0.01$ and weight: $F_{1,16}=1.86$, $p=0.191$, $r^2=0.10$, respectively) nor was the condition index ($F_{1,16}=0.36$, $p=0.558$, $r^2=0.02$). A positive relationship existed between the relative area of MAs and muscle THg concentrations across lakes ($F_{1,16}=20.89$, $p=0.0003$, $r^2=0.57$) after exclusion of one significant outlier (Fig. 3). Further, a positive relationship ($F_{1,16}=81.59$, $p<0.0001$, $r^2=0.84$) between relative area of MAs and number of MAs in the liver was observed and a weaker, positive relationship also existed between muscle THg concentrations and numbers of MAs in the liver for all fish ($F_{1,16}=6.87$, $p=0.019$, $r^2=0.30$).

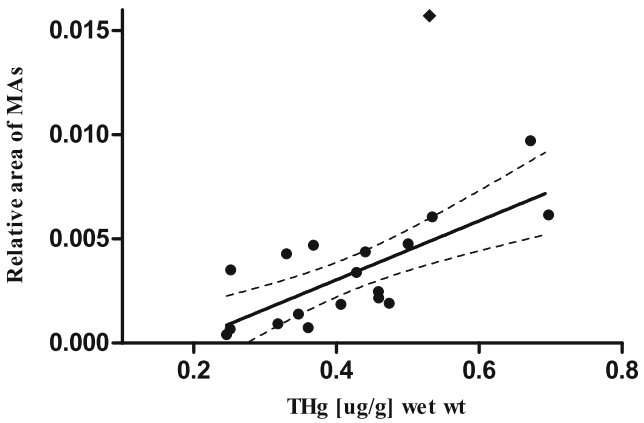

Fig. 3 Linear regression of relative tissue area occupied by macrophage aggregates in the liver and total muscle Hg concentrations (μg g^{-1} wet wt) for yellow perch (*Perca flavescens*) from four lakes in Kejimkujik National Park and National Historic Site, Nova Scotia, Canada. *Dashed lines* indicate 95th percentile and *rhomb symbol* indicates a significant outlier

Liver ultrastructure in relation to the muscle THg concentrations of the fish

This study analyzed ultrastructural alterations in the hepatocytes of yellow perch in relation to the muscle THg concentrations. Because all lakes in Kejimkujik are characterized by Hg contamination, the ultrastructural analysis was based on the comparison of fish with lower muscle THg to fish with higher muscle THg concentrations. As mentioned previously, fish from North Cranberry Lake had high muscle THg (0.5–0.7 μg g^{-1} wet wt) and differed significantly from the other sites, especially from Upper Silver, where the lowest THg concentrations (0.25–0.31 μg g^{-1} wet wt) in fish muscle were found.

The liver parenchyma of fish from Upper Silver had a homogenous appearance and was composed of hepatocytes, endothelial cells, Ito cells, biliary epithelial cells, extracellular spaces including the space of Dissé, sinusoids, and bile canaliculi. In addition, the livers of yellow perch from this lake contained MAs as well as individual extravascular phagocytes, although the relative area occupied by MAs in the liver was lowest in fish from Upper Silver when compared to all other lakes (see above).

The ultrastructure of hepatocytes of fish from Upper Silver Lake was characterized by regularly parallel, stacked cisternae of rough endoplasmic reticulum (RER) in close proximity to a central, mostly round shaped nucleus often possessing a dense area of heterochromatin (Fig. 4). The mitochondria, usually associated with RER, were small, relatively electron dense, and of spherical or elongated shape. Small-sized lysosomes occurred frequently in the cytoplasm. They were highly heterogenic in morphology and varied from white and gray to dark electron dense granules (Fig. 4). There was little glycogen storage in the hepatocytes. Lipid droplets were present but generally at low numbers. Due to this general low amount of energy storage material (glycogen, lipid) in the hepatocytes, a conspicuous compartmentation of the cytoplasm into a perinuclear organelle-rich and a marginal organelle-poor area of energy storage material was not evident. The intercellular space was frequently widened and often contained membranous structures (Fig. 5).

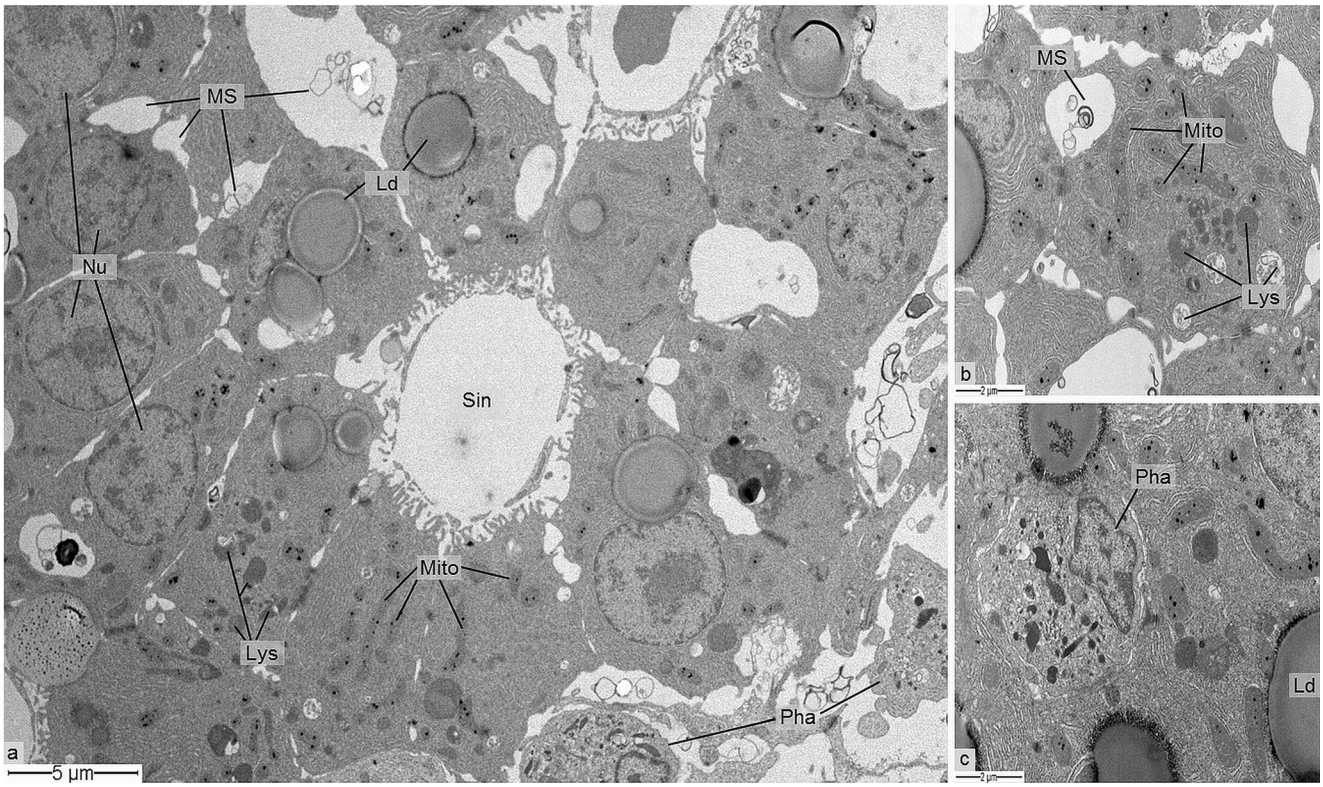

Fig. 4 Transmission electron micrograph of yellow perch (*Perca flavescens*) hepatocytes from Upper Silver Lake, Kejimkujik National Park and National Historic Site, Nova Scotia, Canada. **a**, **b** The hepatocytes were regularly arranged alongside the sinusoid (*Sin*), and a central, spherical nucleus (*Nu*) was enclosed by parallel stacks of rough endoplasmic reticulum (*RER*). RER was also found in association with mitochondria (*Mito*) in the cytoplasm. No glycogen storage was observed, but some prominent lipid droplets (*Ld*) were found. Heterogeneous, small lysosomes (*Lys*) were present in the cytoplasm. In some areas of the liver, the intercellular space was widened, often containing membranous structures (*MS*). Extravascular phagocytes (*Pha*) were regularly observed in the parenchyma (**c**)

Livers of yellow perch with higher THg muscle concentrations, such as those from fish caught in North Cranberry Lake, displayed a clearly increased size and heterogeneity of the intrahepatocellular lysosomes when compared to perch from Upper Silver Lake (Fig. 5). However, the frequency of lysosomes did not clearly differ between livers of higher and lower Hg fish. Semiquantitative analysis of the number and size of lysosomal elements in the hepatocytes corroborated that lysosomes were enlarged in fish from North Cranberry Lake with highest muscle THg concentrations compared to all other lakes. The change in the lysosomal compartments was also confirmed by light microscopy (Table 2). Moreover, the presence of extravascular phagocytes in the parenchyma was clearly increased in fish from Upper Silver over fish from North Cranberry. Phagocytes in the livers of North Cranberry perch contained high amounts of dark, electron-dense, spherical structures (Fig. 6), whereas those from Upper Silver contained heterogenic, small, and round shaped to acicular structures. Widening of intercellular space was also observed in tissues of fish from North Cranberry, but not as often as in samples from Upper Silver. Similar to fish from Upper Silver, the liver ultrastructure of fish from North Cranberry was characterized as follows: polygonal hepatocytes with regular, stacked cisternae of rough endoplasmic reticulum surrounding the central, spherical nucleus; the numerous mitochondria often in close association with RER; low levels of glycogen; and few lipid droplets (Fig. 6).

The general ultrastructural appearances of the MAs between livers of fish with higher and lower THg muscle concentrations were analyzed; however, no differences were evident. The MAs were clearly separated from the hepatic tissue and contained high amounts of granules varying from gray to dark and electron dense comparable with hepatic lysosomes. Also, few degenerated hepatocytes were observed within the MAs (Fig. 7).

Electron energy loss spectrometry

EELS was used to investigate whether the MAs were a site of intrahepatic Hg accumulation. A strong positive signal for Hg was detected in MAs as well as in hepatic lysosomes. However, the hepatic cytosol itself revealed only a weak signal for Hg and no other intracellular compartments showed a comparable strong signal for Hg. In both lysosomes and MAs, the strongest signal for Hg was detected in the dark electron dense granules followed by gray colored areas, whereas electron lucent areas showed no signal for Hg (Fig. 8).

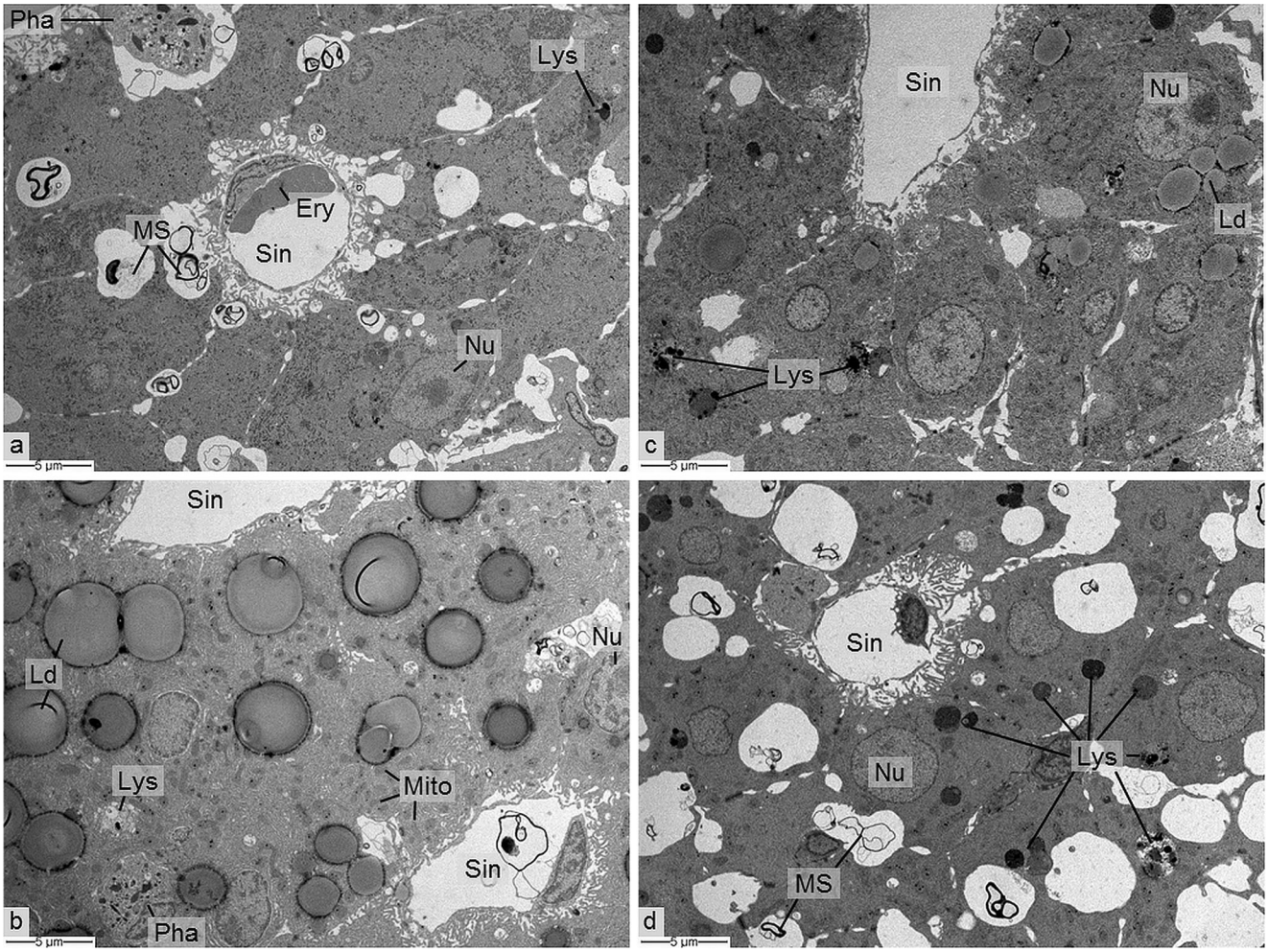

Fig. 5 Ultrastructural organization of yellow perch (*Perca flavescens*) hepatocytes from Upper Silver (**a**, **b**) and North Cranberry (**c**, **d**) Lakes, Kejimkujik National Park and National Historic Site, Nova Scotia, Canada. Hepatocytes from fish of both lakes were regularly arranged alongside the sinusoid (*Sin*) and characterized by a central nucleus (*Nu*) surrounded by rough endoplasmic reticulum, spherical to elongated mitochondria (*Mito*) in the cytoplasm, low glycogen content, and prominent lipid droplets (*Ld*). Lysosomes (*Lys*) were found in the cytoplasm and were highly heterogenic in morphology. But, in contrast to Upper Silver, lysosomes in samples from North Cranberry were larger in size and were mostly conspicuous dark and electron dense. Intercellular space was widened in samples from both lakes often containing membranous structures (*MS*). *Pha* phagocyte, *Ery* erythrocyte

Discussion

In the present study, the relationship between relative MA area in the liver and muscle THg concentrations of yellow perch in KNPNHS was quantitatively evaluated. The study further assessed whether the increase in MA area of the liver or fish Hg concentrations was associated with pathological alterations of the ultrastructure of the liver. We used a novel method to quantify MAs and found that the relative liver area occupied with MAs was positively related to fish muscle THg concentrations. Moreover, the lysosomes tended to be larger in fish from North Cranberry Lake, which had significant higher Hg concentrations compared to all other lakes. Further, Hg was detected within the MAs and hepatic lysosomes using EELS. This study is one of a few examining effects of elevated Hg concentrations on liver histopathology of a wild fish species and the quantification of those changes (e.g., MAs), combined with semiquantitative evaluation of alterations in the liver ultrastructure.

Muscle THg concentrations for yellow perch from KNPNHS were similar to those found previously in the same species from these lakes (0.08–2.13 μg g^{-1} wet wt, Batchelar et al. 2013), and the mean muscle concentration (0.351 μg g^{-1} wet wt) reported for yellow perch across northeastern North America (Kamman et al. 2005) was exceeded in 65 % of the sampled fish from KNPNHS. Furthermore, the 0.2 μg g^{-1} threshold (wet wt whole body) for adverse effects of Hg on fish health, equivalent to 0.33 μg g^{-1} wet wt in muscle tissue (Beckvar et al. 2005, Wyn et al. 2009), was exceeded by 70 % of the sampled fish in this park. Despite the elevated THg concentrations in these fish, general health endpoints such as Fulton's condition index were not adversely affected, as

Table 2 Analysis of lysosomal increase in size and frequency in yellow perch (*Perca flavescens*) hepatocytes in relation to muscle THg concentrations (μg g^{-1} wet wt)

Lake	Lysosome increase in size		Lysosome frequency	Muscle THg (μg g^{-1}) wet wt
	Light microscopy	Electron microscopy	Electron microscopy	
North Cranberry	+++	+++	++	0.697
North Cranberry	+++	++	+	0.672
North Cranberry	+++			0.534
North Cranberry	+++	+++	+++	0.530
Puzzle	+			0.500
Hilchemakaar	+			0.459
Cobrielle	+++	+++	+	0.459
Cobrielle	+			0.428
Puzzle	+	++	+	0.368
Hilchemakaar	+	-	+	0.347
Puzzle	+			0.330
Upper Silver	++	+	++	0.318
Upper Silver	++			0.303
Cobrielle	+			0.252
Upper Silver	+	+	++	0.251
Upper Silver	++	++	+++	0.246

Lysosome increase in size was classified via light and electron microscopy using the following categories: − no observed alterations, + mild alterations, ++ moderate alterations, and +++ strong alterations. Lysosome frequency was only estimated via electron microscopy

previously reported by Batchelar et al. (2013). A possible explanation might be the small range of fish sizes in the current study. This is in contrast to the negative relationship between condition and whole body THg (0.03 to 0.23 μg g^{-1} wet wt) reported for yellow perch (Suns and Hitchin 1990) and for walleye (*Sander vitreus*) (0.2 to 0.38 μg g^{-1} wet wt in muscle, Munn and Short 1997). Results from the current study suggest that condition index is a less sensitive indicator of Hg exposure compared to other effects (e.g., pathologies, oxidative stress).

Both this study and that by Batchelar et al. (2013) found a positive relationship between MAs in the liver and fish Hg, despite the use of different methods of MA quantitation. The image analysis software herein used for MA quantification allows for high-throughput screening and enabled us to analyze both number and area of MAs over the entire liver section, while still revealing MA distribution and variability among different liver sections. A 20-fold higher area per liver section was analyzed in comparison to previously applied methods (Barst et al. 2011; Batchelar et al. 2013; Drevnick et al. 2008). The fact that our findings agree with the previous study indicates the positive correlation between hepatic MAs and Hg concentrations is robust in yellow perch from KNPN HS. Previous work found that THg concentrations (0.13–6.04 μg g^{-1}wet wt) were higher in the liver than those in the muscle (Batchelar et al. 2013). Due to the liver perfusion used in the current study, quantification of Hg in this organ was not possible. However, liver THg concentrations for the current fish are assumed to be in a similar range as those reported by Batchelar et al. (2013), who found that THg concentrations in the liver were positively related to relative volume of MAs in that tissue.

MAs are mainly responsible for detoxification and recycling of exogenous as well as endogenous material such as damaged cells and indigestible materials (Agius and Roberts 2003; Wolke 1992). MA size and frequency are reported to increase with fish age (Agius and Roberts 2003; Brown and George 1985; Schwindt et al. 2006) and starvation (Agius and Roberts 1981; Mizuno et al. 2002), as well as with contaminant exposure (Fishelson 2006; Fournie et al. 2001; Passantino et al. 2014). These are all processes associated with enhanced cell degradation and catabolism. Since fish in this study were of rather uniform size, age is unlikely to be a determinant of MA size and frequency; this is further corroborated by the lack of a relationship between MAs and fish size (Brown et al. 2009; Purchase 2004; Purchase et al. 2005). A positive relationship between hepatic MAs and Hg tissue concentrations was reported for a variety of fish species, including pike (*Esox lucius*) with muscle Hg ranging from 0.21 to 0.82 μg g^{-1} wet wt (Meinelt et al. 1997), spotted gar (*Lepisosteus oculatus*) (0.01–1.1 μg g^{-1} wet wt in muscle, Barst et al. 2011), and trahira (*Hoplias malabaricus*) (1.45 μg g^{-1} wet wt in muscle, Mela et al. 2007). These findings suggest that the MAs function as a sink for the accumulated Hg. Moreover, this assumption is supported by literature reports that MAs contain higher Hg concentrations than surrounding liver tissue (Barst et al. 2011; Batchelar et al. 2013) as well as by the EELS analyses of the present study.

Another question addressed in this study was whether ultrastructural organization of yellow perch hepatocytes was adversely affected by elevated Hg accumulation and increased MA presence. Our findings do not provide evidence for degenerative alterations of hepatocyte fine structure, at least not within the range of fish Hg concentrations examined. The fact that these findings are in contrast to the altered liver ultrastructure reported in laboratory studies accounts for the complexity in understanding the health of fish from natural habitats. Dilation of the nuclear envelope as well as the RER was evident in the hepatocytes of sea bass 48 h after exposure to 501 μg L^{-1} waterborne inorganic Hg. Moreover, the RER appeared to be divided into vesicles and swelling of mitochondria was most frequently observed (Giari et al. 2008). Trahira hepatocytes showed leukocyte infiltration in combination with necrotic areas, changes in nuclear shape and

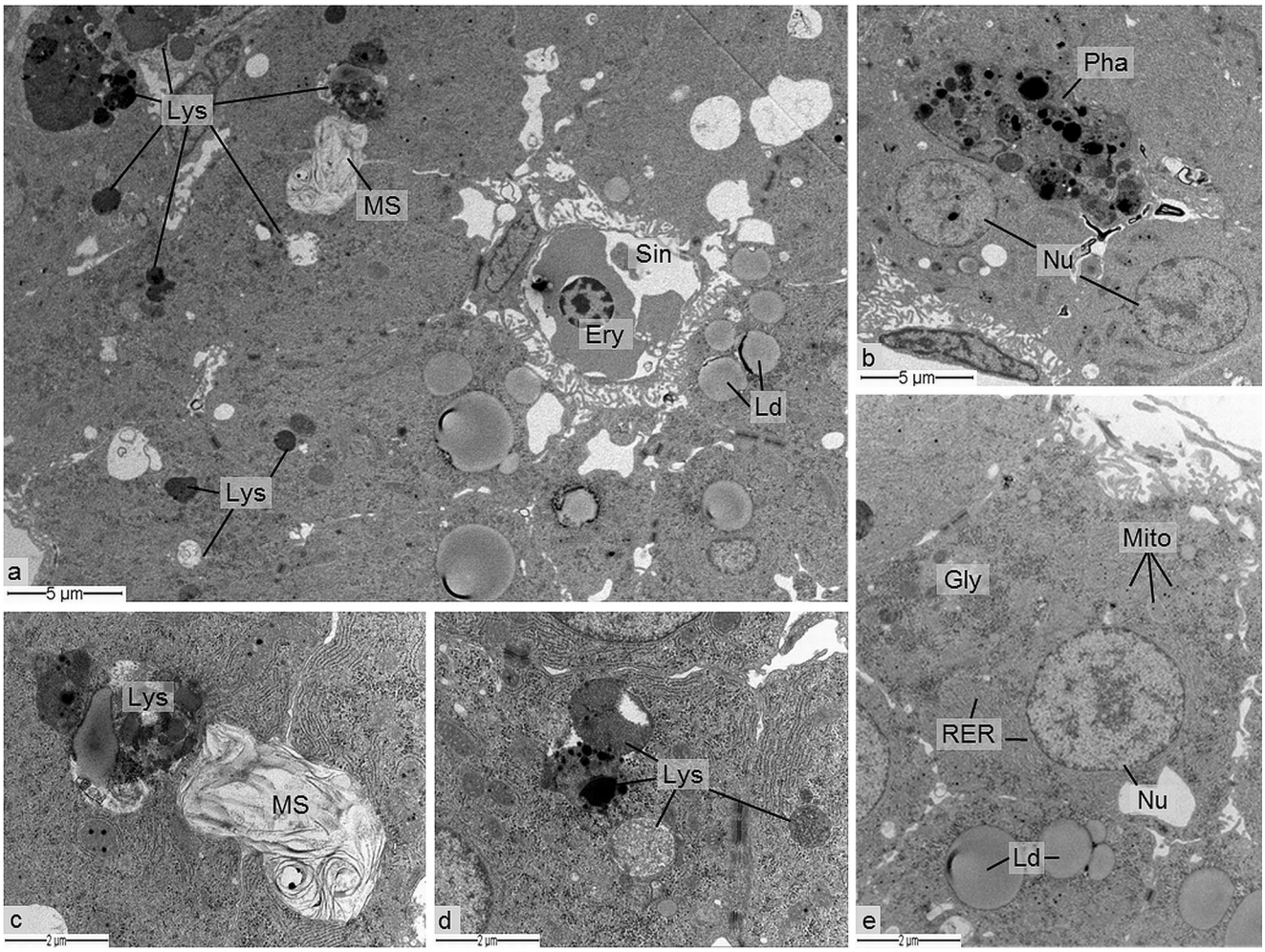

Fig. 6 Ultrastructure of yellow perch (*Perca flavescens*) liver from North Cranberry Lake, Kejimkujik National Park and National Historic Site, Nova Scotia, Canada. **a** Polygonal hepatocytes alongside the sinusoid (*Sin*), with regular ultrastructural organization: **e** stacked cisternae of rough endoplasmic reticulum (*RER*) in the vicinity of a central, spherical nucleus (*Nu*) and in association with mitochondria (*Mito*) in the cytoplasm. **a**, **c**, **d** Lysosomes (*Lys*) were conspicuous dark and electron dense. **b** Few phagocytes (*Pha*) consisting of dark, electron dense granules. *Ery* erythrocyte, *Gly* glycogen, *Ld* lipid droplet, *MS* membranous structures

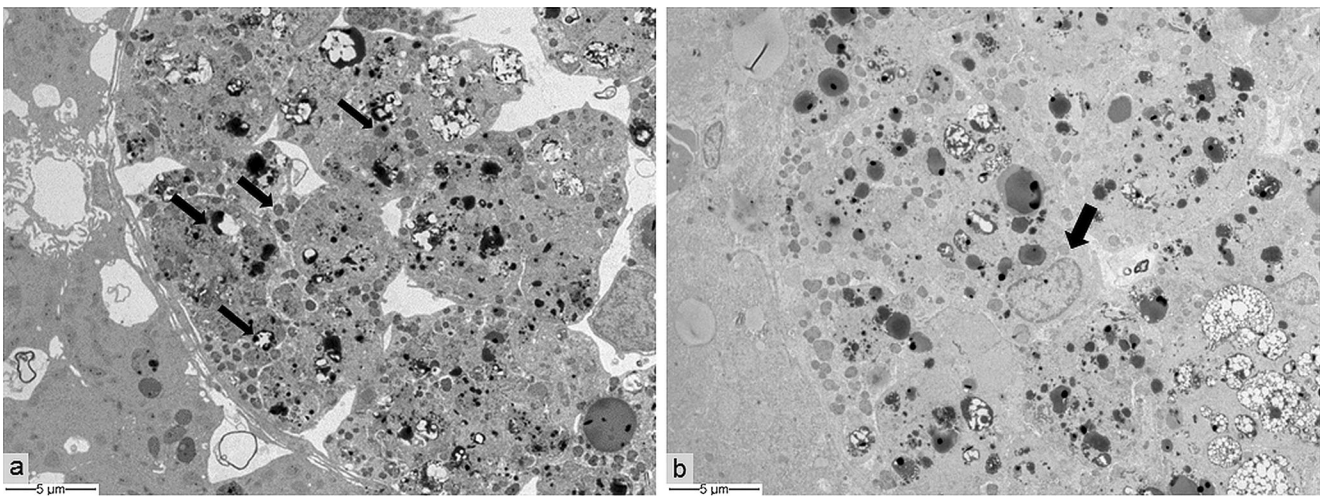

Fig. 7 Transmission electron micrograph of macrophage aggregates (MAs) in yellow perch (*Perca flavescens*) liver from Kejimkujik National Park and National Historic Site, Nova Scotia, Canada. **a** MAs strongly loaded with dark electron dense granules (indicated by *black arrow*) and **b** MAs with degenerated hepatocyte (indicated by *black arrows*)

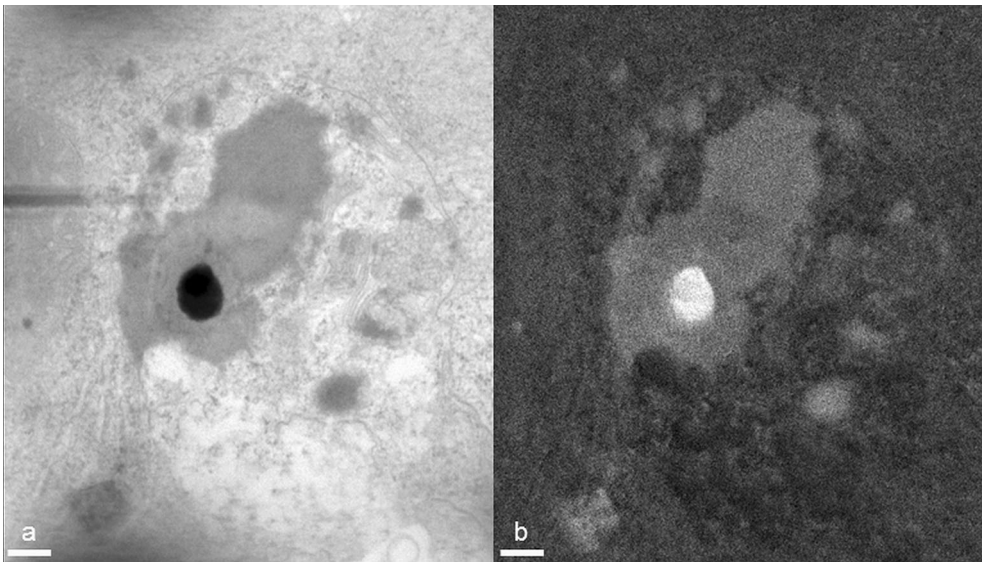

Fig. 8 Transmission electron micrograph of a hepatic lysosome in yellow perch (*Perca flavescens*) from Kejimkujik National Park and National Historic Site, Nova Scotia, Canada (**a**). Electron spectroscopic imaging revealed a strong signal for Hg indicated by *white color* within the dark electron-dense hepatic lysosome (**b**). A weaker signal for Hg was detected within the *gray areas* of hepatic lysosomes indicated by *gray color. Scale bar*=0.2 μm

heterochromatin distribution, and damage of Dissé's space after 70 days of dietary exposure to 0.075 μg g^{-1} MeHg wet wt (1.45 μg g^{-1} muscle THg concentration, Mela et al. 2007). However, the presence of atypical cytoplasmic electron dense granules was observed in Trahira and comparable in morphology to the lysosomes found in this study.

While overall hepatocyte fine structure displayed no pathological alterations, two observations were unusual. The first was the wider intercellular spaces in the hepatocytes. However, the frequency and severity of this phenomenon were not related to muscle THg concentrations. A second observation was that the lysosomes appeared to be enlarged and highly heterogenic in fish with highest muscle THg concentrations. By means of EELS, the lysosomes were found to contain Hg. Accumulation of Hg in hepatic lysosomes was also reported for rainbow trout (*Oncorhynchus mykiss*) after 7 weeks of dietary exposure to MeHg (Baatrup and Danscher 1987). They also observed an accumulation of inorganic Hg in the lysosomes over time and speculated that inorganic Hg is derived from MeHg through demethylation and that lysosomes are a site of biotransformation in fish liver. Batchelar et al. (2013) reported for yellow perch from Kejimkujik that in the liver, both inorganic and MeHg increased along with THg concentrations, but the percentage present as MeHg decreased with higher THg concentration. This was also reported for northern pike (Drevnick et al. 2008) and striped bass (Cizdziel et al. 2003). Detailed knowledge of detoxification of MeHg in fish is scarce, and in contrast to inorganic Hg, MeHg does not induce, bind to, or undergo detoxification by metallothionein (Barghigiani et al. 1989). As mentioned above, one hypothesis derived from those studies is, although it is not yet proven, that in vivo MeHg demethylation may occur in the liver of fish. Another possible explanation is that both inorganic and MeHg are sequestered within the hepatic lysosomes and MAs removing Hg from cellular processes. The fact that Hg was only detected within the hepatic lysosomes, but no other hepatic organelles, and MA area increased with THg concentrations in this study provides evidence to support either of the hypotheses.

Our findings indicate that neither the general health of yellow perch from KNPNHS nor the general hepatocyte ultrastructure was adversely affected by elevated THg concentrations in yellow perch of KNPNHS lakes. However, MA area in the liver increased along with muscle THg concentrations and MAs were shown to contain Hg. The findings imply that yellow perch tolerated the elevated Hg concentrations and MAs in the liver function as a sink for the accumulated Hg. Hepatic lysosomes might also be involved in sequestering or detoxification of the Hg in the fish liver (Baatrup and Danscher 1987; Batchelar et al. 2013, this study). Further research is needed to investigate whether Hg is detoxified in the liver of fish and confirm this hypothesis. Within the range of THg concentrations occurring in fish in this study, metal sequestration was apparently efficient enough to protect the liver from pathological tissue damage. In addition, the costs of the likely detoxification did not result in trade-offs in growth and condition of the fish. An important unknown to address is the Hg concentration at which the protective capacity of the fish is surpassed and adverse effects develop.

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