International Journal for Parasitology 49 (2019) 647–656

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Schistosoma mansoni does not and cannot oxidise fatty acids, but these are used for biosynthetic purposes instead

Michiel L. Bexkens [a], Mirjam M. Mebius [a], Martin Houweling [b], Jos F. Brouwers [b], Aloysius G.M. Tielens [a,b], Jaap J. van Hellemond [a,*]

[a] Dept. Medical Microbiology & Infectious Diseases, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands
[b] Dept. Biochemistry & Cell Biology, Fac. Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

ARTICLE INFO

Article history:
Received 4 November 2018
Received in revised form 15 March 2019
Accepted 24 March 2019
Available online 3 June 2019

Keywords:
Beta-oxidation
Energy metabolism
Helminths
Lipid metabolism
Schistosomiasis
Genome analysis

ABSTRACT

Adult schistosomes, parasitic flatworms that cause the tropical disease schistosomiasis, have always been considered to be homolactic fermenters and, in their energy metabolism, strictly dependent on carbohydrates. However, more recent studies suggested that fatty acid β-oxidation is essential for egg production by adult female *Schistosoma mansoni*. To address this conundrum, we performed a comprehensive study on the lipid metabolism of *S. mansoni*. Incubations with [^{14}C]-labelled fatty acids demonstrated that adults, eggs and miracidia of *S. mansoni* did not oxidise fatty acids, as no $^{14}CO_2$ production could be detected. We then re-examined the *S. mansoni* genome using the genes known to be involved in fatty acid oxidation in six eukaryotic model reference species. This showed that the earlier automatically annotated genes for fatty acid oxidation were in fact incorrectly annotated. In a further analysis we could not detect any genes encoding β-oxidation enzymes, which demonstrates that *S. mansoni* cannot use this pathway in any of its lifecycle stages. The same was true for *Schistosoma japonicum* and all other schistosome species that have been sequenced. Absence of β-oxidation, however, does not imply that fatty acids from the host are not metabolised by schistosomes. Adult schistosomes can use and modify fatty acids from their host for biosynthetic purposes and incorporate those in phospholipids and neutral lipids. Female worms deposit large amounts of these lipids in the eggs they produce, which explains why interference with the lipid metabolism in females will disturb egg formation, even though fatty acid β-oxidation does not occur in schistosomes. Our analyses of *S. mansoni* further revealed that during the development and maturation of the miracidium inside the egg, changes in lipid composition occur which indicate that fatty acids deposited in the egg by the female worm are used for phospholipid biosynthesis required for membrane formation in the developing miracidium.

1. Introduction

The blood dwelling parasite *Schistosoma mansoni* is a causative agent of the neglected tropical disease schistosomiasis that affects over 200 million people worldwide (Colley et al., 2014). Throughout the life-cycle of this helminth, *S. mansoni* encounters various environments and adapts its energy metabolism accordingly. The free-living stages, cercariae and miracidia, live on their endogenous glycogen stores which they completely oxidise to carbon dioxide using Krebs cycle activity and oxidative phosphorylation (Van Oordt et al., 1989; Tielens et al., 1991). Within the mammalian host, adult *S. mansoni* reside in the mesenteric veins, where male and female worms live paired and acquire everything they need directly from the blood of the host. These adult schistosomes have a mainly fermentative metabolism as only a small part of the glucose obtained from the host is fully oxidised to carbon dioxide using Krebs cycle activity and oxidative phosphorylation, while the major part is degraded to lactate and excreted as such (Bueding, 1950; Schiller et al., 1975; van Oordt et al., 1985). Lipids are also obtained from the host. The lipid metabolism of schistosomes is rather compromised, as schistosomes cannot synthesise sterols or free fatty acids de novo and must use complex precursors from the host (Brouwers et al., 1997). Until recently it was generally accepted that schistosomes do not catabolise lipids for ATP production. However, in 2009 the genome of *S. mansoni* was published, including an automated annotation which indicated that genes for all enzymes used in β-oxidation of fatty acids are present

* Corresponding author at: Erasmus MC, University Medical Center Rotterdam, Dept. Medical Microbiology & Infectious Diseases, PO Box 2040, 3000 CA Rotterdam, The Netherlands.
E-mail address: j.vanhellemond@erasmusmc.nl (J.J. van Hellemond).

https://doi.org/10.1016/j.ijpara.2019.03.005
0020-7519/

(Berriman et al., 2009). Automated annotations of genomes are, however, inherently prone to misannotations and are therefore continuously revised. Currently, several of the *S. mansoni* genes earlier annotated as coding for fatty acid β-oxidation enzymes are no longer annotated as such in GeneDB and in databases such as Biocyc.org, WormBase and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

In 2012 Huang et al. (2012) reported that oxidation of fatty acids acquired from the host is essential for egg production by female *S. mansoni* worms. Thereafter fatty acid β-oxidation has received a lot of attention and most general reports on schistosomiasis now state that fatty acid oxidation is essential for egg production in female schistosomes (Colley et al., 2014; Guigas and Molofsky, 2015; Pearce and Huang, 2015; Oliveira et al., 2016). Subsequently, the postulated fatty acid β-oxidation process was a subject of studies on gene expression in schistosomes (Buro et al., 2013; Li et al., 2017) and of studies that aimed to identify novel drugs for schistosomiasis (Edwards et al., 2015; Timson, 2016).

However, the assumption that fatty acid oxidation occurs in schistosomes is still controversial as fatty acid oxidation has never been demonstrated directly in *S. mansoni*, nor in any other parasitic trematode for that matter (Rumjanek and Simpson, 1980; Saz, 1981; Frayha and Smyth, 1983). This prompted us to perform a comprehensive analysis of the lipid metabolism of *S. mansoni*. We incubated adult worm pairs as well as eggs and miracidia with [^{14}C]-labelled glucose and [^{14}C]-labelled fatty acids to determine the metabolic fate of these substrates in *S. mansoni*. Furthermore, a genomic analysis was performed to examine the possible presence of genes in the *S. mansoni* genome encoding enzymes involved in oxidation of fatty acids. To further investigate the role of fatty acids in eggs we performed a lipidome analysis of eggs during their development.

2. Materials and methods

2.1. Parasites and chemicals

A Puerto Rican strain of *S. mansoni* was maintained in Golden hamsters with animal ethics approval (licence EUR1860-11709). Animal care and maintenance were in accordance with institutional and governmental guidelines. Adult *S. mansoni* worms were isolated from isoflurane anaesthetised hamsters 7 weeks p.i. Worms were collected from the portal vein following heart perfusion with S_{20} medium (20 mM HEPES, 85 mM NaCl, 5.4 mM KCl, 0.7 mM Na_2HPO_4, 1 mM $MgSO_4$, 1.5 mM $CaCl_2$, 25 mM $NaHCO_3$ and 20 mM glucose pH 7.4) (Tielens and van den Bergh, 1987). *Schistosoma mansoni* eggs were obtained from livers of infected hamsters. These livers were homogenised in 1.8% (w/v) NaCl using a MACS homogeniser (Miltenyi Biotec, San Diego, USA). The liver homogenate was treated with 1% trypsin in 1.8% NaCl (BD, New Jersey, USA) for 1 h at 37° C, after which eggs were isolated by filtration over three sieves with decreasing mesh sizes (Dresden and Payne, 1981). The eggs were collected and rinsed with sterile water. When indicated (see Section 2.4) the total isolated egg fraction was separated into immature and mature eggs via Percoll density centrifugation by the method described by Ashton et al. (2001). All chemicals used were from Sigma Aldrich, St. Louis, MO, USA unless otherwise specified.

2.2. Metabolic incubations

Schistosoma mansoni worms (10 pairs per incubation) were incubated in 25 ml Erlenmeyer flasks for 2.5 h at 37 °C, 95% O_2, 5% CO_2 in 5 ml of S_5^+ medium (20 mM HEPES, 85 mM NaCl, 5.4 mM KCl, 0.7 mM Na_2HPO_4, 1 mM $MgSO_4$, 1.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 5 mM glucose and 1% (v/v) delipidated BSA pH 7.4). All incubations were started with the addition of one of the labelled substrates (all from PerkinElmer, Boston, MA, USA): D-[6-^{14}C] glucose (5 mM, 5 μCi), [1-^{14}C] octanoic acid (210 μM, 5 μCi) or [1-^{14}C] oleic acid (210 μM, 5 μCi). Radioactive incubations were stopped by acidification of the medium to pH 2 by addition of HCl through the septum of the sealed Erlenmeyer flask. Carbon dioxide was trapped for 1.5 h in 200 μl of 4 M KOH in a centre well suspended above the incubation medium. Afterwards, the trapping solution was transferred to a vial containing water and Luma gel (Lumac*LCS, Groningen, The Netherlands), after which radioactivity was measured in a scintillation counter. Worm pairs were removed from the incubation medium and stored at −20 °C until further analysis. The acidified supernatant was neutralised by the addition of 6 M NaOH. The labelled metabolic end products in the supernatant were analysed by anion exchange chromatography on a Dowex 1X8, 100–200 mesh column (Serva) (60 × 1.1 cm) in chloride form (Tielens et al., 1981). The column was eluted successively with 200 ml of 5 mM HCl and 130 ml of 0.2 M NaCl. All fractions were collected and radioactivity was measured in Luma gel. All values were corrected for blank incubations.

Schistosoma mansoni eggs ($4.3 * 10^4$–$6.2 * 10^4$) were freshly isolated from infected livers and subsequently transferred to a 25 ml Erlenmeyer flask containing 5 ml of a 1 mM glucose solution supplemented with 100 μg of penicillin and 100 units of streptomycin per ml. After addition of D-[6-^{14}C] glucose (5 mM, 5 μCi) or [1-^{14}C] octanoic acid (210 μM, 5 μCi) eggs were incubated for 20 h at 22 °C while shaking gently at 125 rpm. Incubations were stopped and analysed as described above.

2.3. Analysis of incorporated lipids after metabolic incubation

To analyze the incorporation of radioactively labelled fatty acids into complex lipids by *S. mansoni* worms and eggs, lipids were extracted from incubated worms and eggs according to the method of Bligh and Dyer (1959). Prior to the lipid extraction, eggs were disrupted by sonication and adult worms were homogenised by a Teflon potter. The isolated lipid fraction was subsequently split into neutral lipids and phospholipids by dissolving the total lipid fraction in chloroform, after which it was loaded on a 2 ml silica gel 60 column (8 cm tall, 0.5 cm in diameter) equilibrated in chloroform. Neutral lipids were eluted with chloroform, followed by elution of phospholipids with methanol. The lipid composition of the neutral lipid and phospholipid fractions was further analysed by thin layer chromatography (TLC) on Silica G by the methods of Freeman and West (1966) and Skipski et al. (1962), respectively. After separation of distinct lipid classes by TLC, the plates were dried and placed in iodine vapour to visualise lipid spots, which were subsequently scraped off. The collected silica was suspended in 1 ml of H_2O and 3 ml of Luma gel were added before radioactivity was measured in a scintillation counter.

2.4. Lipidome analysis of immature and mature eggs

Approximately 1000 freshly isolated *S. mansoni* eggs were stained with Nile red lipophilic stain (1 mg/ml) for 20 min, shaking at 1200 rpm at 25 °C in 1 ml of 0.9% (w/v) NaCl. After staining, the eggs were washed twice with 1 ml 0.9% (w/v) NaCl and mounted for microscopy. Phase contrast images were captured at 200× magnification. For each bright field image, a corresponding fluorescent exposure was recorded. Eggs were classified by size and developmental stage as described by Jurberg et al. (2009).

In order to analyze the lipid content in immature and mature eggs, lipids were extracted as described in Section 2.3. Subsequently, the phospholipid content in mature and immature eggs

was quantified by the method of Rouser et al. (1970), and the ratio of phospholipids over neutral lipids as well as the lipid species composition was determined by Liquid Chromatography coupled to Mass Spectrometry (LCMS). The extracted lipids were loaded on a hydrophilic interaction liquid chromatography (HILIC) column (2.6 μm HILIC 100 Å, 50 × 4.6 mm, Phenomenex, Torrance, CA, USA) and eluted at a flow rate of 1 mL/min with a gradient from acetonitrile/acetone (9:1, v/v) to acetonitrile/H_2O (7:3, v/v) with 10 mM ammonium formate. Both elution solutions also comprised 0.1% (v/v) formic acid. The column outlet of the LC was connected to a heated electrospray ionisation (HESI) source of an LTQ-XL mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA). Full scan spectra were collected from *m/z* 450–1050 at a scan speed of three scans/s. For analysis, the data were converted to mzXML format and analysed using XCMS version 1.52.0 running under R version 3.4.3 (Smith et al., 2006; R Development Core Team: A language and environment for statistical computing. R Foundation for Statistical Computing, 2016). Principle Component Analysis (PCA) provided by the R package pcaMethods (Stacklies et al., 2007) was used to visualise the multidimensional LC–MS data.

2.5. Identification strategy to detect genes possibly encoding enzymes required for fatty acid oxidation in the S. mansoni genome

In order to detect genes within the *S. mansoni* genome that are possibly involved in fatty acid oxidation, genes known to be involved in fatty acid oxidation (KEGG pathway 00071) were retrieved from six model reference species: *Caenorhabditis elegans*, *Crassostrea gigas*, *Danio rerio*, *Drosophila melanogaster*, *Homo sapiens* and *Mus musculus*. The protein sequences of these genes were used as a query in a forward BlastP search against the *S. mansoni* genome with an *E*-value cut-off of 10^{-20}. This forward BlastP search resulted in the identification of 14 *S. mansoni* protein sequences. These proteins possibly involved in lipid metabolism were further investigated by the following annotation strategy. First, using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm (Edgar, 2004), the corresponding proteins of these identified *S. mansoni* genes were aligned to the amino acid sequences of the best hit from the forward BlastP query with the six model organisms. Second, these alignments were further investigated to determine whether obvious conserved regions in the proteins of the model organisms are present in the corresponding schistosomal proteins. Third, the *S. mansoni* proteins were used as a query in a reversed BlastP search against the six model organisms to check their identity and to verify whether the model organism contains a protein with more similarity to the schistosomal protein than the one used in the forward BlastP where only the proteins involved in fatty acid oxidation were used as a query. As a control we also searched in the *S. mansoni* genome for the presence of the enzymes of KEGG pathways 00010 (glycolysis and gluconeogenesis) and 00020 (Citrate Cycle).

3. Results

The lipid metabolism of *S. mansoni* adult worms and that of eggs and miracidia was studied by incubations with ^{14}C-labelled fatty acids, after which the metabolic fate of these fatty acids was determined by analysis of ^{14}C-labelled excreted end products to detect catabolic processes and by analysis of ^{14}C-labelled lipids to detect incorporation of fatty acids in anabolic processes. Normal physiological functioning of *S. mansoni* can only be studied in paired worms as female worms need males for normal functioning. Female-specific gene expression is dependent on pairing with male worms. To maintain female vitelline cell development there must be direct contact between the male and the female, and they cease egg laying after removal of the accompanying male (Loverde et al., 2004). Therefore we intentionally studied paired worms as our radioactive method is sensitive enough to detect fatty acid oxidation even if it would occur to a significant extent in female worms only.

3.1. Lipid metabolism in adult S. mansoni worm pairs

In order to use fatty acids for the production of ATP, fatty acids must be oxidised to carbon dioxide, as fatty acids are too reduced to be fermented. To detect fatty acid oxidation by adult *S. mansoni* worm pairs, the worms were incubated with [1-^{14}C] oleic acid, or with [1-^{14}C] octanoic acid, a medium chain-length fatty acid that easily crosses membranes. After these incubations we analysed the formation of $^{14}CO_2$. We could not detect any fatty acid oxidation by adult *S. mansoni* worm pairs, as the production of ^{14}C-labelled CO_2 from [1-^{14}C] octanoic acid as well as from [1-^{14}C] oleic acid was below the detection limit of 0.05 nmol of CO_2 per h (Table 1). In a simultaneously performed control experiment with [6-^{14}C] glucose, approximately 60 nmol of CO_2 were produced per h by 10 worm pairs, which is comparable with earlier studies (Tielens et al., 1989). This result showed that the parasites were metabolically active and that if production of ^{14}C-labelled CO_2 from fatty acids had occurred, it would have been detected by our assay system. All together these results showed that the adult worms did not oxidise fatty acids under standard incubation conditions.

Table 1
Analysis of end product formation and/or incorporation of labelled substrate in neutral lipids and phospholipids by *Schistosoma mansoni* worms or eggs plus miracidia. Organisms were incubated with labelled glucose or fatty acid for up to 20 h, after which an end product formation was analysed in the headspace or supernatant of the incubation. *S. mansoni* worms or eggs and miracidia were then analysed for incorporation of labelled substrate.

	End product formation				Incorporation of labelled substrate			
	CO_2 (nmol/h)		Lactate (nmol/h)		Neutral lipids (nmol/h)		Phospholipids (nmol/h)	
Substrate	worms[a]	eggs + miracidia [b]	worms[a]	eggs + miracidia [b]	worms[a]	eggs + miracidia [b]	worms[a]	eggs + miracidia[b]
[6-^{14}C]-Glucose	62.3 ± 10.4[c]	7.13[d]	534 ± 74[c]	na	N.D.	0.49[e]	N.D.	0.19[f]
[1-^{14}C]-Octanoic Acid	N.D.	N.D.	na	na	N.D.	33.81[g]	N.D.	N.D.
[1-^{14}C]-Oleic Acid	N.D.	N.D.	na	na	15.99 ± 3.14[c]	na	5.51 ± 2.39[c]	na

N.D., not detectable, below detection limit of assay.
na, not analysed.
[a] 10 worm pairs per incubation.
[b] Values are expressed per 50,000 (eggs + miracidia).
[c] All values represent the mean in nmol per h per 10 worm pairs and S.D. of three independent experiments.
[d] Mean of two independent experiments (6.98 and 7.28).
[e] Mean of two independent experiments (0.53 and 0.44).
[f] Mean of two independent experiments (0.19 and 0.18).
[g] Mean of two independent experiments (29.9 and 37.73).

Previous research has shown that *S. mansoni* cannot synthesise lipids de novo, and therefore, adult worms take up fatty acids from their environment, after which the fatty acids can be modified by elongation before incorporation in triacylglycerol (TAG) species and phospholipids (Meyer et al., 1970; Brouwers et al., 1997). To investigate the anabolic fate of the exogenous supplied ^{14}C-labelld fatty acids in adult *S. mansoni* worms, we determined the incorporation of [1-^{14}C] octanoic acid and [1-^{14}C] oleic acid into phospholipids and neutral lipids by adult worm pairs in the above mentioned incubations. This showed that adult worm pairs incorporated oleic acid in both neutral lipids and phospholipids, at rates of approximately 16 nmol and 5.5 nmol per h per 10 worm pairs, respectively (Table 1). Incorporation of octanoic acid was not detected (Table 1). From these results and earlier reports (Meyer et al., 1970; Brouwers et al., 1997), it can be concluded that the lipid metabolism of *S. mansoni* adult worms is fairly limited because adult worms do not de novo synthesise nor oxidise fatty acids. Adult schistosomes have to obtain fatty acids from their environment and can modify those, after which they are used as building blocks for the synthesis of phospholipids and neutral lipids such as TAG.

3.2. Lipid metabolism of S. mansoni eggs and miracidia

We also investigated the lipid metabolism of *S. mansoni* eggs and miracidia by incubation of isolated *S. mansoni* eggs in water with trace amounts of ^{14}C-labelled substrates. Although eggs were isolated from liver tissue in 1.8 % (w/v) NaCl and in the dark, a situation in which only a few eggs will hatch and release miracidia (Xu and Dresden, 1990), the subsequent metabolic incubation was performed in water and during the 20 h incubation a substantial part of the eggs hatched and released miracidia into the medium. Therefore, the results of these incubations reflect in fact the combined metabolic activities of eggs plus miracidia. The *S. mansoni* eggs plus miracidia were incubated with [1-^{14}C] oleic acid, [1-^{14}C] octanoic acid and with [6-^{14}C] glucose. After overnight incubation the production of $^{14}CO_2$ was determined, but *S. mansoni* eggs plus miracidia did not produce detectable amounts of $^{14}CO_2$ from the ^{14}C-labelled fatty acids, whereas in the simultaneously performed control incubation with [6-^{14}C] glucose 7.1 nmol of CO_2 was produced per h per 50,000 organisms (Table 1).

Since the supplied ^{14}C-labelled fatty acids were not used for fatty acid oxidation by *S. mansoni* eggs and miracidia, we analysed whether the incubated eggs and miracidia had taken up [1-^{14}C] octanoic acid and incorporated it in complex lipids. This revealed that octanoic acid was incorporated in neutral lipids at a rate of 34 nmol per h per 50,000 *S. mansoni* eggs plus miracidia (Table 1). The incubations performed in parallel with [6-^{14}C]-glucose demonstrated that not only fatty acids were incorporated into neutral- as well as into phospholipids, but intermediates of glucose metabolism were also incorporated (Table 1). As *S. mansoni* cannot synthesise fatty acids de novo, the incorporation of ^{14}C-label from glucose in lipids is most likely the result of elongation of fatty acids with acetyl-CoA or of the incorporation of glycerol-3-phosphate as a backbone for TAG or phospholipid biosynthesis. These results showed that *S. mansoni* eggs and/or miracidia take up fatty acids and are capable of incorporating those in complex lipids, but they do not oxidise fatty acids to CO_2.

To perform a more in-depth analysis of the lipid metabolism of the *S. mansoni* egg during development, freshly isolated eggs were separated into mature and immature eggs (Ashton et al., 2001), after which we analysed the distribution and composition of phospholipids and neutral lipids in the immature and the mature egg fraction. Microscopic analysis of Nile red stained *S. mansoni* eggs showed a differential distribution and content of lipids during the development of the egg (Fig. 1). Immature eggs (Fig. 1A–D)

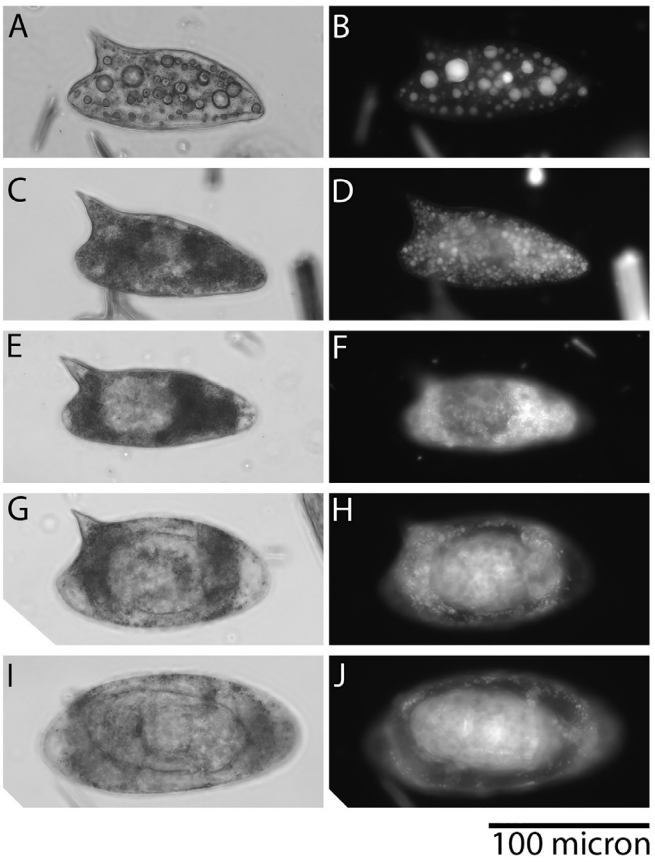

Fig. 1. Differential distribution of lipids in developing *Schistosoma mansoni* eggs. Freshly isolated live *S. mansoni* eggs were stained with Nile Red lipophilic stain, after which representative phase contrast images (A, C, E, G and I) and their corresponding fluorescent images of Nile Red lipophilic stain (B, D, F, H and J) were taken. Eggs are placed in order of maturation; from immature eggs at the top to fully mature eggs with a moving miracidium at the bottom of the figure.

contained many large lipid droplets, which disappeared during maturation of the egg (Fig. 1G–H), suggesting that the amount of neutral lipids decreased during development. To analyze the changes in lipid composition during maturation in more detail, the lipid composition in the immature and the mature eggs was analysed by LC–MS. A significant difference in the phospholipid content in immature versus mature eggs was observed, as the phospholipid content almost doubled from 3.2 pmol to 5.2 pmol of fatty acids per egg after maturation and the amount of neutral lipids decreased, although this difference was not significant (Fig. 2). PCA of the lipidomic data demonstrated that the lipid composition between mature and immature eggs differed substantially (Fig. 3A), as the relative abundance of many different lipid species from all lipid classes differs between mature and immature eggs (Fig. 3B). For instance, the phosphatidylserine species (40:4) and phosphatidylinositol species (38:4) were more abundantly present in immature eggs, whereas the phosphatidylcholine species 34:1 and 36:1 were more abundantly present in immature eggs. However, no significant differences in the overall phospholipid class distribution, fatty acid chain length and degree of unsaturation were observed (data not shown). All together, these results showed that the lipidome of *S. mansoni* eggs changes during egg development.

3.3. Analysis of lipid metabolism at a genomic level.

As the investigated stages of *S. mansoni* apparently did not oxidise fatty acids under standard incubation conditions, the question

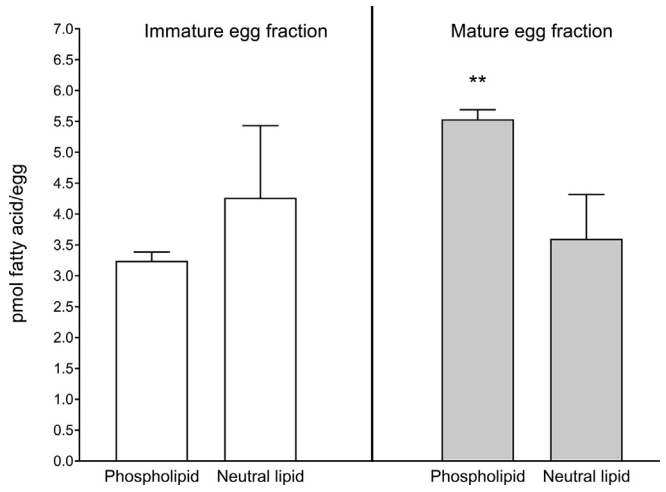

Fig. 2. The amount of fatty acids in phospholipids and neutral lipids in *Schistosoma mansoni* eggs changes during maturation. The phospholipid and neutral lipid content of *S. mansoni* eggs were analysed in immature and mature eggs and expressed as picomoles of fatty acid per lipid fraction. Shown is the average and S.D. of three independent experiments, each performed in duplicate. **A one-tailed paired *t*-test showed a statistically significant increase in the phospholipid content (pmol per egg) of mature eggs versus immature eggs ($P < 0.0054$).

arises whether *S. mansoni* has the genomic capacity to do so at all. To investigate the possible presence of genes in the *S. mansoni* genome which could encode the enzymes required for fatty acid oxidation, we queried the *S. mansoni* genome using genes known to be involved in fatty acid oxidation. Genes annotated in KEGG map00071 "fatty acid degradation" were retrieved from six well characterised model organisms: *C. elegans*, *C. gigas*, *D. rerio*, *D. melanogaster*, *H. sapiens* and *M. musculus*. This forward BlastP search resulted in the identification of 15 *S. mansoni* protein sequences. Of these 15 *S. mansoni* proteins, two identifiers were protein isoforms of the same gene, leaving 14 *S. mansoni* genes that might encode enzymes involved in fatty acid oxidation (Table 2). Ten of these 14 genes seemed to encode acyl-CoA synthetases. Of the four remaining genes, three genes seemed to code for homologs of mitochondrial β-oxidation enzymes (acyl-CoA dehydrogenase, enoyl-CoA hydratase and 3-keto-acyl CoA thiolase) and the protein encoded by the last gene seemed to be a homolog of both carnitine palmitoyl transferase 1 and 2 (CPT-1 and CPT-2). However, the similarity of these last four *S. mansoni* proteins to their corresponding proteins in the six model organisms is probably low, as the *E*-values were rather high ($10^{-20} > E > 10^{-80}$, Supplementary Tables S1, S2). Using this approach, no schistosomal protein was found with a significant homology to 3-hydroxyacyl-CoA dehydrogenase (enzyme 6 in Fig. 4).

To further investigate whether or not the retrieved schistosomal genes really encode the corresponding enzymes involved in fatty acid oxidation, we aligned the found schistosomal proteins with the best hit for each model organism (Supplementary Fig. S1). The alignments for the identified *S. mansoni* genes possibly encoding acyl-CoA synthetases (Fig. 4, enzyme 1) indicated that these *S. mansoni* proteins are true homologues of acyl-CoA synthetases, because the identified *S. mansoni* proteins were highly similar to the ones that encode acyl-CoA synthetases in the six model organisms (Supplementary Fig. S1). However, the alignments of the other five retrieved proteins showed low homology to the corresponding proteins of the six model organisms and regions highly conserved in the proteins of the model organisms seemed to be missing in the schistosomal proteins (Supplementary Fig. S1).

We further analysed these alignments to investigate whether known conserved regions in the proteins in question are indeed absent in the corresponding schistosomal proteins. To this end we produced aligned barcodes of each enzyme where a black bar is produced only when an amino acid is identical in *all* six model organisms and these bar codes were then compared with the found schistosomal proteins (Fig. 5). This analysis clearly showed that the schistosomal acyl-CoA synthetases are indeed highly similar to the ones of the model organisms, as their barcodes are real look-a-likes of those of the model organisms. In contrast, the 100% conserved regions in the model organisms of the other five proteins are not mirrored in the schistosomal ones, despite the large gaps the algorithm introduced in three schistosomal proteins (2–4) to produce the best alignment.

As a final check in this genomic analysis, we performed a reverse BlastP query where the found schistosomal proteins were

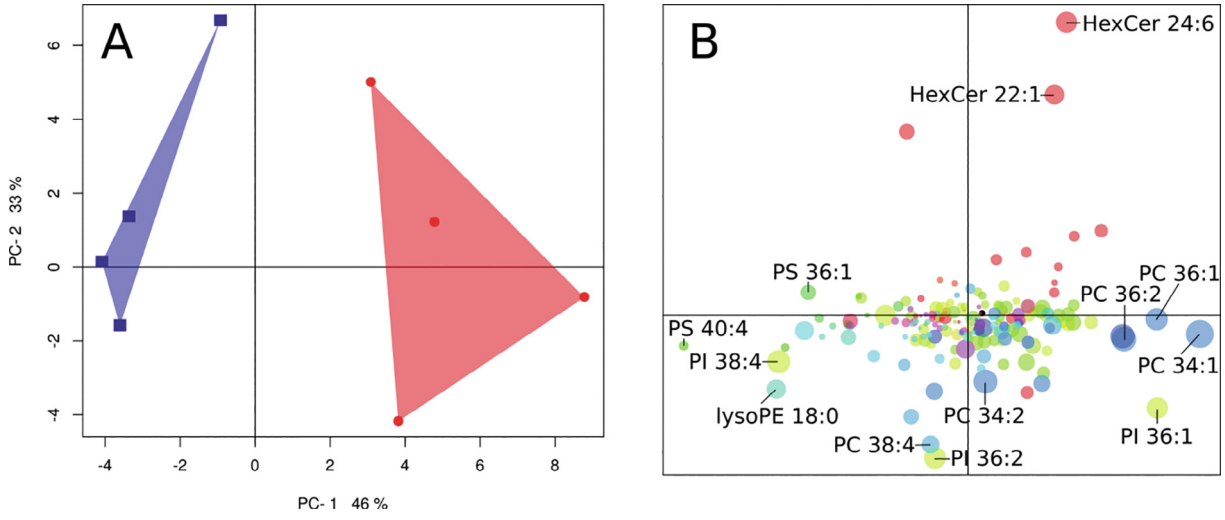

Fig. 3. Lipidome analysis of mature and immature *Schistosoma mansoni* eggs. (A) Principal Component Analysis (PCA) on the lipidomic data of mature (blue (dark grey)) and immature (red (light grey)) eggs revealed different lipid fingerprints for both, as could be concluded from their distinct and non-overlapping score plots. The value of Principal Component 1 (PC-1, representing 46% of total data variance) was found to correspond with the maturation state of the eggs and shows that the difference between mature and immature eggs is reflected in many different lipid species from all major phospholipid classes. (B) Lipids with positive loadings on PC-1 (e.g. PC 34:1, Pi 36:1) were more abundant in immature eggs, lipids with negative loadings on PC-1 (e.g. PS 40:4, PI 38:4) were more abundant in mature eggs. HexCer, hexosylceramide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine.

Table 2
Analysis and comparison of BlastP results with genome annotations of genes coding for enzymes present in the *Schistosoma mansoni* (Smp) genome. A BlastP analysis was performed using proteins known to be involved in fatty acid oxidation in six model organisms. Results were then ordered by enzyme numbers 1–7 as in Fig. 4, representing steps in lipid metabolism and beta-oxidation. Identified genes were compared with earlier studies and analysed using NCBI SMARTblast.

Enzyme	Enzyme code	Potential homologs identified	Earlier ID (Berriman et al., 2009)	Best hit of reversed BlastP against model organisms	Enzyme code of best hit
1. Acyl-CoA synthetase	EC 6.2.1.3	Smp_103160.1	Smp_103160	Acyl-CoA synthetase	EC 6.2.1.3
		Smp_103160.2			
		Smp_175090.1	Smp_175090	Acyl-CoA synthetase	
		Smp_175090.2			
		Smp_175090.3			
		Smp_266800.1	Smp_138660	Acyl-CoA synthetase	
		Smp_125560.1	Smp_125560	Acyl-CoA synthetase	
		Smp_209040.1	Not mentioned	Acyl-CoA synthetase	
		Smp_165850.1	Not mentioned	Acyl-CoA synthetase	
		Smp_244300	Smp_142680, Smp_039870	Acyl-CoA synthetase	
2. CPT-1	EC 2.3.1.21	Smp_146190	Not mentioned	Choline O-acetyltransferase	EC 2.3.1.6
3. CPT-2	EC 2.3.1.21				
4. Acyl-CoA dehydrogenase	EC 1.3.8.-	Smp_122930	Smp_122930	Acyl-CoA dehydrogenase family member 9	EC 1.3.99.-
5. Enoyl-CoA hydratase	EC 4.2.1.17	Smp_024930	Smp_024930	Enoyl-CoA hydratase domain-containing protein 3	–
6. 3-Hydroxyacyl-CoA dehydrogenase	EC 1.1.1.35	ng	Smp_151030	–	–
7. 3-Ketoacyl-CoA thiolase	EC 2.3.1.16	Smp_267310	Smp_197330	Acetyl-CoA acetyltransferase, cytosolic	EC 2.3.1.9

CPT, carnitine palmitoyl transferase; ng, no gene found with significant homology ($E > 10^{-20}$)

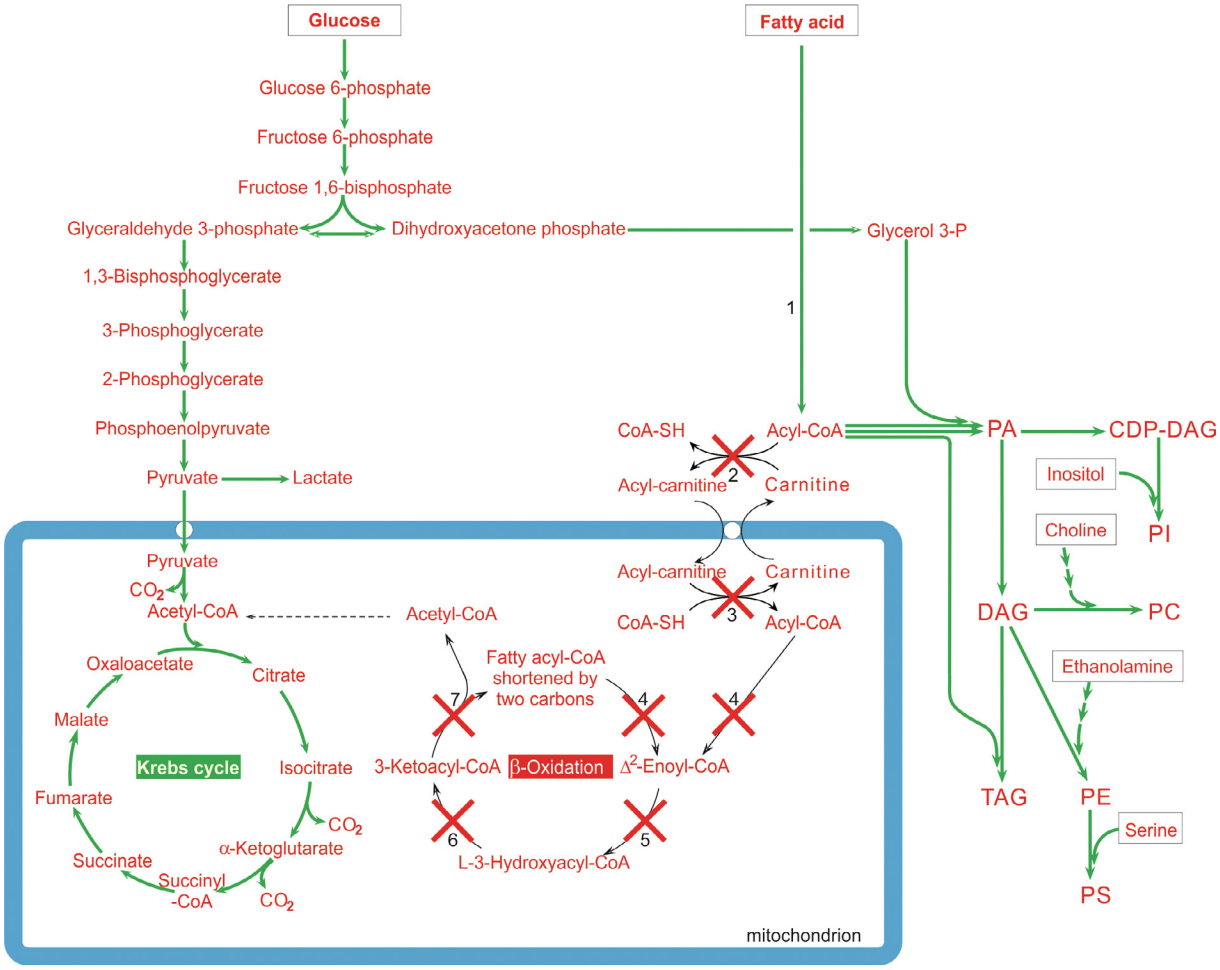

Fig. 4. Metabolic map of the glucose and fatty acid metabolism of *Schistosoma mansoni*. Shown are the glycolytic pathway, Krebs cycle, fatty acid β-oxidation and the anabolic processes of triacylglycerol (TAG) and phospholipid synthesis. Genes encoding the enzymes for glycolysis, Krebs cycle and the anabolic processes are present (thick green (dark grey) arrows), while genes encoding enzymes for fatty acid β-oxidation are absent in the genome of *S. mansoni* (thin black lines and red (black) crosses). CDP-DAG, cytidine diphosphate diacylglycerol; DAG, diacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidyl serine. Boxed substrates are supplied by the host. Enzymes: 1, Acyl-CoA synthetase; 2, Carnitine-palmitoyltransferase 1; 3, Carnitine-palmitoyltransferase 2; 4, Acyl-CoA dehydrogenase; 5, Enoyl-CoA hydratase; 6, 3-hydroxyacyl-CoA dehydrogenase; 7, 3-ketoacyl-CoA thiolase.

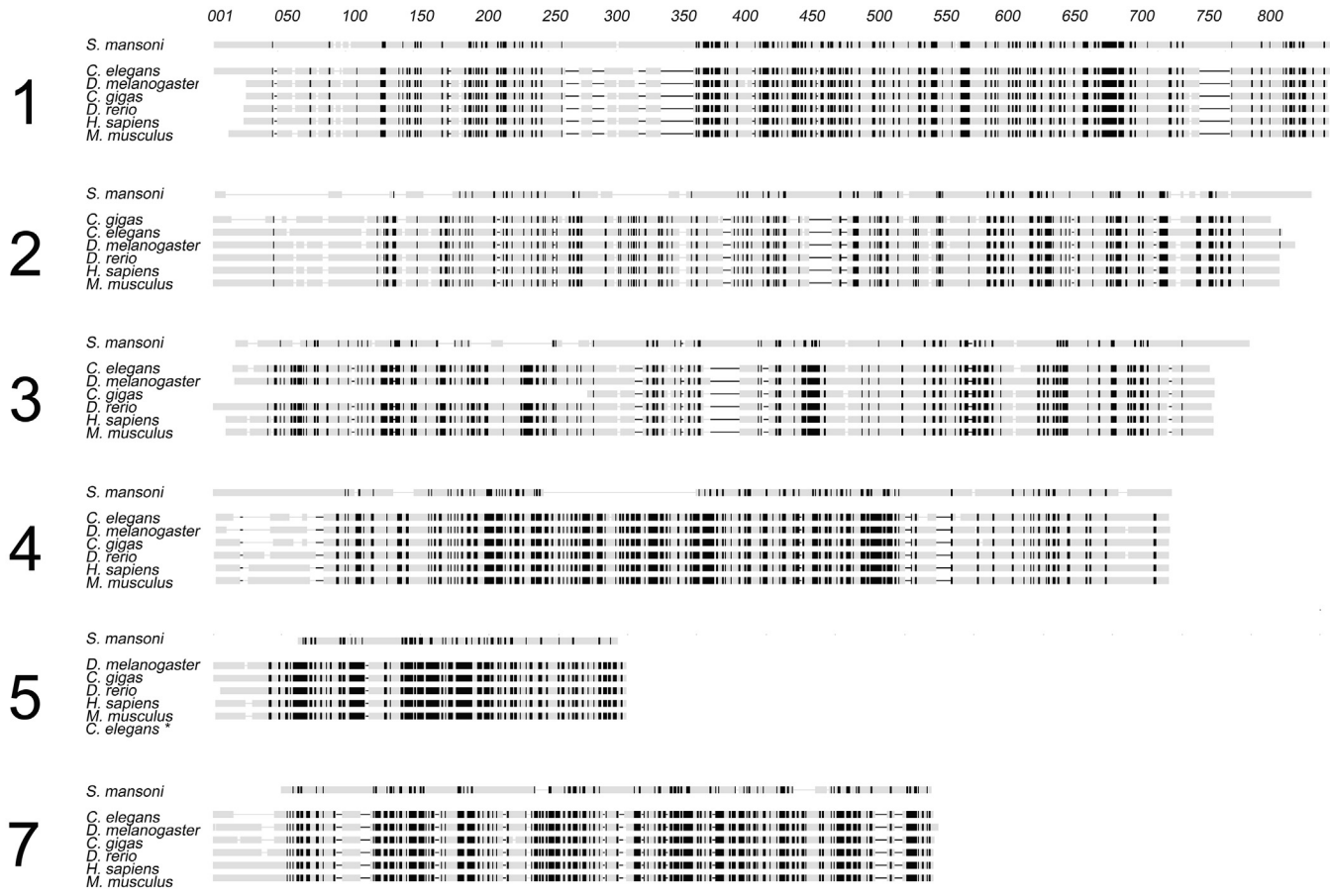

Fig. 5. Barcode alignment of *Schistosoma mansoni* proteins against proteins involved in fatty acid oxidation. Conserved residues (100% identity) present throughout all six model organisms (*Caenorhabditis elegans*, *Drosophila melanogaster*, *Crassostrea gigas*, *Danio rerio*, *Homo sapiens*, *Mus musculus*) have been marked as black bars after which it was analysed whether these residues were also identical in the most similar *S. mansoni* sequences. The resulting barcode shows conserved domains present or absent in *S. mansoni* proteins. The large numbers on the left correspond with the numbering used in Fig. 4 and Table 2. For enzyme number 6, 3-hydroxyacyl-CoA dehydrogenase, no protein with significant homology was detected within the *S. mansoni* genome. The complete alignment is shown in Supplementary Fig. S1. *No gene found with significant homology.

used in a BlastP search against the six model organisms to investigate whether the model organisms contain a protein with a higher similarity to the schistosomal protein than the protein used in the forward BlastP where only the enzymes involved in fatty acid oxidation were used as a query. This reverse BlastP search revealed that the presumed schistosomal acyl-CoA synthetases indeed show the best homology to the acyl-CoA synthetases of the model organisms (Table 2). However, this reversed BlastP analysis also revealed that the presumed homologs of three β-oxidation enzymes, and CPT-1 and CPT-2, were in fact coding for other proteins, or were at least more similar to those other proteins than to the enzymes involved in fatty acid oxidation (Table 2).

As a positive control, the same genomic analysis was performed for KEGG pathway 00010 (glycolysis and gluconeogenesis) and KEGG pathway 00020 (Citrate Cycle), pathways that are both present in *S. mansoni*. This analysis demonstrated that genes could be detected in the *S. mansoni* genome for all enzymes of both pathways. In addition, the identified *S. mansoni* genes were highly homologous to their corresponding genes in the six model organisms, as demonstrated by the low *E*-values ($E < 10^{-80}$, not shown).

All together, the results of this genomic analysis showed that the *S. mansoni* genome contains genes coding for acyl-CoA synthetases, which are enzymes that activate fatty acids by coupling those to coenzyme-A (Fig. 4, enzyme number 1). The resulting acyl-CoAs are the beginning of several anabolic pathways that are known to be present in *S. mansoni* (Fig. 4). The genome of *S. mansoni*, however, does not contain genes with a reasonable homology to genes encoding enzymes for fatty acid β-oxidation in the six model organisms. In addition, no genes were found for the two enzymes required for import of fatty acids into the mitochondrion: the cytosolic CPT-1 and mitochondrial CPT-2. This is in itself no longer surprising: an import system for fatty acids is superfluous when the mitochondria contain no enzymes for fatty acid β-oxidation. We also performed this analysis on the published whole genomes of *Schistosoma japonicum*, *Schistosoma haematobium*, *Schistosoma mattheei*, *Schistosoma rodhaini*, *Schistosoma curassoni* and *Schistosoma margrebowiei*, which led to the same result, i.e. none of those parasites possess enzymes for fatty acid β-oxidation (not shown).

4. Discussion

In this study we performed a comprehensive analysis of the lipid metabolism of *S. mansoni* with a special focus on the debated presence or absence of fatty acid β-oxidation in adult worms. Metabolic incubations with the [^{14}C]-labelled fatty acids octanoic acid (C8:0) and oleic acid (C18:1) were used to examine the metabolic fate of fatty acids taken up by adult worms and eggs plus miracidia. In addition to a physiologically relevant fatty acid (oleic acid), octanoic acid was studied as well, due to its high bio-availability as it is a medium-chain fatty acid that can pass through membranes

relatively easily and independent of carnitine shuttles (Schönfeld and Wojtczak, 2016). These experiments showed that fatty acids are taken up from the environment and are subsequently incorporated in complex lipids such as TAG and phospholipid species, which is an observation that is in agreement with multiple earlier publications (Meyer et al., 1970; Saz, 1970; Rumjanek and Simpson, 1980; Frayha and Smyth, 1983; Brouwers et al., 1997). Despite the high sensitivity of our assay in which radioactive fatty acids were used, we could not detect the production of carbon dioxide from fatty acids, neither by adult worms nor by eggs and miracidia. This showed that these *S. mansoni* stages do not oxidise fatty acids, which confirms an earlier study which demonstrated that miracidia consume their glycogen reserves but not their endogenous TAG stores (Tielens et al., 1991).

As our present study showed that fatty acids were not oxidised, we re-examined whether schistosomes possess the necessary enzymes to do so. In the first automated genome annotation in 2009, it was reported that the genes for the enzymes required for fatty acid β-oxidation are present in the genome of *S. mansoni* (Berriman et al., 2009). Our analysis to resolve the possible presence of genes coding for fatty acid β-oxidation enzymes in *S. mansoni* demonstrated that the genes coding for enzymes required for fatty acid β-oxidation are absent in the *S. mansoni* genome. This discrepancy with earlier results is caused by the inherent impreciseness of the automated annotation performed earlier. The earlier identified genes indeed encode enzymes related to lipid metabolism with some of the conserved domains present in enzymes required for fatty acid β-oxidation, but these conserved domains are also present in enzymes involved in other processes such as lipid binding and biosynthetic thiolase reactions. The presence of these shared domains explains why these proteins were earlier accidently annotated as being involved in β-oxidation of fatty acids (Berriman et al., 2009). During the review process of this manuscript, an analysis of all available genomes of helminths was published by the International Helminth Genomes Consortium. In that new analysis it is also concluded that schistosomes indeed lack genes of the enzymes necessary for β-oxidation of fatty acids (Coghlan et al., 2019). Genes coding for acyl-CoA synthetases, on the other hand, are present in the genome of *S. mansoni*, but these enzymes activate fatty acids by coupling those to coenzyme-A and the resulting acyl-CoAs are the beginning of several anabolic pathways that are known to be present in *S. mansoni* (Fig. 4).

As *S. mansoni* does not possess the enzymes necessary for fatty acid oxidation, how is it then possible that experiments seemed to indicate that fatty acid oxidation is essential for egg production by female *S. mansoni* (Huang et al., 2012)? That study used inhibitors and RNA interference (RNAi) to study fatty acid metabolism, while none of the experiments provides direct evidence for fatty acid oxidation, i.e. production of carbon dioxide from fatty acids. The first set of experiments showed that fecund female schistosomes use oxidative phosphorylation and that this process is essential for egg production. Oxidative phosphorylation by itself, however, is no indication for fatty acid oxidation. It has been shown earlier that in adult *S. mansoni* the degradation of glucose to carbon dioxide via Krebs cycle activity and oxidative phosphorylation produces at least one-third of the ATP produced by glucose breakdown, the remainder is produced during the production of lactate, the major end product which is excreted (Van Oordt et al., 1985). This explains why tampering with oxidative phosphorylation has a profound effect on energy consuming processes such as egg laying and vice versa: interference with energy consuming processes will affect oxidative phosphorylation. The second type of experiments was related to CPT1 (enzyme 2 in Fig. 4), an essential component of the pathway used for the import of fatty acids into mitochondria. This enzyme can be inhibited by etomoxir and it was observed that addition of this inhibitor resulted in a decrease in the oxygen consumption rate and in a decrease in the production of eggs in vitro (Huang et al., 2012). However, *S. mansoni* does not possess CPT1 (Figs. 4, 5; Supplementary Table S3) and etomoxir is known to inhibit not only CPT1 but also diacylglycerol acyltransferase (Xu et al., 2003), an anabolic enzyme catalysing the final reaction in the synthesis of TAG, a process known to occur in schistosomes (Fig. 4). The importance of this enzyme for female schistosomes in the process of egg laying is discussed further below. In the third type of experiments RNAi and an inhibitor were used to study the effect of a decrease in activity of two enzymes on the rate of oxygen consumption and egg laying. The observation that inhibition or RNAi of acyl-CoA synthetase (enzyme 1 in Fig. 4) influences egg laying and oxygen consumption by female *S. mansoni* is not surprising in view of the importance of anabolic processes that occur during this energy consuming process. A correlation between activity of acyl-CoA activity and egg laying is therefore no indication for fatty acid oxidation. The observed slight inhibition of egg laying after RNAi of acyl-CoA dehydrogenase (enzyme 4 in Fig. 4) is enigmatic as schistosomes do not possess a gene for that enzyme (Figs. 4, 5; Table 1; Supplementary Table S3). As a fourth line of evidence for the occurrence and importance of fatty acid oxidation in egg-laying females, the presence of large lipid reserves in the vitellarium of fecund females is mentioned, together with the observation that the decline of these lipid reserves correlates with egg laying and the rate of oxygen consumption. These observations are, however, no indication for fatty acid oxidation and are the result of the use of lipid by female schistosomes to stuff it into the eggs. The role of lipids in the process of egg laying by the female and also during the further development of the egg is discussed below.

As fatty acids do not function in schistosomes as substrate for β-oxidation and therefore have no role as fuel in ATP production, the question arises as to what their real function in *S. mansoni* is. In general, fatty acids are present in cells, mainly in two forms: they can be stored as TAGs and they are used as building blocks of phospholipids and glycolipids. In this respect it should be realised that female worms produce approximately 350 eggs per day (Cheever et al., 1994) and each egg contains fatty acids present in TAG and phospholipids (Fig. 2). Hence, the female worm not only requires energy and amino acids for egg production, it also requires substantial amounts of fatty acids, and therefore their daily uptake and digestion of red blood cells is approximately eight times higher than that of males (Lawrence, 1973; Cheever et al., 1994; Skelly et al., 2014). These ideas are in agreement with an earlier report of Newport and Weller (1982) who demonstrated that fatty acids are an absolute requirement for egg laying. The main lipid constituent of the immature egg is TAG (Fig. 2), which is synthesised from diacylglycerol by the enzyme diacylglycerol acyltransferase (Smp_158510). Expression of this gene was shown to be strongly (10×) upregulated in fecund females in a bi-sex infection, in comparison with virgin females in a single sex infection (Fitzpatrick et al., 2005; Anderson et al., 2015; Lu et al., 2017). By whole mount in situ hybridisation and RNA-seq it was shown that this enzyme is expressed exclusively in the vitellarium, which is the site of egg production (Wang and Collins, 2016). These results showed that female worms expel a large amount of fatty acids in lipids present in the excreted eggs and explains why fatty acids are essential for egg production. Fatty acids are essential components that, although they cannot be synthesised by the parasite itself, are used in anabolic processes. This explains why anabolic lipid metabolism in the female worm is crucial for egg production and why interference with it or with ATP generating processes such as oxidative phosphorylation will affect egg production (Huang et al., 2012).

As it was shown that fatty acids are essential for egg production and that interference with lipid metabolism affects egg production (Huang et al., 2012), we investigated the lipidome of eggs during

maturation. As mature and immature eggs can be separated by density (Ashton et al., 2001), it is likely that a change in lipid composition occurs during egg development. Microscopic evaluation of developing eggs revealed that immature eggs contain many large lipid droplets (Fig. 1), which confirms earlier observations (Neill et al., 1988). Mature eggs, on the other hand, have far less lipid droplets and instead more cellular membranes were stained (Fig. 1). The lipidomes of mature and immature eggs was then analysed by LCMS, which demonstrated that mature eggs contain significantly more phospholipids than immature eggs. On the other hand, mature eggs contained less neutral lipids, albeit not significantly so. These observed changes in lipid composition of eggs prompted us to postulate that TAG in lipid droplets in the immature egg serves as a fatty acid storage that is used during egg maturation for phospholipid biosynthesis, which is needed for the formation of new membranes in the dividing cells of the developing miracidium.

In addition to the increase in phospholipid content, the phospholipid composition changes during egg maturation. The lipidomic 'finger print' of immature eggs was shown to be different from that of mature eggs, as indicated by the distinct and non-overlapping score plots in the PCA (Fig. 3A). The corresponding PCA loadings plot (Fig. 3B) showed that the difference between mature and immature eggs was reflected in many different lipid species from all phospholipid classes. These results showed that lipid metabolism is important during egg maturation as the lipid composition is substantially adjusted during development.

In conclusion, our results show that *S. mansoni* adults take up fatty acids and incorporate those in phospholipids and neutral lipids such as TAG. Use of fatty acids by female schistosomes in these anabolic processes is crucial for egg production as the secreted eggs contain large amounts of fatty acids provided by the host. However, adult worms, eggs and miracidia do not oxidise fatty acids, and therefore, fatty acid catabolism does not occur and thus does not contribute to ATP production. Genome analysis showed that previously postulated genes for fatty acid β-oxidation were incorrectly annotated or attributed and that *S. mansoni* in fact lacks genes encoding the enzymes required for fatty acid β-oxidation. Hence, *S. mansoni* does not and cannot oxidise fatty acids (nor can *S. japonicum* or any other schistosome species that has been sequenced).

Acknowledgements

This research did not receive any specific grants from funding agencies in the public, commercial, or not-for-profit sectors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2019.03.005.

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