Supplementary Methods

Reporter and expression plasmids

The Atoh7 reporter plasmids were described in\textsuperscript{1}. The CMV-MitoDsRed2 (pDsRed2-Mito) and CMV-MitoGFP (pAcGFP1-Mito) plasmids were from Clonetech. Atoh7-MitoDsRed2 was designed using UGene software (Unipro). The GFP sequence was excised from Atoh7-GFP plasmid using AgeI and KpnI restriction enzymes. The mitoDsRed2 sequence from the CMV-MitoDsRed2 plasmid was amplified with PCR and extremities were digested with Xmal and KpnI restriction enzymes. Ligation was performed using Quick Ligation Kit (New England BioLabs).

Retina electroporation

Embryonic eyes were collected at E5 and the pigmented epithelium (RPE) was removed. Stripped eyes were electroporated in electroporation cuvettes (BT 640, BTX) with the reporter plasmid CMV-GFP, Atoh7-RFP, or CMV-MitoDsRed2 at 0.1µg/µl. Electroporation was performed in 100µl using 5 pulses of 5V and 50ms, separated by 1s interval with BTX ECM830 electroporator.

Tissue culture

Electroporated retinas were cultured in DMEM (ThermoFisher, 41965-039) complemented with 10% Fetal Bovine Serum (ThermoFisher) and 1% Penicillin-Streptomycin (ThermoFisher) for 8h, 24h, or 48h at 37°C in an incubator with 5% CO\textsubscript{2}.

Tissue dissociation

After 8h or 24h of tissue culture, electroporated retinas were washed with HBSS not containing Ca\textsuperscript{2+} or Mg\textsuperscript{2+} (ThermoFisher) and dissociated mechanically and enzymatically using Trypsin 0.05% (ThermoFisher) for 15-20min at 37°C. Trypsin enzyme activity was deactivated by addition of 10% Fetal Bovine Serum (ThermoFisher).

Fluorescence Activated Cell Sorting
After dissociation, cells were pelleted and resuspended in DMEM (Amimed). Cells were sorted by FACS Aria II for GFP positive cells, or with FACS Astrios for GFP and red fluorescent protein (RFP) positive cells. Cells were pelleted and resuspended at 1-2 x (10^6) cells / ml. 100-200μl was plated on a poly-dl-ornithine (Sigma-Aldrich) coated permanox chamber slide (Lab-Tek). Cells were left 30min at 37°C and 5% CO_2 for adhesion, then were fixed with 4% paraformaldehyde for 20min. Finally, after DPBS washing, DABCO was added and the slide was sealed with a coverslip for imaging.

**Confocal imaging**

Dissociated cells were plated on permanox chamber slides (Lab-Tek) coated with poly-dl-ornithine, left 30min for adhesion in a 37°C 5% CO_2 incubator, and then fixed for 20min with 4% paraformaldehyde. Coverslips were sealed after addition of DABCO (Sigma-Aldrich) with 50% glycerol (Sigma-Aldrich) in DPBS (Thermo-Fisher). Imaging was done with a Leica Sp5 Laser scanning Confocal microscope in photon counting mode using a Leica 20x multi-immersion objective (0.7 N.A.) in Leica type F-type immersion oil of refractive index 1.518. An Argon laser was used for 488nm GFP excitation and DPSS561 for 561nm RFP excitation. Optical sections of 1μm were acquired with a 2-4x line accumulation. Confocal images were processed with ImageJ/Fiji softwares for counting.

**Time-Lapse imaging**

Electroporated E5 retinas were flattened by removing the lens and vitreous body 24 hours following electroporation, and were placed on a 35-mm glass bottom dish (Pelco, Wilco Wells) where they were incorporated in collagen prepared as described in^2, allowing axon growth to be documented in the horizontal plane. The dish was incubated at 37°C to allow collagen polymerization. Culture medium composed of DMEM without phenol red, and complemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin was added on top of the collagen matrix. Retinas were imaged using a Leica Widefield AF6000LX inverted microscope with a Leica 40x dry long-distance objective (0.55 N.A.) for up to 72h in the Green and Red channel using Leica GFP and Rhodamine filter cubes. Stacks of images separated by 1μm for a total of 40-60microns were used with a time interval of 20min, 2min or 20s. Data were saved as *.lif file.

**RTqPCR**
RNA from FACS sorted cells or from whole retinas was extracted using TRIzol reagent (ThermoFisher) according to the product manual in triplicate. Primers were designed using NCBI primer blast and Primer3 websites and sequence stored in UGene software. Primers were ordered from Microsynth. Primers were tested using RNA from total retina extracted with TRIzol. RNA quantification was done with spectrophotometer and Qubit 2.0 (ThermoFisher) and RNA quality was checked using BioAnalyzer (Agilent). RT was performed using Takara PrimeScript reverse transcriptase prior to qPCR. Normalization was done using housekeeping genes, indicated in italic in Supplementary Table S2.

**mtDNA quantification by qPCR**

DNA of FACS sorted cells or dissected retinas was extracted according to the DNeasy Blood & Tissue DNA extraction kit (Qiagen). Primers were designed for targets on gDNA and mtDNA. qPCR was performed and gDNA was used for normalization. All the primers are described in Supplementary Table S1.

**In vivo proton (1H) magnetic resonance spectroscopy (1H-MRS)**

**In vivo measurement**

Hen eggs (n=6) and pigeon eggs (n=6) were individually scanned in ovo (in the egg) at embryonic stage E6, and then rescanned ex ovo (out of the egg) at embryonic stage E8 in a horizontal 14.1T/26cm Varian magnet (Agilent Inc., USA). Briefly, eggs were positioned on a holder and maintained at hatching temperature (37-37.5°C) during the scans using a circulating water heating system. A 17mm transmit-receive 1H quadrature surface coil fitting the shape of the egg was placed as close as possible to the chick embryo (i.e. on the top surface). T2-weighted fast spin-echo (FSE) images (Figures S3A and S3B) were used for localization and proton spectra were acquired with the SPECIAL sequence (TE/TR=2.8/4000ms) with OVS and VAPOR water suppression3,4. Shimming was performed using FAST(EST)MAP with the typical linewidth (full width at half maximum; FWHM) of water signal ranging between 5 and 10Hz depending on the size and position of the volume of interest (VOI), expressed in microliters. As 1H-MRS requires a minimal volume of tissue (1µl) for acquiring an adequate (i.e. above 8) signal-to-noise ratio (SNR), spectra were acquired from E6 onwards. The eyes of the embryo were identified by the recognizable lens that appears dark on T2-weighted images due to its oval shape and low water content (Supplementary Figures S1A and S1B). The scan duration, from minutes to hours, reflected VOI size selected for the full eye and vitreous as follows: vitreous (Chick E6: 4.5µl; E8: 20µl
and Pigeon E6: 1μl; E8: 16μl) and full eye (Chick E6: 60μl; E8: 160μl and Pigeon E6: 18μl; E8: 80μl). Scan time ranged between 20min for large voxels (full eye) and several hours for small voxels (vitreous) to reach adequate SNR. Embryos start showing spontaneous muscular contraction leading to intense movements of the whole body at embryonic stage E8. Given the high motion-sensitivity of localized ¹H-MRS, embryos were therefore scanned out of the egg, i.e. ex ovo at E8 by maintaining the organism alive in cold DPBS medium in a sealed bag. As the bird embryo’s respiration is based on oxygen diffusion in the egg, this setup provided a physiological mimetic of the embryonic environment which allowed maintaining the embryo alive for the 1h scan and prevented motion-induced artifacts. The use of the term “in vivo” herein refers to the intact embryo, regardless of whether it was inside or outside of the egg.

**In vivo spectra quantification**

¹H-MRS allows measuring non-invasively metabolite concentrations above ~1mM in avian eyes, both in ovo and ex ovo (Figures S3C and S3D). Spectra were corrected for B₀ drift and analyzed using the jMRUI (v.5.2) software package⁵. Metabolite spectra were quantified with QUEST routine of jMRUI using a simulated basis set including several metabolites as well as main lipid resonances. The water signal for each voxel were integrated using the Hankel Lanczos singular values decomposition (HLSVD) routine in JMRUI, normalized, and gain- and proton corrected. Absolute quantification of metabolites was performed using the water signal as an internal reference, assuming its concentration to be 99% in the vitreous and 80% in the full eye⁶,⁷. Upon quantification of the spectra, the Cramér-Rao lower bounds (CRLB) was determined to assess the reliability of the fitting process. SNR was calculated in the time domain with jMRUI after water residual removal. Spectra acquisition of whole eye in vivo led to an adequate SNR and fitting of lactate, as indicated by the following (CRLB/ SNR) values obtained: in chick, 7.9±1.6% / 27±7 (E6) and 21.8±8.5% / 57±7 (E8); in pigeon, 14.9±3.7% / 26±5 (E6) and 21.1±8.1% / 40±9 (E8)). The quantification of lactate in vitreous spectra was associated with a small fitting error as well (CRLB/ SNR: chick, 6.6±1.8% / 30±12 (E6) and 1.7±0.1% / 87±8 (E8); pigeon, 14.9±7.0% / 8±2 (E6) and 9.1±3.3% / 36±4 (E8)).

**In vivo pH measurement**

Citrate chemical shift (δ) - pH dependence was measured in phantoms on the 14.1 Tesla scanner by dissolving 5mM citrate and lactate buffered at several values of pH ranging from 6.0 to 7.5. After scaling
the spectra using lactate as internal reference, the center of the methylene resonances was measured and plotted against pH. The pH was calculated by fitting the resulting data with a second order polynomial function ($\delta=0.01587\cdot pH^2-0.227\cdot pH+3.4$).

**High resolution proton ($^1$H) nuclear magnetic resonance ($^1$H-NMR) spectroscopy**

**Metabolite extracts measurement**

Retina, vitreous and lens (n=5-7) were individually dissected from embryos (chick E6, E8 and E15; pigeon E6, E8, E12 and E15) and frozen in liquid nitrogen. Retinal pigment epithelium was carefully separated and removed from the retina to avoid potential contamination. Tissue samples were ground on a mortar using liquid nitrogen, weighed, and metabolites were subsequently extracted using the methanol/chloroform Folch-Pi extraction technique. Briefly, 400μl methanol was added on the powdered tissue, followed by subsequent addition of 400μl Millipore water and 400μl chloroform. The mixture was left for 30min at 4°C with stirring and centrifuged until phase separation (10min at 400rpm and 4°C). The aqueous phase containing hydrophilic metabolites was then lyophilized and dissolved in 1ml deuterium oxide and 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) as internal water-soluble reference at 0.1μM. The lipophilic (chloroform) phase was evaporated on ice using a nitrogen flow and dissolved in 1ml deuterated chloroform with tetramethyl silane (TMS) as internal chloroform-soluble reference. Fully relaxed $^1$H-NMR spectra were acquired using a DRX-600 spectrometer (Bruker BioSpin, Fällanden, Switzerland). Each sample lead to a 520-spectra scan with a pulse-acquired sequence (30° pulse) using a pulse delay of 5s and an acquisition time of 2.7s.

**Metabolite spectra quantification**

Spectra were referenced to the internal reference (DSS or TMS), and phase and baseline corrected manually using the MestReNova software. Selected metabolite peaks, with minimal adjacent resonance overlap, were integrated manually using MestReNova software. These peaks were proton corrected and normalized to the DSS methyl singlet at 0ppm and referenced to the tissue sample mass in amounts of metabolite extracted per mass of wet tissue [µmol/g]. The following resonances ($\delta$, in ppm) were considered (number of protons, spectral pattern): lactate $\delta$ 1.31 (1H, d), alanine $\delta$ 1.47 (3H, d), creatine $\delta$ 3.03 (3H, s), N-acetyl-aspartate $\delta$ 2.00 (1H, s), aspartate $\delta$ 2.80 (1H, dd) glutamate $\delta$ 2.33 (2H, m), GABA $\delta$ 2.28 (2H, t), glutamine $\delta$ 2.43 (2H, m), citrate $\delta$ 2.60 (2H, m), oxaloacetate $\delta$ 2.38 (2H, s), scyllo-inositol $\delta$ 3.33 (6H, s), myo-inositol $\delta$ 3.52 (2H, dd), taurine $\delta$ 3.42 (2H, t) and alpha glucose $\delta$ 5.22 (1H,
d) as 36% of total glucose (spectral patterns are described as: s: singlet; d: doublet; t: triplet; dd: doublet of doublet; m: multiplet). Lactate in the lens was quantified by normalizing the lactate signal to the NAA singlet peak (δ=2.00ppm); for the sake of comparison with the retina, the same quantification approach was also performed for retinal lactate. The water-soluble lipid peak at 1.28ppm was quantified by normalizing its height to the creatine (3.03ppm) peak height. Lipid spectra were quantified using the methyl terminal peak of fatty acyl chains (CH₃) δ 0.88 (3H, t) as reference. Due to shorter lipid T₂ relaxation times that lead to broader linewidths, the peak height rather than the integral was considered relevant for the selected measured resonances: Fatty acyl chain (CH₂)n δ 1.28 (2H, m), total cholesterol C18 δ 0.68 (3H, s), double unsaturated fatty acyl δ 2.80 (2H, m), phosphodiesters 4.00 (2H, d), glyceryl backbone of triglycerides δ 4.15/4.29 (2H, m), glyceryl backbone of triglycerides δ 5.20 (1H, m) and mono unsaturated fatty acyl δ 5.30 (2H, m). Each peak height was measured and normalized to the 0.88ppm peak after dividing the height by the number of protons.

Statistical analysis
Statistical analysis was performed using prism 5 software (Graph pad) and results are represented as mean±SEM, unless otherwise indicated. Two-group comparisons were analyzed with unpaired two-tailed Student’s t-tests. Comparisons of the in vivo groups at two embryonic stages were calculated with a paired two-tailed Student’s t-test. Two-group comparisons of in vivo pH measurements were analyzed with non-parametric Mann-Whitney test. More detail on statistical analysis is given in the associated figure legends. The proportion and standard deviation of cells positive for mitochondria fluorescence were calculated, and a Chi-squared test was used to compare two groups. Because the chi-squared test assumes independence of experimental units, we also confirmed our p-values by permutations of the population data and calculated the difference in mean between the two groups (10'000 permutations). From this, a normal distribution was derived and used to calculate the probability to observe a difference equally or more extreme than the experimental difference. In all cases, this additional test, which does not require independence of observations, yielded very similar p-values. The R script will be made available.
References


Supplementary figures

(A) Quantification of the 1.28 ppm peak marker of neuronal progenitor cells (NPC) at different embryonic stages in chick and pigeon. Signal of hydrophilic lipid from the aqueous phase of metabolite extraction at 1.28 ppm corresponds to methylene resonances. Peak height was normalized to the signal of total creatine (tCr; cellular marker) or NAA (neuronal marker) (*p<0.05; n.s., not significant; unpaired Student’s t-test, n=6-8 per group). (B) Typical high-resolution 1H-NMR spectra of retina extract used for metabolite quantification. Identified metabolites included: lactate, glucose, alanine, creatine, N-acetyl aspartate (NAA), choline containing compounds (CCC), myo-inositol, scyllo-inositol, taurine, acetate, ...
glutamate, aspartate, γ-aminobutyric acid (GABA). The NMR 0 ppm reference used was the methyl resonance of 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS). (C) Quantification of remaining metabolites extracted from the retina (n=6-8 per group). (D) Lactate content in the lens as compared to the retina in chick embryo at E6, E8 and E12 (*p<0.05; unpaired Student’s t-test; n=4-7 per group). (E) Quantification of remaining metabolites extracted from the vitreous (chick vs. pigeon, *p<0.05,**p<0.005; chick, §§p<0.05; pigeon, *p<0.05, ###p<0.0005; n.s., not significant; unpaired Student’s t-test; n=5-7 per group). All data are represented as mean±SEM.
Supplementary Figure S2: Lipid synthesis does not account for the vitreous citrate decrease at the onset of RGC maturation.

Lipidic profile of the retina changes both in chick and in pigeon during development. (A) Spectra of lipidic phase of retina metabolic extracts show marked cholesterol resonance (0.68ppm) increase in both chick and pigeon. Assignment of cholesterol (B, left) and other lipid (B, right) resonances used for quantification. (C) Quantification of lipid $^1$H-NMR resonances in the lipidic phase from dissected retina of chick ("p<0.05, **p<0.005; unpaired Student’s t-test; n=6-8 per group). (C) Quantification of lipid $^1$H-NMR resonances in the lipidic phase from dissected retina of pigeon ("p<0.05, **p<0.005; ***p<0.0005; unpaired Student’s t-test; n=5-6 per group). All data are presented as mean±SEM.
Supplementary Figure S3: In vivo MRI images of the embryo and quantification of remaining whole eye metabolites in vivo

(A) Full set of T2-weighted images of chick embryo in ovo at E3 and E6 as well as ex ovo at E8. (B) T2-weighted images of pigeon in ovo at E3, E6 and E8. (C) Fit of the 1H-MRS spectrum used for metabolite quantification in a spectrum of chick at embryonic stage E6. Typical basis set for the eye included alanine, aspartate, choline-containing compounds (CCC; phosphorylcholine, glycerophosphorylcholine and choline), citrate, creatine+phosphocreatine, glucose, glutamate, glutamine, lactate (Lac), myo-inositol, scyllo-inositol, N-acetylaspartate (NAA), taurine, as well as main lipid resonances. (D) Table with quantifications of in vivo 1H-MRS spectra (mean±SEM; unpaired Student’s t-test; *p<0.05; n=6-8 per group).

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Chick E6 [mM]</th>
<th>Chick E6 CRLB [%]</th>
<th>Pigeon E6 [mM]</th>
<th>Pigeon E6 CRLB [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>3.40±0.39</td>
<td>2.05±0.36</td>
<td>2.65±0.58</td>
<td>7.69±0.67</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.14±0.06</td>
<td>0.56±0.16</td>
<td>0.29±0.23</td>
<td>0.28±0.08</td>
</tr>
<tr>
<td>Citrate</td>
<td>1.16±0.22*</td>
<td>0.47±0.14</td>
<td>0.18±0.12</td>
<td>0.42±0.13</td>
</tr>
<tr>
<td>OAA/pyr</td>
<td>1.31±0.14</td>
<td>0.97±0.16</td>
<td>0.97±0.03</td>
<td>0.76±0.08</td>
</tr>
<tr>
<td>tCreatine</td>
<td>3.27±0.39*</td>
<td>0.47±0.14</td>
<td>7.53±0.17</td>
<td>0.42±0.13</td>
</tr>
<tr>
<td>NAA</td>
<td>3.40±0.39</td>
<td>0.97±0.16</td>
<td>0.42±0.13</td>
<td>0.42±0.13</td>
</tr>
<tr>
<td>Aspartate</td>
<td>0.68±0.11</td>
<td>1.00±0.13</td>
<td>0.97±0.22</td>
<td>0.67±0.31</td>
</tr>
<tr>
<td>Glutamate</td>
<td>1.87±0.23</td>
<td>1.41±0.16</td>
<td>3.85±0.12</td>
<td>3.85±0.12</td>
</tr>
<tr>
<td>Glutamine</td>
<td>0.28±0.09</td>
<td>0.11±0.04</td>
<td>0.13±0.03</td>
<td>0.13±0.03</td>
</tr>
<tr>
<td>CCC</td>
<td>1.37±0.38</td>
<td>1.84±0.44</td>
<td>0.83±0.30</td>
<td>4.71±0.05</td>
</tr>
<tr>
<td>Myo-Inos</td>
<td>0.84±0.19</td>
<td>0.97±0.30</td>
<td>0.97±0.12</td>
<td>0.97±0.12</td>
</tr>
<tr>
<td>Scyllo-Inos</td>
<td>1.47±0.34</td>
<td>0.83±0.30</td>
<td>0.97±0.12</td>
<td>0.97±0.12</td>
</tr>
<tr>
<td>Taurine</td>
<td>0.91±0.20</td>
<td>0.85±0.13</td>
<td>4.33±1.33</td>
<td>4.33±1.33</td>
</tr>
</tbody>
</table>
Supplementary Table S1 - List of primers for qPCR

Primers with the prefix “MT” target mt-DNA, and primers with the prefix “G” target genomic DNA.
### Supplementary Table S2 - List of primers for RTqPCR

Normalization genes are indicated in italic.