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1 **Re-oxygenation after anoxia induces brain cell death and memory loss in the**
2 **anoxia tolerant crucian carp**

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24 **Short title:** Brain damage in anoxic crucian carp

25 **Keywords:** *fish; caspase 3; cell proliferation; gene expression; learning;*

26 **Summary statement**

27 This study shows that anoxia-tolerant crucian carp sustain brain cell death during early re-
28 oxygenation, as well as impaired memory, though damage is transient and does not diminish
29 learning ability.

30 **Abstract**

31 Crucian carp (*Carassius carassius*) survives without oxygen for several months, but it is unknown
32 if they are able to protect themselves from the cell death normally caused by absence, and
33 particularly re-entrance, of oxygen. Here, we quantified cell death in brain tissue from crucian carp
34 exposed to anoxia and re-oxygenation using the terminal deoxy-nucleotidyl transferase dUTP nick-
35 end labelling (TUNEL) assay, and cell proliferation by immunohistochemical staining for
36 proliferating cell nuclear antigen (PCNA) as well as PCNA mRNA expression. We also measured
37 mRNA and protein expression of the apoptosis executer protease caspase 3, in laboratory-exposed
38 fish and fish caught in their natural habitat over the year. Finally, a behavioural experiment was
39 used to assess the ability to learn and remember how to navigate in a maze to find food, before and
40 after exposure to anoxia and re-oxygenation. The number of TUNEL-positive cells in the
41 telencephalon increased after one day of re-oxygenation following 7 days of anoxia, indicating
42 increased cell death. However, there were no consistent changes in whole-brain expression of
43 caspase 3 in either laboratory or naturally exposed fish, indicating that cell death might occur via
44 caspase-independent pathways or necrosis. Re-oxygenated crucian carp appeared to have lost the
45 memory of how to navigate in a maze (learnt prior to anoxia exposure), while the ability to learn
46 remained intact. PCNA mRNA was elevated after re-oxygenation indicating increased
47 neurogenesis. We conclude that anoxia tolerance involves not only protection from damage but also
48 repair after re-oxygenation.

49 **Introduction**

50 The ability of the crucian carp (*Carassius carassius*) to survive anoxia for several months, during
51 overwintering in ice-covered lakes of northern Europe, is well established (e.g. Vornanen et al.,
52 2009). This fish species has therefore been studied intensely, and its survival strategy has been
53 shown to include several elements. The crucian carp can maintain anaerobic adenosine triphosphate
54 (ATP) production for much longer periods than other vertebrates due to the build-up of
55 exceptionally large tissue glycogen stores, primarily in the liver, in the summer and fall (Vornanen
56 et al., 2009; 2011), and by entering a hypo-metabolic state during anoxia exposure (e.g. Nilsson,
57 1992; 2001). In addition, the crucian carp produces ethanol as the major end-product of glycolysis
58 (Johnston and Bernard, 1983). The release of ethanol to the water circumvents the accumulation of
59 anaerobic end-products, i.e. lactate and protons, which cause severe disturbances to acid-base
60 balance in animals deprived of oxygen, as observed in the closely related common carp (*Cyprinus*
61 *carpio*; Johnston and Bernard, 1983). Lastly, the heart of the crucian carp can maintain cardiac
62 output without any oxygen (Stecyk et al., 2004), making it possible for the fish to maintain blood
63 circulation and thereby activity (Nilsson, 2001; Nilsson and Lutz, 2004), while allowing for a
64 sufficient release of ethanol over the gills (Stecyk et al., 2004; Farrell and Stecyk, 2007), thus
65 preventing intoxication.

66 In most other vertebrates, brain ATP levels plummet within minutes of anoxia exposure,
67 leading to a stop in ion pumping, followed by a general depolarization of cells and loss of ion
68 homeostasis, eventually resulting in cell death through necrosis (premature cell death) or apoptosis
69 (programmed cell death) (Lipton, 1999). It has recently become clear that the traditional view of
70 necrosis as a single disorderly process is oversimplified, and the term 'regulated necrosis' has been
71 proposed to describe the increasing network of non-apoptotic yet controlled cell-death pathways
72 (e.g. Berghe et al., 2014; Linkermann and Green, 2014). Regardless, necrotic cells can have fatal
73 consequences for neighbouring tissues because they induce tissue inflammation (e.g. Proskuryakov
74 et al., 2003), whereas in apoptosis the tissue constituents are taken care of in an orderly fashion and
75 inflammation is avoided (see reviews by Elmore, 2007; Campo, 2009). Although apoptosis is a
76 controlled process, it can be just as devastating as necrosis because it can be caused by relatively
77 brief periods of anoxia and result in progressive cell death for days after an anoxic insult has ended
78 (Lutz et al., 2003). Apoptosis caused by hypoxia, anoxia, and other cellular stressors is primarily
79 initiated in the mitochondria through a process referred to as the intrinsic pathway (Elmore, 2007).
80 Specifically, the ATP deficit resulting from hypoxia and anoxia cause depolarization of the

81 mitochondrial membrane and Ca^{2+} overload, and subsequently, pores form in the inner and outer
82 mitochondrial membranes through which so-called death factors, such as cytochrome C and
83 apoptosis-inducing factor (AIF), are released. Many forms of apoptosis are dependent on a special
84 group of proteases (i.e. enzymes targeting other proteins) known as caspases, which are activated by
85 for example cytochrome C (Green and Reed, 1998; David et al., 2009). The executer caspases 3 and
86 9 have been directly linked to apoptosis in human cells induced by anoxia (Santore et al., 2002) and
87 hypoxia/re-oxygenation (Ho et al., 2006). Additionally, cell death can be induced shortly after re-
88 oxygenation as a result of the oxidative damage to proteins and DNA caused by an increased
89 production of reactive oxygen species (ROS). Indeed, increased ROS production and apoptosis, as
90 well as regulated necrosis, have been linked to hypoxia and ischemia-reperfusion injuries (Neumar,
91 2000; White et al., 2000; Zhao et al., 2015), for example in rat brain (Zhu et al., 2003) and human
92 (Nohl et al., 1993) and mice (Zhang et al., 2016) cardiomyocytes. If cell death occurs during
93 hypoxia/anoxia or following re-oxygenation, regardless of whether this is through apoptosis or
94 necrosis, it is characterised in the later stages by the degradation of DNA. This feature makes it
95 possible to detect cell death using the terminal deoxynucleotidyl transferase dUTP nick end
96 labelling (TUNEL) assay (Gavrieli et al., 1992; Ansari et al., 1993; Charriaut-Marlangue and Ben-
97 Ari, 1995; Grasl-Kraupp et al., 1995).

98 Contrary to mammals and most other vertebrates, the crucian carp is able to maintain
99 sufficient ATP levels during anoxia to meet the slightly lowered demand (Johansson and Nilsson,
100 1995; Nilsson, 2001). It has therefore generally been presumed that the fish avoids anoxia induced
101 cell death. While no studies have been conducted on crucian carp, there are studies suggesting that
102 some anoxia-tolerant freshwater turtles (*Trachemys* sp. and *Chrysemys* sp.) are actually able to
103 prevent cell death during anoxia and re-oxygenation (Kesaraju et al., 2009; Nayak et al., 2010;
104 Pamenter, 2014). These animals exhibit an extreme suppression of ATP demand through decreased
105 neuronal activity (‘spike arrest’; Perez-Pinzon et al., 1992; Fernandes et al., 1997), which is
106 mediated through increased GABAergic activity (Nilsson and Lutz, 1993; Pamenter et al., 2011), as
107 well channel arrest mediated through suppressed glutamatergic activity (Buck and Bickler, 1995;
108 1998; Pamenter et al., 2008). Furthermore, the increased ROS activity observed in mammalian
109 brain during re-oxygenation (Granger and Kvietys, 2015; Coimbra-Costa et al., 2017) is absent
110 from the turtle brain (Milton et al., 2007; Hogg et al., 2015). However, the crucian carp maintains
111 neuronal activity and hence brain energy consumption to a much larger extent than turtles (Lutz and
112 Nilsson, 1997). It can therefore not be taken for granted that it is equally able to protect itself from

113 apoptotic or even necrotic brain cell death, either during anoxia as a result of mitochondrial
114 depolarization due to the lack of a terminal electron receptor (i.e. oxygen), or during re-oxygenation
115 as a result of increased ROS production.

116 Consequently, the overall objective of the present study was to examine if anoxic and re-
117 oxygenated crucian carp suffer from cell death in the brain accompanied by detrimental effects at
118 the behavioural level, or if the fish protects its brain against cell death during exposure to anoxia
119 and re-oxygenation. To do so, we investigated three hypotheses:

120 1) Exposure to anoxia in the laboratory leads to increased cell death in the brain of crucian carp,
121 either during anoxia or during recovery in normoxia (re-oxygenation), or both, and that cell
122 proliferation is increased during re-oxygenation to counteract the increased cell death.

123 2) The hypothesised increase in cell death is apoptotic and involves caspase 3 activity. Hence, an
124 increased expression of caspase 3 is expected in the brain of crucian carp exposed to anoxia in the
125 laboratory and in the brain of wild crucian carp during seasonal exposure to anoxia and re-
126 oxygenation.

127 3) The brain damage hypothesized to occur with anoxia and/or re-oxygenation is reflected in
128 changes in behaviour, revealed as impaired memory or spatial learning ability.

129 **Methods and materials**

130 *Experiments*

131 The hypotheses were tested with three sets of experiments:

132 1) Cell death (identified by the TUNEL assay) and cell proliferation (identified by
133 immunohistochemical staining for proliferating cell nuclear antigen (PCNA) as well as
134 measurement of PCNA mRNA expression) were quantified in the telencephalon of crucian carp.
135 The telencephalon is the brain area proposed to contain the fish homologue of the mammalian
136 hippocampus - a key area for learning and memory (Rodríguez et al., 2002). The measurements
137 were done on crucian carp exposed to normoxia, anoxia or anoxia followed by re-oxygenation in
138 the laboratory

139 2) The mRNA and protein expression of the protease caspase 3 were measured in whole brain from
140 another set of crucian carp exposed to normoxia, anoxia or anoxia + re-oxygenation for the same

141 durations as in the first experiment. The mRNA and protein expression of caspase 3 was also
142 measured in whole brain of crucian carp sampled from a small lake at five different time points over
143 the year when oxygen levels in the lake cycled from normoxic to anoxic conditions (October,
144 December, May, June, September).

145 3) The effect of anoxia + re-oxygenation on the spatial learning and memory abilities of crucian
146 carp was assessed by a series of maze trials.

147 *Animals*

148 Crucian carp (*Carassius carassius* L. 1758) were obtained from the small lake Tjernsrudtjernet,
149 near Oslo, Norway (59°55'18.4"N, 10°36'32.9"E), and the fish studied in the laboratory were kept
150 in a 750 L holding tank (experiment 1 and 2) or multiple smaller glass aquaria (experiment 3) at the
151 aquarium facilities at the Department of Biosciences, University of Oslo. The holding tanks were
152 continuously supplied with aerated and de-chlorinated tap water (~2 L min^{-1}), the temperature of
153 which varied with season (9-12°C). Light was kept at a 12D:12L cycle. The fish were fed daily with
154 commercial carp food from at least two months and up to a year prior to experimentation to allow a
155 build-up of sufficient glycogen stores, but food was withheld 24 h prior to the tissue sampling
156 experiments and for three days prior to the commencement of the maze experiments. All
157 experiments were conducted on adult fish of both sexes.

158 In the laboratory, five separate sets of anoxia exposures were conducted for the present
159 study: one for immunohistochemical measurements (hypothesis 1, experiment 1), one for protein
160 and gene expression (hypotheses 1 and 2, experiments 1 and 2), and three for behavioural
161 measurements (hypothesis 3, experiment 3). Additionally, crucian carp were collected in
162 Tjernsrudtjernet on five different occasions (hypothesis 2, experiment 2): October 2010, December
163 2010, May 2011, June 2011, and September 2011. The anoxia-exposure experiments were approved
164 according to Norwegian animal research guidelines at an approved animal facility (Norwegian
165 Animal Research Authority, approval no. 155/2008). The sample size in the different groups in the
166 different experiments was kept to the minimum that allowed robust statistical comparison.

167 *General protocol for laboratory exposure to anoxia*

168 Two identical cylindrical dark tanks (25 L) were equipped with a flow-through of water and air
169 bubbling, one tank serving as the normoxic control tank and the other as the anoxia/re-oxygenation
170 tank. Approximately twenty-five fish were acclimated to the tanks with a flow-through of aerated

171 water for approximately 24 h. Only half of the fish were sampled from the normoxic tank, but
172 having the same fish density in each tank made the environment, except for the oxygen level, equal
173 for the two groups. The fish were not fed during the experiment. To induce anoxia, nitrogen gas
174 (AGA A/S, Oslo, Norway) was bubbled directly into the holding tank as well as into the incoming
175 water through a long column connected to the tank with gastight tubing. Oxygen level and
176 temperature were monitored daily in both tanks using a galvanic oxygen meter (Oxi3310, WTW,
177 Weilheim, Germany). In all three experiments, the oxygen level was above 95% air saturation in
178 the normoxic tank and below 0.1% air saturation in the anoxic tank, which was considered to be
179 anoxia (Nilsson, 1989; Stensløkken et al., 2014). Following the anoxic period, nitrogen bubbling
180 was replaced with air bubbling, resulting in re-oxygenation of the water and the fish. The normoxic
181 control group and the anoxia group were sampled after 7 days (N7 and A7, respectively). The
182 remaining fish in the anoxia group were sampled after 1 day of normoxia (A7R1) or 7 days of
183 normoxia (A7R7), following the initial 7 days of anoxia. Upon termination of the experiment the
184 fish were stunned with a blow to the head, followed by cutting of the spinal cord and removal of the
185 whole brain within one minute. The brain was frozen immediately in liquid nitrogen and stored at -
186 80°C until further use, unless otherwise indicated.

187 *Immunohistochemistry experiment and sampling (hypothesis 1, experiment 1)*
188 These experiments were conducted in January 2009. Crucian carp (N = 24, body mass (± s.d.) = 43
189 ± 11 g) were exposed to normoxia, anoxia, and re-oxygenation as described under *General protocol*
190 *for laboratory exposure to anoxia*. The fish were killed by decapitation, and the whole brain was
191 dissected out and drop-fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS).
192 After 24 h the brains were transferred to 20% sucrose solution for another 24 h, and finally placed
193 in 30% sucrose solution for 24 h. The brains were embedded in Tissue-Tek® O.C.T- medium
194 (Sakura Finetek, Inc., Torrance, CA, USA), and frozen in melting isopentane. The frozen brains
195 were stored at -80 °C, until sectioned at a thickness of 25 µm using a cryostat (Microm HM 560,
196 Thermo Scientific, ThermoFisher Scientific Inc., Waltham, MA, USA) and mounted on
197 SuperFrost™ Plus (Thermo Scientific) slides. The slides were air dried at room temperature for 48
198 h and kept at -80°C for storage.

199 *TUNEL assay*
200 Unless otherwise stated, all procedures were performed at room temperature. The slides were
201 thawed, washed in PBS (pH 7.4) for 5×5 min, post-fixed in paraformaldehyde (4%, 15 min), and

202 then washed (PBS, 3x5 min). Epitope retrieval was performed by incubation for 30 min in sodium
203 citrate (0.1 M, pH 6) containing 0.1% Triton X-100 (Sigma-Aldrich® Norway A/S, Oslo, Norway).
204 After incubation the sections were rinsed in PBS (3x5 min), and treated with 3% H_2O_2 (Sigma) to
205 inhibit peroxidase activity (10 min). After washing with PBS (3x5 min), 50 µL of terminal
206 deoxynucleotidyl transferase (TdT) reaction buffer (25 mM Tris-HCl, 200 mM sodium cacodylate,
207 0.25 mg mL^{-1} bovine serum albumin, 1 mM cobalt chloride) was added to each section, and they
208 were incubated for 10 min. Hereafter, the sections were incubated with 50 µl each of TdT reaction
209 mixture [1600 U mL-1 TdT and 3.6 µM biotin-16-dUTP (Roche Diagnostics) in TdT reaction
210 buffer] at 37°C in a humidity box for 1 hour. The reaction was stopped by washing with a stop
211 buffer (300 mM NaCl, 30 mM sodium citrate) followed by 3x5 min in PBS. The sections were then
212 incubated with 50 µl Streptavidin-HRP (streptavidin protein conjugated to horseradish peroxidase,
213 Thermo Scientific) for 20 min and washed 3x5 min in PBS. Finally, peroxidase activity was
214 visualized using DAB [3,3'-diaminobenzidine 0.01 M (PanReac Applichem, AppliChem GmbH,
215 Darmstadt, Germany) and 0.01% H_2O_2 in PBS, 7 min], washed 3×5 min with distilled water
216 (dH_2O), and cover-slipped using a permanent mounting medium. Positive controls were made by
217 incubating sections with 50 µL DNase I (20 U mL^{-1}, Invitrogen, ThermoFisher Scientific Inc.),
218 prior to the labelling step. Negative controls were made by omitting TdT from the reaction mixture.

219 *PCNA staining*

220 The slides were thawed, washed in PBS (5×5 min), and post-fixed in paraformaldehyde (4%, 10
221 min). Epitope retrieval was performed using a citric acid buffer (10 mM, pH 6.0, 85 °C, 60 min).
222 Slides were washed in PBS and unspecific binding blocked with 6% skim milk powder (Acumedic)
223 and 0.03% Triton X-100 (Sigma) in PBS. Sections were treated with primary antibody for 24 h at
224 4°C [1:50, Rabbit Anti-PCNA (Dako, Agilent Technologies Inc., Santa Clara, CA, USA) in PBS
225 with 0.6% skim milk powder and 0.03% Triton X-100] and washed 3×5 min with PBS. This
226 antibody has been shown to work for crucian carp in a previous study (Sollid et al., 2005).
227 Endogenous peroxidase activity was blocked with 3% H_2O_2 (Sigma, 15 min) and the sections were
228 then washed 3×5 min with PBS. Slides were incubated with secondary antibody for 30 min
229 (EnVision+® System Labelled Polymer-HRP, Anti-Rabbit, Dako) and washed 3×5 min with PBS.
230 Finally peroxidase activity was visualized using DAB [3,3'-diaminobenzidine 0.01 M (Applichem)
231 and 0.01% H_2O_2 in PBS] for 15 min, washed 2×5 min with dH_2O and cover-slipped using
232 permanent mounting medium.

233 *Image preparation and analysis*

234 For quantification of TUNEL- and PCNA-positive nuclei, an Olympus BX50WI microscope with a
235 ColorView camera (1288 x 966 pixels resolution), and Olympus Cell B software were used.
236 Pictures were taken at 20 x magnification and merged using Olympus Cell B and Photoshop CS3
237 software. Every fourth section (one 25 µm section per 100 µm) throughout the telencephalon was
238 analysed, and pre-optic areas were excluded when they appeared in the same sections. Rather than
239 analysing sections throughout the whole brain, the telencephalon was chosen because of its
240 proposed homology to the mammalian hippocampus. Only nuclei with intact nuclear membranes as
241 well as positive TUNEL staining were counted. The analysed volume was found from the area of
242 each section, determined using Photoshop CS3 software, and the section thickness (25 µm). The
243 number of TUNEL- and PCNA-positive cells per analysed tissue volume was calculated by
244 dividing the total number of stained nuclei for all sections counted in the telencephalon from each
245 fish by the total analysed volume for each fish.

246 *Gene and protein expression measurements (hypotheses 1 and 2, experiments 1 and 2)*

247 The experimental animals (N = 40, body mass (± s.d.) = 33 ± 13 g) for this experiment were caught
248 in Tjernsrudtjernet in August 2012 and the exposure conducted in September 2013 as described
249 under *General protocol for laboratory exposure to anoxia*. Brain tissue was also obtained from fish
250 of both sexes that had been sampled after capture at Tjernsrudtjernet (N = 40, body mass = 49 ± 11
251 g) on October 31, 2010 (n = 8), December 9, 2010 (n = 8), May 2, 2011 (n = 8), June 16, 2011 (n =
252 7), and September 14, 2011 (n = 8). Both temperature and oxygen concentration were measured in
253 the pond upon sampling (using a WTW Oxi3310 oxygen meter) and captured fish were
254 immediately transported to the University of Oslo in plastic bags containing pond water. Fish were
255 maintained in the bags until sampled (maximum 2 h after capture), during which time water
256 temperature and oxygen level were regulated to be consistent with the natural conditions. Low
257 temperature was maintained by placing the bags on ice, and hypoxic or anoxic conditions were
258 maintained by bubbling the water at an appropriate rate with N_2. Whole brain tissue was sampled as
259 described under *General protocol for laboratory exposure to anoxia*.

260 *Protein extraction, total RNA extraction, and cDNA synthesis*

261 Total RNA and total protein from the brain tissue was extracted with the PARIS™ Kit (Life
262 Technologies, Carlsbad, California, United States) according to the manufacturer's protocol, except
263 that 5% isoamyl alcohol (Merck Millipore, Billerica, Massachusetts, United States) was added to

264 the cell disruption buffer prior to homogenization, to reduce foaming. All samples were randomized
265 upon extraction. An external RNA control (20 pg mw2060 per mg of tissue) was added during RNA
266 isolation (Ellefsen et al., 2008). Due to the viscous condition of the solution, all samples were
267 drawn through a 27G syringe (BD, New Jersey, United States). Total RNA was DNase treated with
268 TURBO DNA-free™ Kit (Life Technologies, ThermoFisher Scientific Inc.) according to the
269 manufacturer's protocol and RNA quantity and quality were assessed using a NanoDrop 2000 UV-
270 Vis Spectrophotometer (Thermo Scientific) and 2100 Bioanalyzer (Agilent Technologies, Palo Alto,
271 CA, USA), respectively. RNA quality was determined from RNA integrity numbers (RINs)
272 calculated by the 2100 Bioanalyzer (range 1–10). The RIN for the tissue samples had a mean (±
273 s.e.m.) of 7.17±0.13, confirming good RNA quality. The cDNA was made from 260.7 ng RNA
274 (based on the lowest concentration) with Oligo(dT)$_{12-18}$ (Life Technologies) and SuperScript™ III
275 Reverse Transcriptase (Life Technologies).

276 *Partial cloning and sequencing of caspase 3*

277 Partial cloning was carried out to obtain the crucian carp mRNA sequence for caspase 3 genes. Two
278 paralogs were already identified in zebrafish, caspase 3A (casp3A) and 3B (casp3B), in the National
279 Center for Biotechnology Information (NCBI) database (Reference Sequence numbers
280 NM_131877.3 and NM_001048066.1, respectively). The proteins encoded by these sequences are
281 roughly the same size, with some very similar and some less similar areas (see Supplementary
282 Information Fig. S1). Partial cloning primers were designed using Primer3 (Rozen and Skaletsky,
283 2000) based on the casp3A and casp3B sequences from zebrafish as well as other sequences from
284 vertebrates available in the NCBI database. The primers (see Table 1A) were synthesized by Life
285 Technologies (Carlsbad, California, United States). The polymerase chain reaction (PCR)
286 (Eppendorf Mastercycler gradient, Eppendorf AG, Hamburg, Germany) was performed on cDNA
287 from crucian carp brains, using Platinum® Taq DNA Polymerase and dNTP mix (Life
288 Technologies) according to the manufacturer's protocol. The following PCR program was used: 1)
289 94°C for 10 min, 2) 94°C for 30 s, 3) 48°C for 1 min (if several fragments or a smear appeared in
290 the agarose gel, 55°C was utilized instead), 4) 72°C for 1 min (predicted fragment size less than 1
291 kb), step 2) – 4) was repeated 39 times, then followed by step 6) 72°C for 10 min, and step 7) hold
292 4°C. PCR products were run on a 1% agarose gel with ethidium bromide (Sigma), 10x BlueJuice™
293 Gel Loading Buffer (Life Technologies), and 1 kb+ DNA ladder (Life Technologies). Fragments
294 with the expected size were ligated into vectors using the pGEM®-T Easy Vector System I
295 (Promega, Fitchburg, Wisconsin, United States) and transformed in $CaCl_2$- competent *E. coli* cells

296 (produced from stock at the University of Oslo) by heat-shock treatment. The bacteria were grown
297 on lysogeny broth (LB) plates containing ampicillin and a mix of Isopropyl β-D-1-
298 thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal)
299 (Promega, Fitchburg, WI, United States). Colony PCR with M13F2 and M13R (Life Technologies)
300 primers and subsequent gel electrophoresis were performed to obtain sequences of the genes of
301 interest. The following PCR program was used: 1) 94°C 10 min, 2) 94°C 30 s, 3) 55°C 1 min, 4)
302 72°C 1 min. Step 2) to 4) was repeated 35 times, followed by step 6) 72°C 10 min, and step 7) hold
303 4°C. The fragments were purified with Illustra ExoProStar 1-Step (GE Healthcare Life Sciences,
304 Little Chalfont, United Kingdom), and sequenced with the M13F primer at the ABI-lab (the
305 Norwegian Sequencing Centre, Department of Biosciences, University of Oslo).

306 *Quantitative real-time PCR of casp3a, casp3b and PCNA*
307 Primers for quantitative real-time PCR (qPCR) were designed based on the sequences obtained
308 from partial cloning and sequencing of the three products found in crucian carp, i.e. casp3ai,
309 casp3aii and casp3b (NCBI GenBank accession numbers MF288604, MF288605, and MF288603,
310 respectively) using the Primer3 online utility (Koressaar and Remm, 2007; Untergasser et al.,
311 2012). The primer pairs were designed to bind at exon-intron boundaries and all primer pairs gave
312 only one melting curve with the LightCycler® 480 SYBR Green I Master (Roche, Basel,
313 Switzerland) detection. The qPCR products were subsequently cloned and sequenced as described
314 above to confirm that the correct sequences were amplified by the qPCR primers. The primer pair
315 used for casp3a picked up both paralogs obtained in the partial cloning experiment (casp3ai and
316 casp3aii). qPCR primers for the proliferation marker PCNA were obtained from Sandvik et al.
317 (2012). Primer pairs used in the qPCR assay are listed in Table 1B. The qPCR was performed using
318 the LightCycler® 480 Real-Time PCR System with LightCycler® 480 SYBR Green I Master
319 according to the manufacturer's protocol. All samples were randomized during the qPCR
320 experiment, and run in duplicate. The following qPCR program was used: 1) Pre-incubation (95°C
321 10 min, 2) three step amplification (95°C 10 s, 60°C 10 s, 72°C 10 s), 3) Melting (95°C 5 sec, 65°C
322 60 sec, 97°C 1 s), and 4) Cooling (1°C 10 s). Step 2) was repeated 42 times. The primer efficiency
323 was calculated with the LinReg software (version 1.0.0.0) (Ruijter et al., 2013), and an average
324 efficiency for each primer pair in each duplicate was utilized in the final calculations of the gene
325 expression levels. All data were normalized by using the relative expression ratio between mw2060
326 and the gene of interest using the second derivative maximum method to calculate the expression

327 level for each gene (Ellefsen et al., 2008), see Eqn. 1 where E is the priming efficiency and CP is
328 the crossing point.
329

330 $$\text{Expressed mRNA level} = \frac{E_{\text{Standard gene}}^{\;CP_{\text{Standard gene}}}}{E_{\text{Target gene}}^{\;CP_{\text{Target gene}}}} \qquad (1)$$

331 *Western blotting of caspase 3a*
332 Protein levels of casp3a were analysed in whole brain from both laboratory-exposed and wild-
333 caught crucian carp by Western blotting. Prior to the experiment, all protein lysates were
334 centrifuged at 12000×**g** for 10 minutes at 4°C, in order to obtain a rough cytoplasmic lysate. Based
335 on a high conservation in amino acid alignment (sequences obtained by cloning were translated to
336 amino acid sequences and aligned with zebrafish casp3A, see Supplementary Information Fig. S1),
337 a primary antibody against zebrafish casp3A p17 (Anti-Caspase-3a (p17) NT, Z-Fish™, cat. no.
338 55371, lot no. NA2301, AnaSpec Inc, Freemont, California, United States), which is situated on the
339 small subunit cleaved from casp3A during activation, was utilized. This antibody is thus expected to
340 bind both to the inactive pro-caspase (showing a band at 32 kDa) and to the cleaved, active form of
341 casp3A (showing a band at 17 kDa). The primary antibody was tested on a positive control,
342 consisting of apoptotic gill tissue from crucian carp exposed to high temperature (25-30°C; Sollid
343 and Nilsson, 2006). The positive control showed the expected band at 17 kDa (cleaved, active
344 caspase 3) and a weaker band at 58 kDa, which was more pronounced in the brain tissue, where
345 both the 17kDa and the 32 kDa (non-cleaved, inactive caspase 3) bands were visible (See
346 Supplementary Information Fig. S2).
347 The protein samples were prepared with 2X Laemmli sample buffer, and added on a
348 NuPAGE® Novex® 4-12% Bis-Tris Protein Gel (Life Technologies). The gels were run with XT
349 MES Running Buffer (Bio-Rad Laboratories, Hercules, California, United States) at 200 V for 35
350 minutes. NuPage® Antioxidant (Life Technologies) was added to the running buffer in the inner
351 chamber to keep the proteins in a reduced state. Proteins separated on the gels were transferred to a
352 0.45 µm polyvinylidene difluoride (PVDF) membrane (Life Technologies), and blotted with 1X
353 transfer buffer at 150 V. The membranes were blocked in a 5% skim milk powder solution
354 dissolved in tris-buffered saline and Tween 20 (TBST). Blots were incubated with a 1:1000 dilution
355 of primary antibody and a 1:10.000 dilution of the secondary antibody [goat anti-rabbit IgG HRP –
356 conjugated (AnaSpec)]. Amersham ECL Prime Western Blotting Detection Reagent (GE
357 Healthcare Life Sciences) was added prior to imaging with Kodak 4000r Pro image station (Kodak,

358 Rochester, New York, United States). The blots were then stained with coomassie brilliant blue
359 (PhastGEL™ BlueR, GE Healthcare Life Sciences) for quantification of total loaded protein.
360 Calculations were performed using the ImageQuant software (version TL 8.1, GE Healthcare Life
361 Sciences). Animals from one of the experimental groups (A7, A7R1, or A7R7 and Oct 2010, Dec
362 2010, May 2011, or Sept 2011) and animals from the control group (N7 or June 2011), were
363 analysed on the same blot in order to compare quantification between different blots. The intensity
364 of each band of interest was normalized to total loaded protein within the same lane (see Eqn. 2),
365 and then normalized to an average of all control ratios for the target protein within the same blot.
366 Only the 17 kDa bands, representing the cleaved and active form of casp3a, were quantified and
367 used for further analysis.

368 $$\text{Normalized protein expression} = \frac{\left(\frac{\text{Band intensity of target protein}}{\text{Intensity of total protein within the same gel}}\right)}{\text{Average ratio for target protein across lanes within the same gel}} \qquad (2)$$

369 *Memory and spatial learning experiments (hypothesis 3, experiment 3)*
370 The experimental animals (N = 53, body mass (± s.d.) = 33 ± 12 g) utilized for these experiments
371 were caught in Tjernsrudtjernet in the fall of 2010 and summer of 2011. Fish were maintained as
372 described above (See *Animals*) at water temperatures that followed the season. Experiments were
373 performed from February to September of 2011, at which time the water temperature ranged
374 between 11°C and 12°C.
375 The effects of anoxia and subsequent re-oxygenation on the ability of the crucian carp to
376 recall a previously learned task, as well as on the spatial learning ability of the fish, were assessed
377 using a maze system based on that of Girvan and Braithwaite (2000) and Sloman (2010). Three
378 mazes were constructed by horizontally dividing a rectangular polyvinyl chloride (PVC) box into
379 four equally sized areas (see Supplementary Information Fig. S3A). Each partition contained two
380 openings; one through which the fish could progress to the next partition and one that led to a dead
381 end. Two separate experiments (schematically represented in Supplementary Information Fig. S3B
382 and 3C and detailed below) were conducted to assess if crucian carp show impaired memory and/or
383 impaired learning after anoxia + re-oxygenation. Preliminary experiments revealed that fish that
384 first experienced the maze individually were hesitant to explore the set-up and unlikely to
385 successfully navigate the maze, even if provided with numerous opportunities to explore the set-up.
386 Thus, both the memory and learning experiments included initial group training sessions, where
387 groups of three (learning experiment) or six (memory experiment) fish were placed together to

388 explore the maze (with the presence of the food reward) for one hour a day for five consecutive
389 days prior to conducting individual trials. During each individual trial, a food reward was placed in
390 the end of the last compartment of the maze, and the fish placed in the first compartment. After a
391 one hour habituation period in the start section (which was closed off from the rest of the maze by a
392 removable screen), the partition was opened and the fish was allowed to explore the maze. Two
393 parameters were measured during each individual trial: 1) the time it took the fish to pass through
394 the maze and reach the food item; and 2) the number of times the fish made a mistake by going
395 through an opening that led to a dead end or turning around and going back through an opening that
396 it had already passed through. Fish were returned to their respective tanks between trials and food
397 was withheld during the period of time that fish partook in the group training and individual trials.

398 *Protocol for the memory experiment*

399 For the experiment designed to investigate how exposure to anoxia and re-oxygenation affected
400 memory of a previously learned behaviour, fish were deprived of food for three days, subjected to
401 group training sessions for five consecutive days, allowed a two day break from the maze and then
402 subjected to five individual trials that occurred every other day over 10 days (see Supplementary
403 Information Fig. S3B). Fish were then exposed to anoxia or control normoxia, as detailed under
404 *General protocol for laboratory exposure to anoxia*, and returned to their respective tanks. Twenty-
405 four hours after the conclusion of anoxia or control normoxia exposure, each fish was tested once in
406 the maze, and the post-exposure time to find food and number of errors were recorded. Fish that
407 failed to find the food in three out of the five individual trials (two out of 9 fish in each group) were
408 excluded from analysis. Since we found most TUNEL-positive cells following one day of re-
409 oxygenation in the previous experiment, fish in this experiment were exposed to five days of anoxia
410 followed by one day of re-oxygenation (anoxia-group, final sample size of n = 7) or five days of
411 control normoxia followed by an additional day of normoxia (normoxia-group, final sample size of
412 n = 7).

413 *Protocol for the learning experiment*

414 For the experiment designed to investigate how exposure to anoxia and re-oxygenation affected
415 learning ability, 35 fish were deprived of food for 24 h, exposed to five days of anoxia or five days
416 of control normoxia, as detailed under *General protocol for laboratory exposure to anoxia*,
417 subjected to group training sessions for five consecutive days (control normoxic and anoxia-
418 exposed fish were not mixed in group training), allowed a two day break from the maze and then

419 subjected to five individual trials that occurred every other day over 10 days (see Supplementary
420 Information Fig. S3C). Anoxia-exposed fish in this experiment were thus exposed to a total of 18
421 days of re-oxygenation and the learning trials occurred from day 8 to 18 of re-oxygenation. As in
422 the memory experiment, fish that failed to successfully navigate the maze in three out of the five
423 individual trials (10 out of 18 normoxic fish and 8 out of 17 anoxic fish) were excluded from
424 analysis.

425 *Assessment of feeding activity after exposure to anoxia*
426 Since the memory and learning experiments relied on food as the incentive for successfully
427 navigating the maze, observations were conducted to assess feeding activity of the anoxia- and
428 normoxia-exposed fish. Crucian carp acclimated to 11-12°C (N = 22, body mass (± s.d.) = 32 ± 7 g)
429 were exposed to anoxia (n = 10, five groups) or control normoxia (n = 12, six groups) for 5 days as
430 detailed under *General protocol for laboratory exposure to anoxia*. Twenty-four hours post-
431 exposure, groups of two normoxia- or two anoxia-exposed fish were randomly placed into one of
432 the separated partitions of the three mazes. After a 1 h habituation period, four food pellets were
433 added to each of the four partitions and the feeding activity of the fish was monitored for 1 h. The
434 feeding trials were repeated every second day over 10 days for a total of five trials and fish were not
435 fed outside of the trials during this period. Feeding activity was quantified as the percentage of fish
436 feeding (number of groups of fish feeding divided by the total number of groups) and the
437 percentage of pellets consumed over the 1 h period (number of pellets consumed divided by the
438 total number of pellets).

439 *Statistics*
440 All data were analysed and figures prepared using Prism v7.02 (GraphPad Software, Inc., La Jolla,
441 CA, USA). The count data for TUNEL- and PCNA-stained cells, as well as the number of
442 navigational errors counted in both of the behavioural experiments, were square root transformed
443 before statistical analysis to obtain normality and variance homogeneity. In other cases of non-
444 normality or variance non-homogeneity data were log10 transformed to fulfil the assumptions of the
445 analysis of variance (ANOVA) (PCNA and casp3a mRNA expression from laboratory-exposed
446 fish, casp3a mRNA and protein expression from wild-caught crucian carp). The TUNEL- and
447 PCNA-positive cell counts as well as mRNA and protein expression from both laboratory-exposed
448 and wild-caught fish were analysed using a one-way ANOVA. All one-way ANOVAs with
449 significant effects were followed by Tukey's multiple comparison tests. A two-way ANOVA

450 without repeated measures (because not all fish completed all five trials) was used to analyse the
451 effect of trial number and treatment group on the time taken to find food and the number of
452 navigational errors, before anoxia exposure in the memory experiment and after anoxia exposure in
453 the learning experiment. A two-way ANOVA with repeated measures was used to compare
454 treatment groups pre- and post-exposure. The two-way ANOVAs were followed by Sidak's
455 multiple comparisons. P-values lower than 0.05 were considered significant.

456 **Results**

457 *Cell death and cell proliferation*

458 There was a pronounced effect of treatment on the number of TUNEL-positive cells in the
459 telencephalon of crucian carp (Fig. 1A; one-way ANOVA, $F_{(3, 20)} = 27.35$, $P < 0.0001$; see also
460 Supplementary Information Fig. S4). The number of TUNEL-positive cells was higher in anoxia-
461 exposed fish that were re-oxygenated for 24 h (A7R1) compared to the control normoxic fish (N7)
462 (Tukey's multiple comparison, $P < 0.0001$), the anoxia-exposed, but not re-oxygenated fish (A7) (P
463 < 0.001), and the fish exposed to anoxia and seven days of re-oxygenation (A7R7) ($P < 0.0001$).
464 However, the increase in the number of TUNEL-positive cells was transient, as it was not
465 significantly different from the normoxic control (N7) after 7 days of re-oxygenation (A7R7) (P =
466 0.1278). The number of cells stained positive for PCNA did not differ significantly between the
467 treatment groups (Fig. 1B; one-way ANOVA, $F_{(3, 20)} = 0.4552$, $P = 0.7166$). The mRNA expression
468 of PCNA, however, was significantly affected by treatment (Fig. 1C; one-way ANOVA, $F_{(3, 35)}$ =
469 4.753, $P = 0.007$), and there was a higher PCNA mRNA expression in A7R7 compared to N7
470 (Tukey's multiple comparison, $P = 0.0062$) and A7R1 ($P = 0.0375$).

471 *Caspase 3 expression in laboratory-exposed and wild-caught crucian carp*

472 Western blotting revealed a weak whole brain expression of the cleaved (active) form of casp3a
473 (17kDa) (Supplementary Information Fig. S5A), which was only marginally affected by treatment
474 (Fig. 2A; one-way ANOVA, $F_{(3, 32)} = 3.251$, $P = 0.0345$), being lower in A7R7 compared to A7R1
475 (Tukey's multiple comparison, $P = 0.044$). The mRNA expression of casp3a, however, was
476 significantly affected by treatment (Fig. 2B; one-way ANOVA, $F_{(3, 36)} = 4.037$, $P = 0.0142$).
477 Contrary to protein expression, the average mRNA expression was lowest during A7R1, and
478 highest in N7 and A7R7, though the differences were only significant between A7R7 and A7R1 (P
479 = 0.0076). The overall mRNA expression of casp3b was two orders of magnitude lower than the
480 casp3a mRNA expression, but was similarly affected by treatment (Fig. 2C; one-way ANOVA, $F_{(3,}$

481 $_{36)}$ = 6.003, P = 0.002). This was due to the A7R7 having a higher expression than N7 (Tukey's
482 multiple comparison, P = 0.004), A7 (P = 0.0073) and A7R1 (P = 0.0295).
483 Oxygen levels and temperature in Tjernsrudtjernet on the dates crucian carp were captured
484 showed a seasonal pattern, being highest in June, decreasing slightly in September, and reaching the
485 lowest levels in October and December, and increasing again in May (Fig. 3A). In the summer,
486 temperature was 13-18°C and daytime oxygen was 120-170% air saturation, suggesting a very high
487 photosynthetic activity. During the winter, temperature was below 4°C and oxygen close to 0% air
488 saturation in December. There was a significant effect of sampling month on the casp3a mRNA
489 expression (Fig. 3B; one-way ANOVA, $F_{(4, 34)} = 23.76$, $P < 0.0001$). Expression was much lower in
490 October and December than in May, June and September (Tukey's multiple comparison, $P < 0.005$
491 for all). As observed for the laboratory exposed fish, the expression of the cleaved casp3a (17kDa)
492 was weak (Supplementary Information Fig. S5B). The protein expression of cleaved casp3a was,
493 nonetheless marginally affected by sampling month (Fig. 3C; one-way ANOVA, $F_{(4, 33)} = 2.701$, P =
494 0.0474), due to slightly higher expression in May compared to October (Tukey's multiple
495 comparisons test, P = 0.0479).

496 *Effect of anoxia on memory and learning*

497 In the memory experiment, both treatment groups were equally able to learn to find food in the
498 maze pre-exposure (Fig. 4A, B). There was no interaction between the individual trial number and
499 treatment group (two-way ANOVA; $F_{(4, 54)} = 0.727$, P = 0.5774), no overall treatment effect ($F_{(1, 54)}$
500 = 1.098, P = 0.2993), and the time to find food (Fig. 4A) decreased progressively with individual
501 trial number ($F_{(4, 54)} = 3.531$, P = 0.0124). Specifically, the time to successfully navigate the maze
502 was shorter in individual trials 3, 4 and 5 compared to individual trial 1 (main effect of individual
503 trial, Tukey's multiple comparisons test; $P < 0.05$ for all). The number of navigational errors (Fig.
504 4B) also appeared to decrease with individual trial number for both treatment groups before
505 exposure, but there was no statistically significant effect of trial number (two-way ANOVA, $F_{(4, 60)}$
506 = 2.185, P = 0.0815), no interaction ($F_{(4, 60)} = 2.077$, P = 0.0951) and no effect of treatment group
507 ($F_{(1, 60)} = 0.5584$, P = 0.4578).
508 When comparing the pre- and post-exposure time taken to successfully navigate the maze
509 and find food between the normoxia- and anoxia-group (Fig. 4C), there was no statistically
510 significant effect of treatment group (two-way ANOVA with repeated measures, $F_{(1, 12)} = 3.336$, P =
511 0.0928), time ($F_{(1, 12)} = 0.8525$, P = 0.374) or interaction ($F_{(1, 12)} = 2.091$, P = 0.1738). Specifically,
512 there was no difference between the two treatment groups pre-exposure (Sidak's multiple

513 comparison, P = 0.9548), or between pre- and post-exposure for the control normoxic fish (P =
514 0.9206). However, the time to find food tended to be longer for the anoxia group post-exposure
515 compared to pre-exposure (P = 0.2251) and compared to the normoxic group post-exposure (P =
516 0.0581), though this effect was likely driven by two individuals performing particularly poorly. On
517 the other hand, when comparing the number of navigational errors before and after exposure (Fig.
518 4D), there was a significant interaction between time and treatment (two-way ANOVA with
519 repeated measures, $F_{(1, 12)}$ = 8.441, P = 0.0132), and an overall significant effect of treatment group
520 ($F_{(1, 12)}$ = 12.03, P = 0.0046) and time ($F_{(1, 12)}$ = 7.384, P = 0.0187). The number of errors was
521 significantly higher in the anoxia group post-exposure compared to pre-exposure (Sidak's multiple
522 comparison, P = 0.0037) and compared to the control normoxic fish (P = 0.0003). There was no
523 difference in number of errors between the pre- and post-exposure trial within the normoxia-group
524 (P = 0.9893), or when comparing anoxia- and normoxia-group pre-exposure (P = 0.9975).

525 The ability to learn to find food in the maze after exposure to anoxia and re-oxygenation
526 (learning experiment) showed a similar pattern as observed before exposure (Fig. 5A). That is, there
527 was a significant effect of individual trial number (two-way ANOVA, $F_{(4, 66)}$ = 6.323, P = 0.0002),
528 but no effect of treatment group ($F_{(1, 66)}$ = 0.6773, P = 0.4135) or any interaction ($F_{(4, 66)}$ = 0.7865, P
529 = 0.5381). The time to find food was significantly higher in individual trial 1 compared to
530 individual trial 3 (main effect of individual trial number, Tukey's multiple comparison test; P =
531 0.0238), 4 (P = 0.0015), and 5 (P = 0.0012). The number of navigational errors (Fig. 5B) also
532 tended to decrease with trial number (two-way ANOVA, $F_{(4, 80)}$ = 2.683, P = 0.0373), though
533 multiple comparison tests failed to identify specific differences. There was no interaction ($F_{(4, 80)}$ =
534 0.4066, P = 0.8034) and no effect of treatment group ($F_{(1, 80)}$ = 0.2823, P = 0.5967).

535 Importantly, both normoxia- and anoxia-exposed fish were feeding after the exposure
536 experiments, though feeding activity was slightly lower in post-anoxia fish (Supplementary
537 Information Fig. S6). At the first day of re-oxygenation, feeding was observed in all groups of
538 normoxic fish and all the food was consumed, while 60% of the post-anoxic groups showed feeding
539 activity and consumed 40% of the food.

540 Discussion

541 Even if crucian carp are extraordinary in their ability to survive without oxygen for extended
542 periods of time, and maintain brain function when anoxic, we here show that they do indeed suffer
543 from moderate brain damage when exposed to anoxia and re-oxygenation. We found that while the
544 number of cells stained positive for TUNEL in the telencephalon of crucian carp did not increase

545 with anoxia *per se*, the number of TUNEL-positive cells was increased by approximately three-fold
546 with 24 h of re-oxygenation. The increased cell death was, however, not clearly associated with an
547 up-regulation of the pro-apoptotic protease caspase 3. The protein expression of active caspase 3
548 was, nonetheless, elevated in some wild-caught individuals in May when oxygen levels were close
549 to normoxic after several months of anoxia, and there seemed to be a marked effect of temperature
550 on overall mRNA expression. At the whole-animal level we found that fish exposed to 7 days of
551 anoxia and then 24 h of re-oxygenation exhibited impaired memory and reduced navigational
552 accuracy in a maze with a food reward. However, the increase in cell death and associated
553 behavioural impairment was transient. After one week of re-oxygenation after anoxia exposure
554 there was no longer any sign of increased cell death, and the number of proliferating cells was
555 maintained while the PCNA mRNA expression was elevated. Moreover, the spatial learning ability
556 was not impaired. Fish that had been exposed to anoxia learned to navigate a maze to a food reward
557 at a similar rate to control-normoxic fish. Combined, the results indicate that the crucian carp is able
558 to repair any neurological damage sustained during anoxia and re-oxygenation.

559 *Brain damage*

560 We hypothesized that exposure to anoxia leads to increased cell death either during anoxia or
561 during the following re-oxygenation. The fact that the number of dying brain cells in the
562 telencephalon was highest during the first day of re-oxygenation indicates that the cell death we
563 observed was more likely to have been induced by ROS, and not the lack of oxygen itself. It has
564 previously been shown that the production of ROS increases during hypoxia and re-oxygenation,
565 due to leakage from the electron transport chain, and that such an increase in ROS can induce cell
566 death (Simon et al., 2000). Similar to the pattern observed in the present study, increased ROS and
567 cell death has been shown to peak after 24 h of re-oxygenation in the rat brain (Coimbra-Costa et
568 al., 2017). Intriguingly, in the present study there was considerable inter-individual variation in the
569 number of TUNEL-positive cells, and some individuals appeared to sustain more brain damage than
570 others. A similar variability was seen in the anoxia + re-oxygenation group in the memory
571 experiment, and even in the wild-caught fish. The indication that crucian carp experience an
572 increase in brain cell death that may be caused by increased ROS production contrasts with
573 observations from anoxia tolerant freshwater turtles, where ROS production is suppressed during
574 anoxia, and return to normal during re-oxygenation (Pamenter et al., 2007). Furthermore, no
575 indications of increased apoptotic activity have been observed in the turtle brain (Milton et al.,
576 2007; Kesaraju et al., 2009; Larson et al., 2014). While crucian carp and freshwater turtles are often

577 grouped together as prime examples of extreme anoxia tolerance, it is important to remember that
578 they utilise markedly different strategies to achieve it. Freshwater turtles are close to comatose, and
579 the spike and channel arrest and suppression of ATP demand likely aids in the protection of the
580 brain. On the contrary, the crucian brain remains functional and active, and it may therefore not as
581 easily protect itself against tissue damage.

582 While there was a clear effect of anoxia and re-oxygenation on the prevalence of TUNEL-
583 positive and hence dying cells, the effect of anoxia and re-oxygenation on caspase 3, a common
584 executer enzyme in apoptosis, was less pronounced. The increase in whole brain expression of
585 caspase 3 mRNA was not concomitant with the increase in number of TUNEL-positive cells at 24 h
586 of re-oxygenation. In addition, while the active (cleaved) form of the protein was present at 24 h of
587 re-oxygenation, it was only weakly expressed and its expression was only marginally higher than
588 that of control normoxic or anoxia-exposed fish. On one hand, this could indicate that the observed
589 cell death might have been caused by necrosis or caspase-independent apoptosis. While caspases
590 have traditionally been viewed as key executers in the process of apoptosis, there are in fact
591 caspase-independent pathways that can also lead to controlled forms of cell death (e.g. Kroemer and
592 Martin, 2005). One such particular form of cell death is referred to as 'parthanatos', to distinguish it
593 from apoptosis (David et al., 2009; Fatokun et al., 2014), while another is referred to as
594 'necroptosis' (Linkermann and Green, 2014), among many others (Berghe et al., 2014).
595 Interestingly, recent studies have indicated that such caspase-independent cell death can occur
596 following DNA damage and oxidative stress (Ma et al., 2016; Wang et al., 2016), and that it might
597 be important after more persistent ischemic events (Manabat et al., 2003), which would be
598 equivalent to the long-term anoxia that crucian carp tolerates. On the other hand, it should be kept
599 in mind that the lack of a clear change in caspase 3 expression is not definite proof that caspase may
600 not be involved. Because caspase 3 is a complex enzyme, which is only active when cleaved into a
601 smaller active and a larger non-active subunit, the cleaved active enzyme may be less stable, and
602 there might be a high turnover which could make it more difficult to detect. It may also be that
603 caspase 3 is activated in the earlier phase of re-oxygenation (e.g. Namura et al., 1998), though in a
604 model of neonatal rat hypoxia–ischemia, Zhu et al. (2000) found co-occurrence of TUNEL- and
605 caspase 3-positive cells at 24 h re-oxygenation. Another less likely explanation is that the cell death
606 was only increased in certain areas of the brain (i.e. the telencephalon), and thus by analysing whole
607 brain any apparent differences in caspase 3 protein expression could have been obscured. However,
608 as anoxia and re-oxygenation conditions would not have been restricted to a particular brain area,

609 there is no reason to suspect that apoptosis would only occur in certain areas, unless the crucian
610 carp is able to control which cells undergo apoptosis and which do not. Surprisingly, in a study on
611 brain tissue from hypoxic/re-oxygenated mice, Chiu et al. (2012) actually found increased activity
612 of caspase 1 in the amygdala, but not in the hippocampus, indicating that some differentiation might
613 be possible, and so a more detailed examination of other areas of the crucian carp brain is clearly an
614 important topic for future investigation.

615 Having assumed that anoxia/re-oxygenation-induced cell death would be a result of caspase
616 3-dependent apoptosis, rather than necrosis or caspase-independent apoptosis, we hypothesized that
617 there would be signs of increased expression of caspase 3 in fish caught in the spring (May), during
618 a time when oxygen levels had returned to normal after a long period of winter anoxia, and thus
619 could have led to oxidative stress and ROS production that could induce caspase 3 activation.
620 Indeed, there was a tendency for support of this hypothesis at the protein level, at least in some of
621 the individuals. Caspase 3 mRNA levels were also significantly elevated in May compared to
622 October and December. However, the elevation persisted during June - September, coinciding with
623 both an increase in temperature from below 4°C to above 10°C and hyperoxic conditions.
624 Additionally, feeding activity and hence dietary status would be much higher during the summer
625 (e.g. Penttinen and Holopainen, 1992). Both elevated temperature and dietary status can be
626 speculated to increase protein turnover, and maintaining protein abundance would therefore require
627 a higher mRNA level, though more detailed experiments are obviously necessary to confirm such a
628 hypothesis. As the change in expression was evident only at the mRNA level, and not at the level of
629 active protein (of which abundance was actually maintained), it at least seems less likely to be
630 indicative of an increase in apoptotic activity due to oxidative stress caused by the hyperoxic
631 conditions in June and September. Furthermore, the blood oxygen partial pressure of crucian carp is
632 likely to be low, as observed in the closely related goldfish (*Carassius auratus*; see Burggren,
633 1982), due to their high haemoglobin oxygen affinity. Moreover, under hyperoxic conditions the
634 interlamellar cell mass has been reported to increase and hence been proposed to serve as a barrier
635 against oxygen entry (Tzaneva et al., 2011). Lushchak et al. (2005) actually found indications that
636 short exposure to hyperoxia resulted in oxidative stress in goldfish tissues, though the level of
637 hyperoxia used was much more severe (300% air saturation) than observed in the present study
638 (120-170% air saturation). A similar observation has been made in mice (e.g. Terraneo et al., 2017).
639 Still, it would be interesting to investigate if hyperoxia itself can cause oxidative stress in crucian
640 carp, even when not preceded by a long period of anoxia.

641 *Regeneration*

642 Death of neurons in the mammalian brain is detrimental and generally irreparable, because of a very
643 limited ability to produce new neurons, except for a few specialized areas such as the hippocampus
644 (Nakatomi et al., 2002; Kokaia and Lindvall, 2003). Fish in general have more plastic brains, with a
645 higher turnover of cells, and as their brain grows continually throughout their lifetime they are
646 obviously able to produce new neurons (Clint and Zupanc, 2001; Zupanc, 2008; Ilies et al., 2012).
647 This plasticity is also indicated in the present data on crucian carp. The number of proliferating
648 cells, contrary to the hypothesis, was not higher during re-oxygenation *per se*, but the number of
649 proliferating cells was still higher than or equal to the number of TUNEL-positive cells, per
650 investigated volume. As there were signs of cell death also in normoxic brains, the combined results
651 would indicate that crucian carp have a natural turnover of cells in the brain, and that cell
652 proliferation continuously replaces damaged cells, readily compensating for a transient increase in
653 cell death caused by anoxia and re-oxygenation. A similar mechanism has been suggested to be
654 utilized by hibernating frogs that sustain increased levels of cell death during hibernation as well as
655 increased cell proliferation, whereby the number of functional cells is maintained (Cerri et al.,
656 2009). However, as we only examined two time-points during re-oxygenation (1 and 7 days), it is
657 also possible that an increase in post-anoxic cell proliferation was lagging behind, occurring later
658 than one day, but having returned to normal levels before day 7 of re-oxygenation. The increased
659 level of PCNA mRNA seen after 7 days of re-oxygenation, though it may seem like a long time, has
660 been observed in a previous study on crucian carp (Sandvik et al., 2012), particularly in the heart,
661 and could reflect that mRNA expression remains elevated longer than the protein itself.

662 *Memory and learning*

663 The finding of increased cell death, as hypothesised, seemed to be reflected also at the functional
664 level. The anoxia-exposed crucian carp that had learned to find food in a maze prior to anoxia
665 exposure made significantly more errors during the post-exposure trial compared to normoxic
666 controls, even though they found the food equally fast (except for two fish). There was an indication
667 that feeding activity was slightly reduced following anoxia, but the fish included in the analyses of
668 behavioural experiments all found the food, indicating that their appetite must have been sufficient
669 to motivate them to navigate the maze. Also, one may have expected that fish lacking appetite
670 would take longer to find the food, rather than finding it fast and making more errors along the way.
671 Arguably, entering dead-ends and turning back more frequently could be interpreted as the fish
672 actively searching for the food. Nonetheless, crucian carp did not exhibit impaired spatial learning

673 ability when given multiple days to recover in normoxic water, indicating that they are able to
674 repair any brain damage caused by anoxia/re-oxygenation, and that this state was therefore
675 transient. While comparable experiments to our knowledge are lacking for both other fish and
676 anoxia tolerant freshwater turtles, it is well established that memory is impaired in mammals
677 suffering from brain ischemia and reperfusion (Shih et al., 2013; Meng et al., 2014; Schimidt et al.,
678 2014). However, the ability of mammals to regenerate neurons is limited and cognitive impairment
679 in neonatal rats exposed to ischemia and reperfusion is carried over into adulthood (Arteni et al.,
680 2003). This contrasts with the transient effect observed in crucian carp, and the difference may to
681 some extent rely on the general difference in plasticity and regenerative ability between mammals
682 and fish. It is also likely that neural repair mechanisms are particularly well developed in the most
683 anoxia-tolerant fish there is - the crucian carp. It is important to note the individual variation, as the
684 ability to navigate the maze after exposure to anoxia was clearly more impaired in some individuals
685 than others, and that this variability fits well with the variability found in cell death.

686 *Perspectives and significance*

687 This study points to the conclusion that while crucian carp survive anoxia for extended periods of
688 time, they are not fully able to protect themselves from the cellular insult that particularly the
689 restoration of oxygen levels poses. While the fish show signs of increased cell death in the
690 telencephalon, and signs that this could affect their brain function and behaviour, it is also evident
691 that they can limit the amount of damage they sustain and recover from the insult, an ability that
692 most other vertebrates lack. This makes the crucian carp an interesting model from a biomedical
693 perspective - while it is unlikely that we will find ways to allow human tissues to survive severe
694 anoxic insults without damage, it is feasible that studies on animals like the crucian carp can
695 provide knowledge for how we can limit and repair the damage. Interestingly, we detected a large
696 inter-individual variation in the magnitude of effects, pointing at the possibility that natural
697 selection may bring about the most anoxia-tolerant crucian carp populations in habitats with the
698 most severe anoxic periods. As far as we know there have been no studies of population differences
699 in anoxia tolerance in the species. Lastly, it is also important to bear in mind the protective role that
700 low temperature might play during the winter in maintaining the damage at a level from which the
701 fish can fully recover. Though the complexity and diversity in cell-death pathways certainly poses a
702 challenge, future studies should attempt to more specifically identify the processes activated in the
703 crucian carp brain during anoxia and particularly re-oxygenation, and how they may differ from the
704 response of hypoxia sensitive species.

Competing interests

No competing interests, financial or otherwise, are declared by the authors.

Author contributions

LYL, MKT, JAWS, CSC, and SL performed the experiments. LYL, MKT, JAWS, and SL analysed the data. SL, IBJ, GEN, KOS and supervised MKT. CS and GEN supervised LYL. SL prepared the first manuscript draft including statistical analysis and figures. GEN, JAWS, CS, SL, KAS, and CSC conceived the experiments. All authors contributed significantly to and approved the submitted manuscript.

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Supplementary Information

Supplementary information is available online at http://jeb.biologists.org/.

List of symbols and abbreviations

AIF	apoptosis inducing factor
ATP	adenosine triphosphate
casp3a	caspase 3a
casp3b	caspase 3b
CP	crossing point
CI	confidence interval
DAB	3,3'-diaminobenzidine
dH_2O	distilled water
HRP	horse-radish peroxidase
IPTG	isopropyl β-D-1-thiogalactopyranoside
LB	lysogeny broth
NCBI	National Center for Biotechnology Information
qPCR	quantitative real-time polymerase chain reaction
PBS	phosphate-buffered saline

735 PCNA proliferating cell nuclear antigen
736 PCR polymerase chain reaction
737 PVC polyvinyl chloride
738 PVDF polyvinylidene difluoride
739 ROS reactive oxygen species
740 RIN RNA integrity number
741 TBST tris-buffered saline Tween 20
742 TdT Terminal deoxynucleotidyl transferase
743 TUNEL Terminal deoxynucleotidyl transferase dUTP nick-end labelling
744 X-Gal 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside

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952 **Table 1:** Primers used for partial cloning of crucian carp casp3a and casp3b (A) and
953 quantitative real-time PCR of mw2060 (external standard) and crucian carp casp3a, casp3b and
954 PCNA (B).

	Gene	Direction	Primer sequence (5´ → 3´)
A	casp3a	F1	AGCCTCAACTACCCCAACAT
		F2	GAACACAGGCATGAACCCAC
		F3	GTTGCCCATGACGATCACAG
		F4	ARCCTCAACTACCCCAACMT
		F5	ACCAGACAGTAGCGCCAGATT
		F6	AGTCGCTGTGCTTCATTTGT
		R1	TCAAGCGCTGAAGTACATCTC
		R2	TGTTTCTTAGAGTCGAAGTCAG
		R5	GAGGAAGTCTGCCTCCACA
	casp3b	F2	CAACGGGACAGACAAGGATG
		R2	CATCAAACCCTGGAAGATTGCA
B	mw2060	F	CTGACCATCCGAGCGATAAT
		R	AGCAAGCTGTTCGGGTAAAA
	casp3a	F	GTGTGTGTGATGCTCAGTCA
		R	CCACACCAGGATCCAGTTCT
	casp3b	F	AGCAACTGTTTACACTCTTCAGA
		R	AACCAGGAACCATTGCCAAC
	PCNA	F	CTTTGGCACTTGTCTTTGAAAC
		R	TTCACCACACAGCTGTATTCCT

955 F = forward primer, R = reverse primer. Several primers for each gene were designed for the partial cloning (A), but
956 this table only show the primers that obtained the correct sequence. The F4 primer for casp3a has a degenerative
957 code where R is G or A, and M is A or C. The primer pairs were chosen for qPCR (B) were based on the lowest
958 crossing point (CP) value, best efficiency, and which primer that gave only one melting curve with the SYBR Green
959 I detection. The primer sequence for PCNA was obtained from Sandvik et al. (2012).

960 **Captions**

961 **Fig. 1: Cell death and proliferation in crucian carp brain.** Number of TUNEL-positive cells per
962 investigated volume in the telencephalon of crucian carp (n = 6 in each group) (A), number of
963 proliferating cells as visualized by immunohistochemical staining for PCNA in the telencephalon (n
964 = 6 in each group) (B) and crucian carp whole-brain mRNA expression of PCNA relative to
965 external standard (n = 10 in each group) (C), after exposure to 7 days normoxia (N7), 7 days anoxia
966 (A7) or 7 days anoxia followed by 1 and 7 days re-oxygenation (A7R1 and A7R7, respectively).
967 Data are mean ± 95% confidence interval (CI) with individual values indicated by dots. Different
968 letters indicate significant differences (Tukey's multiple comparison).

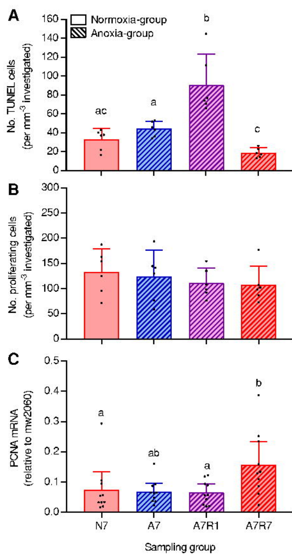
969 **Fig. 2: Caspase 3 expression in crucian carp brain.** Protein expression (normalized to loaded
970 protein as well as normoxic (N7) intensity within each blot) of the cleaved, active casp3a (A), and
971 mRNA expression (relative to external standard) of casp3a (B) and casp3b (C), in crucian carp after
972 exposure to 7 days normoxia (N7), 7 days anoxia (A7) and 7 days anoxia followed by 1 and 7 days
973 re-oxygenation (A7R1 and A7R7, respectively). Sample size was 10 in each group unless otherwise
974 indicated (in parentheses). Data are mean ± 95% CI with individual values indicated by dots.
975 Different letters indicate significant differences (one-way ANOVA followed by Tukey's multiple
976 comparison).

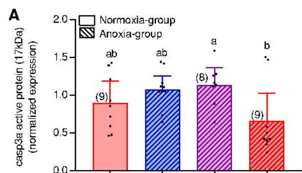
977 **Fig. 3: Seasonal changes in temperature, oxygen level and caspase 3 expression in wild-caught**
978 **crucian carp.** Temperature and oxygen level in Tjernsrudtjernet at the time of sampling (A),
979 mRNA expression of casp3a relative to external standard (B) and protein expression of cleaved,
980 active casp3a normalized to total protein loading and June levels within each blot (C) in whole brain
981 from crucian carp exposed to natural temperature and oxygen levels. Sample size was 8 in each
982 group, unless otherwise indicated (in parentheses). Data in B and C are mean ± 95% CI with
983 individual values indicated by dots. Different letters indicate significant differences (Tukey's
984 multiple comparison tests).

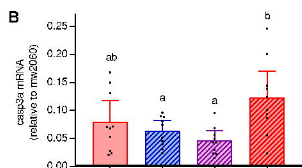
985 **Fig. 4: Effect of anoxia and re-oxygenation on crucian carp memory.** Time taken to find food
986 (A) and number of errors made (B) in a maze in five consecutive trials before anoxia exposure
987 (shaded bar) or control-normoxia exposure for the normoxia-group (unshaded bar) and pre- and
988 post-exposure comparison of time taken to find food (C) and number of errors (D). The pre-
989 exposure values in C and D are from the average of pre-exposure trial 4 and 5 before anoxia

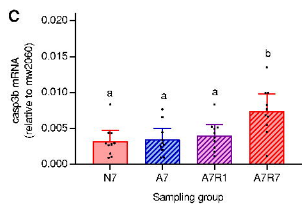
990 exposure (control-normoxia exposure for the normoxia-group) and post-exposure values are from
991 the trial carried out after one day of re-oxygenation after the anoxia exposure (control-normoxia for
992 the normoxia). Data are mean ± 95% CI with individual values indicated by dots (n = 7 in each
993 treatment). In A and B there was no effect of treatment, therefore the difference between trials was
994 assessed on the combined data and different letters indicate significantly different groups (main
995 effect of individual trial number, Tukey's multiple comparison). In C and D, an asterisk indicates a
996 significant difference between the normoxia-group and the anoxia-group within each time point,
997 while lower and upper case letters indicate a difference between pre-exposure and post-exposure for
998 the normoxia- and anoxia-group, respectively (Sidak's multiple comparisons).

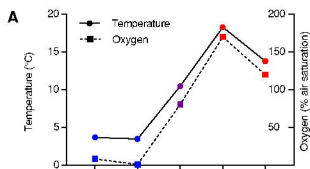
999 **Fig. 5: Effect of anoxia and re-oxygenation on crucian carp learning ability.** Time taken to find
1000 food (A) and number of errors made (B) in a maze in five consecutive individual trials with each
1001 fish (n = 10 in the normoxia group and n = 8 in the anoxia-group, but note that not all fish
1002 completed all trials and the sample size therefore varies between trials) after anoxia exposure
1003 (control-normoxia exposure for the normoxia-group). Note that the first trials were carried out after
1004 8 days of normoxia following the five days of anoxia. Data are mean ± 95% CI with individual
1005 values indicated by dots. As there was no effect of treatment in either measurement, the difference
1006 between trials was assessed on the combined data from both groups, and different letters hence
1007 indicate a significant difference between trial numbers (main effect of trial number, Tukey's
1008 multiple comparison).

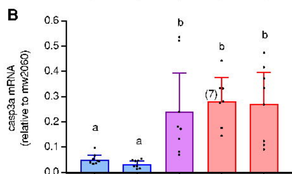

A
B
C
Normoxia-group
Anoxia-group
No. TUNEL cells
(per mm^{-3} investigated)
No. proliferating cells
(per mm^{-3} investigated)
PCNA mRNA
(relative to mw2060)
ac
a
b
c
ab
N7
A7
A7R1
A7R7
Sampling group

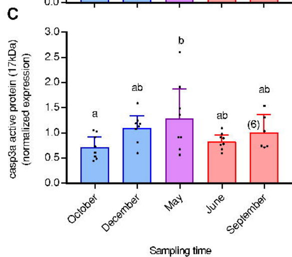

A
Normoxia-group
Anoxia-group
casp3a active protein (17kDa) (normalized expression)
2.0
1.5
1.0
0.5
0.0
ab
ab
a
b
(9)
(8)
(9)

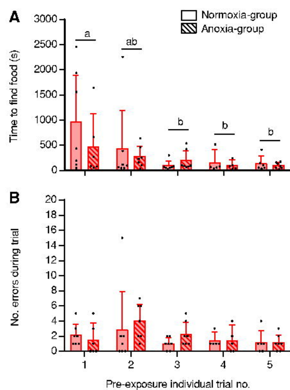

B
casp3a mRNA (relative to mw2060)
0.25
0.20
0.15
0.10
0.05
0.00
ab
a
a
b

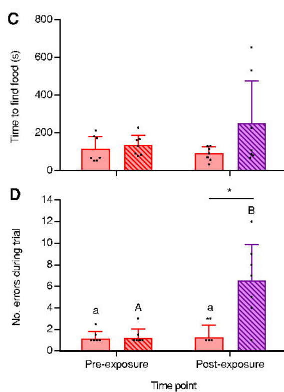

C
casp3b mRNA (relative to mw2060)
0.020
0.015
0.010
0.005
0.000
a
a
a
b
N7
A7
A7R1
A7R7
Sampling group

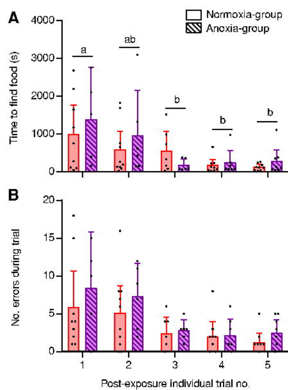

A
Temperature
Oxygen
Temperature (°C)
Oxygen (% air saturation)
20
15
10
5
0
200
150
100
50


B
casp3a mRNA
(relative to mw2060)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
a
b
b
b
(7)


C
casp3a active protein (17kDa)
(normalized expression)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
ab
b
ab
ab
(6)
October
December
May
June
September
Sampling time


A
B
Normoxia-group
Anoxia-group
Time to find food (s)
No. errors during trial
a
ab
b
b
b
Pre-exposure individual trial no.

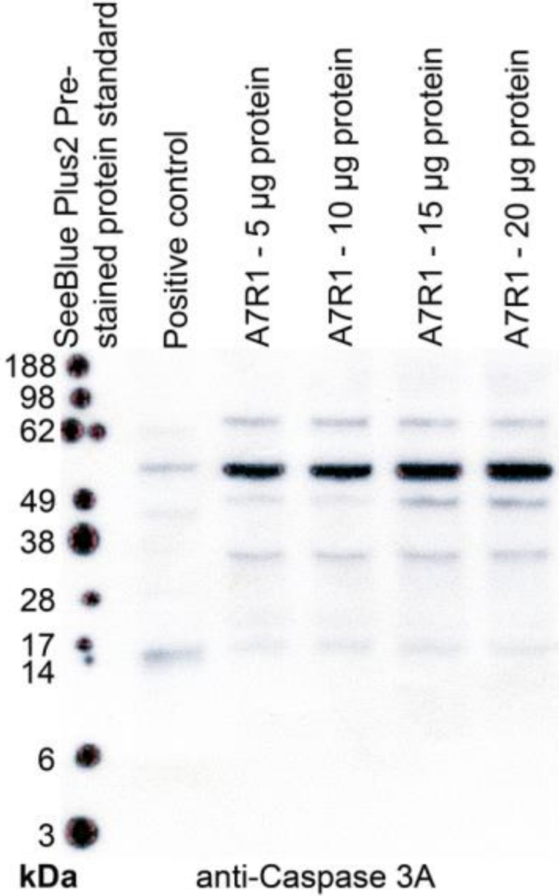

C
D
Time to find food (s)
No. errors during trial
*
B
a
A
a
Pre-exposure
Post-exposure
Time point


A
B
Normoxia-group
Anoxia-group
Time to find food (s)
4000
3000
2000
1000
0
a
ab
b
b
b
No. errors during trial
20
15
10
5
0
1
2
3
4
5
Post-exposure individual trial no.

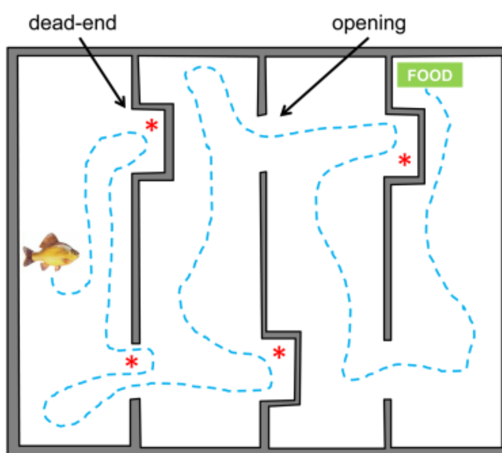
Supplementary Figure S1: Alignment of amino-acid sequences for zebrafish (*Danio rerio*, Dr) casp3A and casp3B (NCBI Reference Sequence number NP_571952.1 and NP_001041531.1, respectively) and crucian carp (*Carassius carassius*, Cc) casp3ai and casp3aii (translated from the sequences obtain through partial cloning), aligned using the Clustal Omega tool (EMBL-EBI). Darker shading indicates higher degree of conservation across isoforms and species.

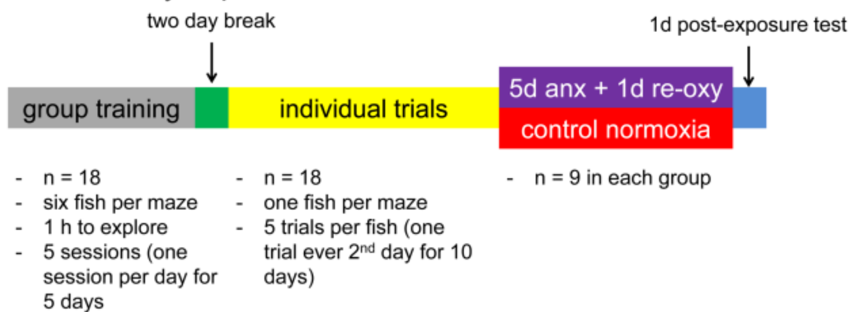
```
casp3A_Dr      1  ------MNGDCVDAKRVDTTDASKDGASASQP--MQVDAK---PQSHAFRYSLNYPNIGHCIIINNKDFDRRTGMNPRNG
casp3ai_Cc     1  ---------------------------------------------------SLNYPNIGHCIIINNKNFDRSTGMNQRNG
casp3aii_Cc    1  --------------------------------------------------------------------------------
casp3B_Dr      1  MSHVKPKGEDTVDARQSDAKQS----SSVTDPGVVQMDAKSHSDDNVDYQYKTNYPNLGQCLIINNKNFHKRTGMGVRNG
casp3b_Cc      1  ------------------------------------------------------------------------------NG

casp3A_Dr     70  TDVDAGNVMNVFRKLGYIVKVYNDQTVAQIMQVLTTVAHDDHSRCASLVCVLLSHGDEGVFFGTDTSVDLKSLTSLFRGD
casp3ai_Cc    30  TDVDAGNVMNVFGKLGYTVKVYNDQTVAQIKQVLTAVARGDHSHCASLVCVMLSHGDEGVFFGTDTSVELKQLTSLFRGD
casp3aii_Cc    1  ------------------------------------VAHDDHRRCASLVCVMLSHGDEGLFYGTDTSVELKTLTSLFRGD
casp3B_Dr     77  TDKDAKKVFETFSQLGFEMKPYNDLTVSQMVALLTKASEEDHSKSAMFACVLLSHGDDGLIYGTDDSIELKRLFAHFRGD
casp3b_Cc      3  TDKDAKNAMETFTNLGFKIKVTNDQTVSQMRDLLAKVSQEDHSQSAMFVCVLLSHGDDGKIYGTDGCIELKQLFTLFRGD

casp3A_Dr    150  RCPSLVGKPKLFFIQACRGTELDPGVETDHPDHPDIPDGRVRIPVEADFLYAYSTVPGYYSWRNTMTGSWFIQSLCEMMT
casp3ai_Cc   110  RCPSLVGKPKLFFIQACRGTELDPGVETDSV------DKSIRIPVEADFLYAYSTVSGYYSWRNTMTGSWFIQSLCEMIT
casp3aii_Cc   45  RCPSLVGKPKLFFIQACRGTELDPGVEADSS------DSSMRIPVEADFLYAYSTAPGYYSWRNTQTGSWFIQSLCEMVA
casp3B_Dr    157  RCTSLVGKPKLFFIQACRGTDLDSGIECDGVGD----EETQRIPVEADFLYAYSTAPGYYAWRNVANGSWFISSLCDMLL
casp3b_Cc     83  RCRSLVGKPKLFFIQACRGSELDGGIEADSVGE----EDTQKIPVEADFFYAYSTPPGYYSRRNVGNGSWIFSLCEMLS

casp3A_Dr    230  KYGSELELLQIMTRVNHKVALDFESTSNMPGFDAKKQIPCIVSMLTKEMYFTP
casp3ai_Cc   184  KYGKELELMQIMTRVNHKVALDFESTSNLPGFDAKKQIPCIVSMLTKEMYFSA
casp3aii_Cc  119  KYGRELDLMQIMTRVNHKVALDFESTSNQPGFDAKKQIPCIVSMLTKEMYFSA
casp3B_Dr    233  KYGKQLEIMQVMTRVNHKVALEFESSCNLPGFDGKKQIPCIVSMLTKELYFPK
casp3b_Cc    159  KYGKELEIMQIMTRVNHKVALDFKSSCNLPGFDE-------------------
```

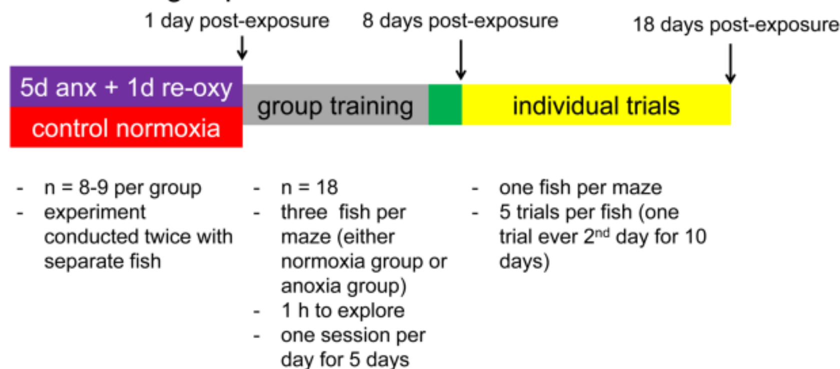
Supplementary Figure S2: Western blotting positive control (gill tissue from crucian carp exposed to 25-30°C) and different protein loading concentrations (brain tissue from crucian carp exposed to anoxia for 7 days followed by one day of re-oxygenation).



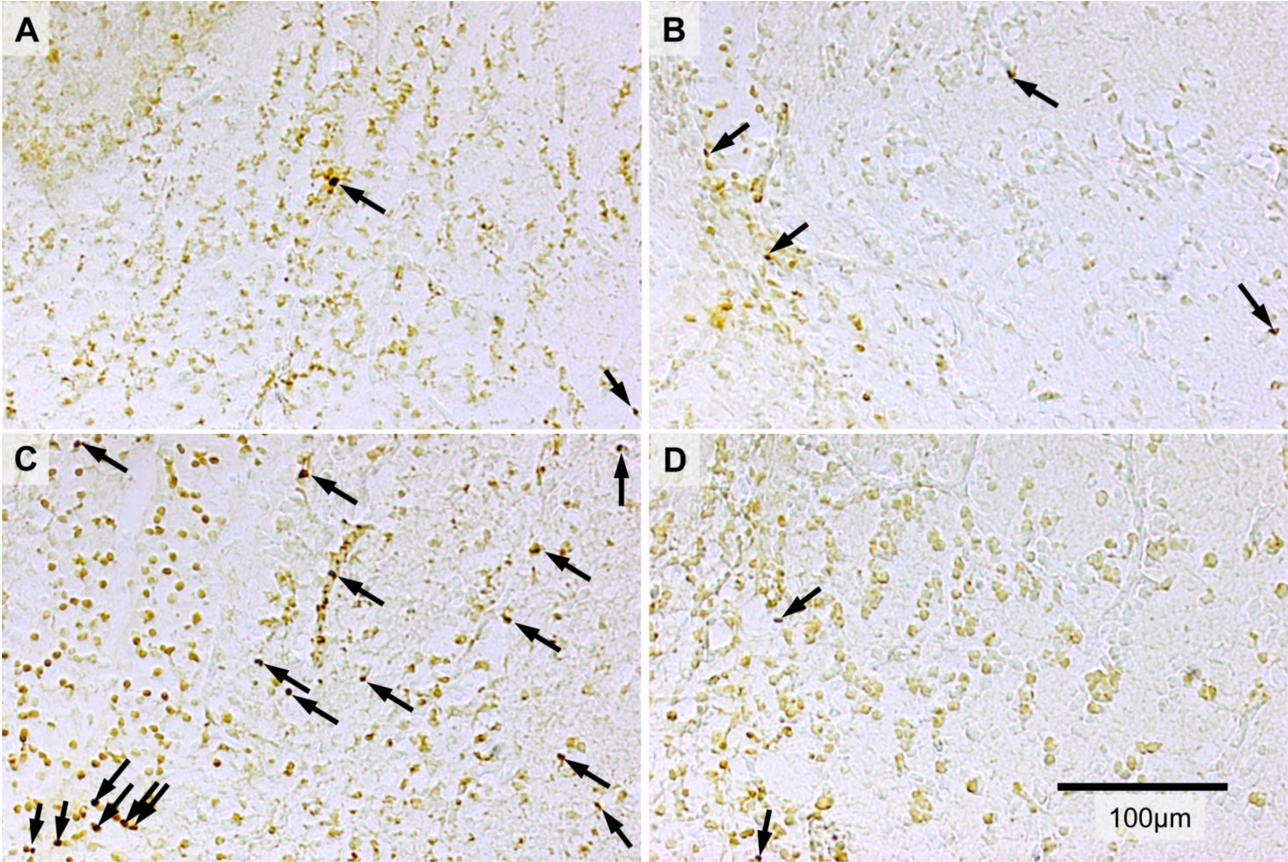
Supplementary Figure S3: Schematic representation of the maze used to assess the effect of anoxia exposure on crucian carp memory and learning (A). The time it took for the fish to reach the food as well as the number of errors made (i.e. going into a dead end or reversing course and returning through an opening, as indicated by asterisks) were recorded during each individual trial. Schematic representation of the daily schedule and protocol for the experiments designed to examine if crucian carp show (B) impaired memory and (C) impaired learning after anoxia/re-oxygenation.



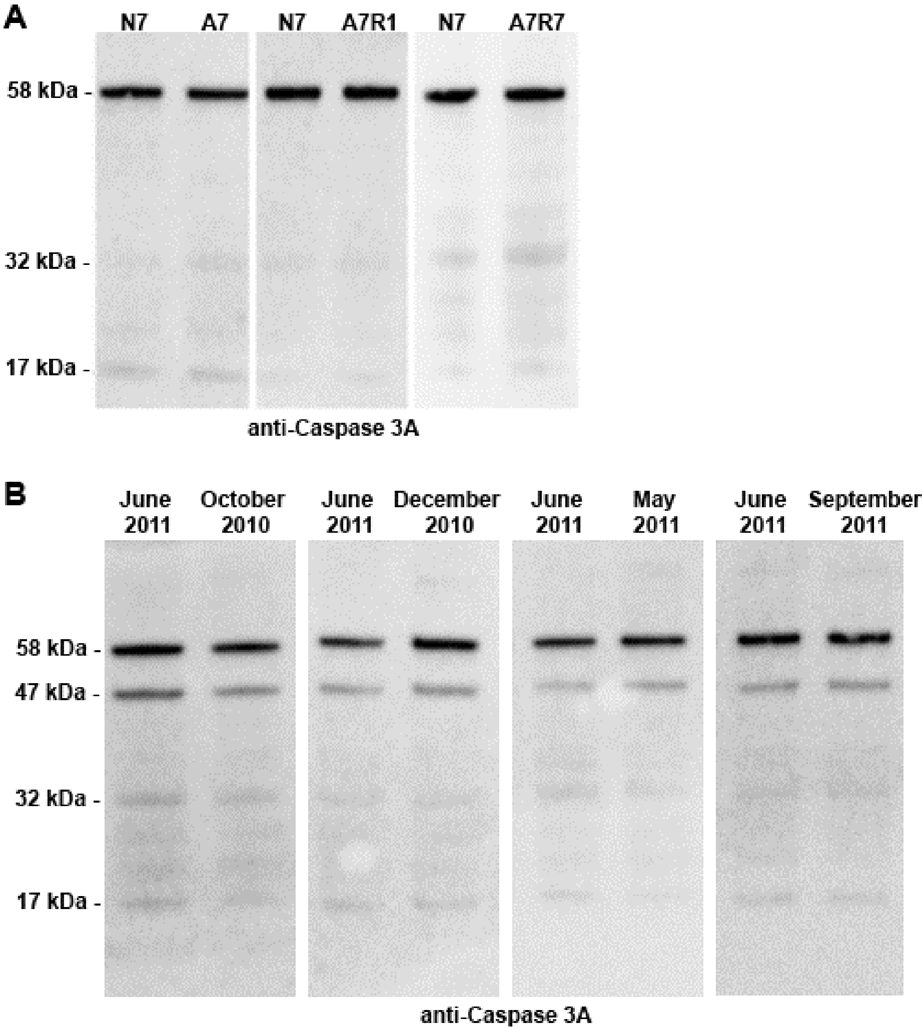




Supplementary Figure S4: Examples of images from TUNEL staining taken at 20X magnification of telencephalon tissue from crucian carp after 7 days normoxia (A), 7 days anoxia (B), 1 day re-oxygenation (C), and 7 days re-oxygenation (D). Apoptotic cell bodies stained by the TUNEL assay are identified with black arrows.



Supplementary Figure S5: Western blotting gel images of casp3a protein expression from brains of crucian carp after 7 days exposure to normoxia (N7) or anoxia (A7), and 7 days anoxia followed by one or seven days of re-oxygenation (A7R1 and A7R7, respectively) (A) and in brains from crucian carp caught in Tjernsrudtjernet at different times of the year (B).



Supplementary Figure S6: Feeding activity measured as the percentage of partitions of the maze with feeding activity (A) and feeding activity as the percentage of pellets consumed (B). Due to the small sample size (n = 10 for the normoxia-group and n = 12 for the anoxia-group) and the nature of the experiment, statistical significance was not assessed, but it was clear that the post-exposure appetite in most of the anoxic fish was maintained.