

Short-term feed and light deprivation reduces voluntary activity but improves swimming performance in rainbow trout *Oncorhynchus mykiss*

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Abstract Rainbow trout *Oncorhynchus mykiss* (~180 g, 16 °C and 5 kg m^{-3}) that were feed deprived and kept in total darkness showed a significant increase in critical swimming speed (U_{crit}) between 1 and 12 days of deprivation (from 3.35 to 4.46 body length (BL) s^{-1}) with no increase in maximum metabolic rate (MMR). They also showed a significant decrease in the estimated metabolic rate at 0 BL s^{-1} over 12 days which leads to a higher factorial aerobic metabolic scope at day 12 (9.38) compared to day 1 (6.54). Routine metabolic rates were also measured in ~90 g rainbow trout that were swimming freely in large circular respirometers at 16 °C. These showed decreasing consumption oxygen rates and reductions in the amount of oxygen consumed above standard metabolic rate (a proxy for spontaneous activity) over 12 days, though this happened significantly faster when they were kept in total darkness when compared to a 12:12-h light–dark (LD) photoperiod. Weight loss during this period was also significantly reduced in total darkness (3.33% compared to 4.98% total body weight over 12 days). Immunological assays did not reveal any consistent up- or downregulation of antipathogenic and antioxidant enzymes in the serum or

skin mucus of rainbow trout between 1 and 12 days of feed and light deprivation. Overall, short periods of deprivation do not appear to significantly affect the performance of rainbow trout which appear to employ a behavioural energy-sparing strategy, albeit more so in darkness than under a 12:12-h LD regime.

Keywords Salmonids · Metabolic rate · Oxygen consumption · Spontaneous activity · Aerobic scope

Introduction

Short- and long-term food deprivation is a common occurrence for both wild and cultured fishes and is often related to seasonal variations in food supply (Love 1975; Wang et al. 2006), migration (Mommsen 2004) or husbandry/slaughter practices in aquaculture (Einen et al. 1998; Álvarez et al. 2008). In the short term, food deprivation requires the utilisation of lipid and glycogen stores to satisfy the minimum energy requirements, i.e. standard metabolic rate (SMR) (Kiessling et al. 1990; Bradford 1993). Depending on species, feeding mode and habitat, it may also lead to various energy-conserving strategies such as decreases in voluntary or spontaneous activity. This has been observed in the roach *Rutilus rutilus* (van Dijk et al. 2002) and in the surfacing behaviour of the obligatory air-breathing channel snakehead *Ophiocephalus striatus* (Pandian and Vivekanandan 1976). Despite reductions in voluntary activity, fish that have been food deprived for 12–48 h have higher maximum aerobic swimming speeds

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(critical swimming speed, U_{crit}) and improved swimming endurance than fed fish (Laurence 1972; Alsop and Wood 1997; Plaut 2001). This is considered to be the result of the reduction in the physiological costs of digestion and assimilation (specific dynamic action, SDA) which results in an expanded capacity for physiological work (Khan et al. 2014b). After an extended period of deprivation, fish should become limited by metabolic fuels (especially active fish) which would reduce swimming endurance or critical swimming speed.

The presence or absence of light is also known to have various effects on fish. In the literature, there has been a particular focus on the effects of photoperiod on the smolting and reproductive cycles of salmonids (Henderson 1963; Hansen et al. 1992; Solbakken et al. 1994; Fjelldal et al. 2011) and moving light stimuli on schooling behaviour and antagonistic interactions (Oppedal et al. 2001; Valdimarsson and Metcalfe 2001; Herbert et al. 2011; Oppedal et al. 2011). Oppedal et al. (2001) found that the voluntary swimming behaviour of Atlantic salmon *Salmo salar* decreased with light intensity and that many fish sought out low-flow zones to maintain position in the darkest treatments. This suggests that low-light levels and darkness could play a significant role in energy conservation in food-deprived fishes. However, there could be other consequences of light deprivation that have to be considered. In particular, changes in photoperiod have been shown to impact the immune response in fish, particularly components of the innate immunity (Zapata et al. 1992; Magnadóttir 2006). It has been documented that a number of defence molecules in fish, such as lysozymes, complements and peroxidases, exhibit diel variations (Ángeles Esteban et al. 2006; Binuramesh and Michael 2011; Lazado et al. 2015, 2016), indicating the strong influence of light–dark changes in the immunological status of fish. As an example, Leonardi and Klempau (2003) found that the expansion of T lymphocytes in rainbow trout *Oncorhynchus mykiss* was significantly suppressed after 60 days on a 24:0-h light–dark (LD) regime, likely due to a chronic stress response. A light-induced stress response was also observed in Atlantic cod *Gadus morhua* reared under continuous light, where higher levels of transcripts coding for antioxidant enzymes and antimicrobial peptides were identified (Giannetto et al. 2014).

As the combination of light and feed deprivation has achieved little attention in the literature, the current

study aims to determine how these factors affect the physiological performance of an important salmonid, the rainbow trout. The primary hypothesis is that swimming performance (U_{crit}) will be highest in the period shortly after feeding has ceased (~2–4 days) and then decrease. The current study also aims to determine if rainbow trout will decrease their metabolism and activity levels to conserve energy during periods of deprivation. This will be conducted under the hypothesis that metabolism and activity will decrease with increasing feed deprivation and that this effect will be greater in fish kept in total darkness compared to those kept under normal light regimes. Humoral and mucosal immune responses in fish exposed to short-term feed and light deprivation will also be assessed to identify the impact on the immunological status of fish under these conditions.

Materials and methods

All handling and experimental procedures were conducted in accordance with national and EU legislation (2010/63/EU) on animal experimentation.

Fish, maintenance, feed and light deprivation

Rainbow trout were obtained from Markmølle Dambrug (Nykøbing, Denmark) as smolts initially weighing ~50 g. Fish were quarantined for 2 weeks at 20‰ salinity, after which they were divided into 16 1.5-m³ holding tanks which were connected to a 200-m³ fresh water, recirculating aquaculture system at the DTU Aqua, North Sea Research Centre, Hirtshals, Denmark. They were held in these tanks for several months whilst all experiments were performed. The entire system was maintained at 16 ± 0.1 °C and densities never exceeded 5 kg m⁻³. During the holding periods, fish were fed a commercial diet (BioMar EFICO Enviro 920 3- and 4.5-mm pellets, 21.9 MJ kg⁻¹ digestible energy) at ~2% of tank biomass daily (as per the recommendations of the feed manufacturer) via a 12-h belt feeder. Oxygen levels never fell below 90% saturation for any extended period.

After 2 weeks of acclimation to the holding tanks, feeding was halted in four adjacent tanks over 4 days, i.e. 1 tank day⁻¹. At the same time that feeding was halted, the tanks were covered with black material so that the fish were in total darkness (DD). Oxygen levels

and temperature remained constant for the entire deprivation period. Each tank was maintained in this way for a total of 12 days. The only disturbance to the darkness was when fish were removed for swim flume respirometry and immune function sampling which occurred after one whole day of being covered and then on days 4, 8 and 12 for each tank (see Table 1). This took no longer than 40 s, and light exposure was kept to an absolute minimum. As this produced only an $n = 4$ for each time point for the swim flume respirometry (1, 4, 8 and 12 days of deprivation, max. 1 fish day⁻¹ throughput), the entire process was immediately repeated on another subset of four holding tanks to produce $n = 8$ for this particular experiment.

Swimming performance with progressive feed and light deprivation

Swimming performance was investigated in rainbow trout (179.61 ± 8.48 g and 226.73 ± 8.18 mm fork length, FL) which had been subjected to light and feed deprivation for either 1, 4, 8 or 12 days ($n = 8$ for each) to resolve the combined effect on swimming performance. The mass specific rate of oxygen consumption (M_{O_2} , mg O₂ kg⁻¹ h⁻¹) was determined in a total of 32 fish over 32 days using a Loligo® Systems 30-L Steffensen-type swim flume respirometer thermostatted to 16 ± 0.1 °C with continuous water exchange. Oxygen saturation in the respirometer was measured continuously using a PreSens® Fibox 3 (PreSens, Germany)

connected to an oxy-dipping probe in a position anterior to the swimming section. The oxygen meter was calibrated at the beginning of every experiment against daily atmospheric pressure. The swim flume was operated using AutoResp™ (V 2.2.0; Loligo Systems, Denmark) which controlled water speed in the swimming section and the cycling between flush, wait and measure periods (5, 1 and 4 min, respectively, a total of 10 min) and calculated M_{O_2} .

Weight, length, depth and width of fish [to compensate for the solid-blocking effect (Steffensen 1989)] were measured, and specimens were placed in the sealed swimming section of the respirometer (550 × 140 × 145 mm). This data was also used in the calculation of the condition factor (Froese 2006). The entire apparatus was then enclosed in curtains to minimise external disturbance. This occurred at 1600 hours and provided fish an overnight period to acclimate to the conditions of the respirometer at 0.5 body length (BL) s⁻¹ and with the system cycling automatically through flush, wait and measure periods. From 0800 hours the following day, a critical swimming speed (U_{crit}) test commenced where the flow speed inside the swimming section was increased by 0.25 BL s⁻¹ every 30 min (i.e. after three 10-min flush–wait–measure cycles). Fish were considered to have stopped swimming when they rested their caudal aspect against the rear of the swimming section or erratic and non-directional burst activity was observed. Fish swimming behaviour was monitored at all times with a Logitech® C525 HD

Table 1 A visual representation of the sampling schedule for the feed and light deprivation trials

	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Tank A	C/F H	1	S/Im	3	4	S/Im	6	7	8	S/Im	10	11	12	S/Im			
Tank B		C/F H	1	S/Im	3	4	S/Im	6	7	8	S/Im	10	11	12	S/Im		
Tank C			C/F H	1	S/Im	3	4	S/Im	6	7	8	S/Im	10	11	12	S/Im	
Tank D				C/F H	1	S	3	4	S	6	7	8	S	10	11	12	S

“C/F H” shows where tanks were covered and feeding was halted, and the period of deprivation is represented by the darkened cells. The numbers in the cells represent the number of days of deprivation experienced by the fish in that particular tank. “S/Im” shows when fish were removed for swimming performance trials (S, $n = 1$) and immunoassays (Im, $n = 3$). Specific protocols relating to these are described in sections “Swimming performance with progressive feed and light deprivation” and “Immunological function with feed and light deprivation”

webcam on an external monitor. Background oxygen consumption levels were measured after the completion of every second trial and confirmed that bacterial respiration was nil. All equipment was cleaned thoroughly between experiments with hot, fresh water.

For each fish, critical swimming speed (U_{crit}) was calculated according to Brett (1964). The 15% quantile method of Chabot and Claireaux (2008) and Franklin et al. (2013) was used to obtain a near-resting value of M_{O_2} from overnight measures at 0.5 BL s^{-1} in order to remove values from periods of excessive activity. This estimate closely matched the first modal peak in M_{O_2} values for the overnight 0.5 BL s^{-1} measurements. Thereafter, the M_{O_2} values obtained from each of the 3 cycles at each speed were averaged to resolve the relationship between swimming speed and M_{O_2} (Korsmeyer et al. 2002; Brown et al. 2011; Khan et al. 2014b). In order to yield an estimate of SMR for every individual fish, average M_{O_2} at all speeds was fitted to an exponential function (Pettersson and Hedenström 2000; Yanase et al. 2012) and extrapolated to estimate M_{O_2} at 0.0 BL s^{-1} . Using all M_{O_2} values from the point that fish first entered the respirometer, maximum metabolic rate (MMR) was calculated using the 99% quantile method of Khan et al. (2014a) as this yielded higher values with less inter-individual variation than M_{O_2} values at U_{crit} . Aerobic metabolic scope (AMS) was calculated by subtracting SMR from MMR for each individual. Factorial AMS was calculated as MMR/SMR for each individual.

Immunological function with feed and light deprivation

The effect of combined feed and light deprivation on immunological function was investigated in rainbow trout that had been subjected to light and feed deprivation for either 1, 4, 8 or 12 days ($n = 9$ at each, only taken from the first three tanks in the sequence, Table 1). Serum and skin mucus samples were collected at each time point. To minimise the effect of daily rhythms in immune response, sample collection was performed between 0800 and 0900 hours. Nine fish were used to represent the initial control group. They were not fed at 24 h prior to the first sample collection. In the succeeding sampling points, three fish were taken from each tank replicate. After euthanising the fish with an overdose of ethylene glycol (Merck, Darmstadt, Germany), skin mucus was obtained by placing the fish in a

polyethylene plastic bag and gently rubbing in a downward motion at least ten times (Lazado et al. 2015). This was performed as gently as possible to avoid fecal contamination. Skin mucus extract was prepared by mixing the collected mucus with 1 volume of sterile phosphate-buffered saline (PBS, pH = 7.4), followed by a 2-min vigorous agitation. The suspension was centrifuged at $10,000g$ for 10 min at $4 \text{ }^\circ\text{C}$ (Sigma 3-18K, Osterode, Germany). The supernatant was collected and kept at $-80 \text{ }^\circ\text{C}$ until analysis. Blood was drawn from the caudal vein using a non-heparinised syringe. It was allowed to clot at room temperature ($\sim 18 \text{ }^\circ\text{C}$) for 2 h and then at $4 \text{ }^\circ\text{C}$ overnight. Serum was collected after centrifugation at $750g$ for 10 min, divided into several aliquots and stored at $-80 \text{ }^\circ\text{C}$ until use. Both serum and skin mucus were analysed for all of the factors described below.

Alkaline phosphatase (ALP) activity was measured following a kinetic assay, employing *p*-nitrophenyl phosphate as a substrate. One unit of activity was defined as the amount of enzyme required to release $1 \text{ } \mu\text{mol}$ of *p*-nitrophenol product in 1 min (Ross et al. 2000). Antiprotease activity was quantified by a trypsin inhibition assay. Activity values were calculated based on a 100% trypsin control (Caipang et al. 2014). *p*-Nitrophenyl myristate was used as a substrate in a kinetic assay to quantify esterase activity. One unit of activity was expressed the same as with ALP (Ross et al. 2000). A microplate-based turbidimetric protocol using *Micrococcus luteus* as the reaction substrate was followed to determine lysozyme activity. One unit of lysozyme activity was defined as the amount of enzyme that resulted in the reduction in absorbance of 0.001 min^{-1} (Sitjà-Bobadilla et al. 2008). Myeloperoxidase activity was measured by utilising 3,3',5, 5'-tetramethyl-benzidine as a chromogenic substrate. Activity value was expressed as absorbance at 450 nm (Caipang et al. 2009). Colorimetric determination of antioxidant enzyme activities was performed using commercially available kits (Cayman, MI, USA) that were previously standardised for fish samples (Lazado et al. 2015). Glutathione peroxidase activity was measured indirectly by a coupled reaction with glutathione reductase. Catalase activity was quantified based on the reaction of enzyme with methanol in the presence of an optimal concentration of H_2O_2 . Superoxide dismutase activity used tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine.

Oxygen consumption and activity with feed and light deprivation

The effect of combined feed and light deprivation on oxygen consumption and estimated routine and spontaneous activity was investigated in rainbow trout over 12 days. Fish from the holding tanks, with no previous exposure to extended darkness or feed deprivation (92.28 ± 4.23 g and 201.88 ± 2.51 mm FL), were food deprived for 24 h prior to experimentation. M_{O_2} was measured in 16 fish (eight in DD and eight in a 12:12-h LD cycle) at 16 °C. Measurements were carried out using two automated intermittent flow respirometers similar to the methodologies outlined by Steffensen (1989), Jordan and Steffensen (2007) and Khan et al. (2014a). Two custom-built 13.01-L acrylic, disc-shaped respirometers (with an Eheim® 1046 submersible flush pump on the one side and an outlet tube extending above the water on the opposite side) were placed in a 250-L reservoir filled with fresh water. The reservoir was supplied with vigorously aerated water from a 600-L system previously described by Frisk et al. (2012) with a water replacement rate of 50 L h^{-1} . Water circulation within the respirometers was provided by an external loop of tubing also containing an Eheim® 1046 submersible pump and a cuvette for the fibre optic oxygen probes which were plumbed perpendicular to the flush pump and outlets. The constant action of the recirculation pumps created a water speed of approximately 0.13 m s^{-1} in the centre of each respirometer. The large size and shape of the respirometers were selected to allow the fish to be spontaneously active rather than confine them.

Oxygen saturation in the respirometer was measured constantly by a PreSens® OXY-4 oxygen meter (PreSens, Germany) connected to a fibre optic dipping probe in the recirculating loop of each chamber. Oxygen saturation was measured constantly across three cycled phases (flushing, wait and measure) that were repeated every 12 min. The dipping probes were calibrated at the beginning of every 12-day experiment.

Fish were weighed in water, and their FL was recorded immediately prior to the start of the experiment. M_{O_2} values were then continually obtained from resting fish over the next 720 h (12 days) either in DD or in an ambient 12:12-h LD cycle (i.e. one fish per respirometer, two respirometers operating under the same light conditions). Fish activity was constantly

observed and recorded using infrared cameras. The fish were then reweighed in water at the end of the experiment to determine weight loss and calculate % body weight loss and specific growth rate (SGR) using the following formula: $\text{SGR} = \ln m_2 - \ln m_1 / t_2 - t_1 \times 100$, where m_1 is the initial weight at the start of the growth period (t_1) and m_2 is the final weight at the end of the growth period (t_2). For every day the fish were in the respirometer, SMR was estimated modally as the first peak in a frequency distribution using $5 \text{ mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$ bins for each individual. After each experiment was complete, background oxygen consumption readings were taken to ensure that it remained negligible. All equipment was cleaned thoroughly between specimens with hot, fresh water. Routine and spontaneous activity was defined as any M_{O_2} above that individual's daily estimate of SMR and was expressed as the amount of oxygen (g) consumed in measurements of M_{O_2} above SMR. As M_{O_2} is a function of both the fish's mass and the net volume of the respirometer [see Steffensen (1989)], estimates of M_{O_2} are ultimately affected by the change in both of these variables over a 12-day period which could introduce a possible source of error. However, the exact body mass at different time points was not known. Loss in body mass ranged from 2.7 to 8.9%, but because weight loss during food deprivation occurs in a non-linear manner, no specific corrections were made.

Statistical analyses

The relationship between days of feed and light deprivation and each of U_{crit} , M_{O_2} at 0.0/1.1/2.3 BL s^{-1} and factorial AMS was analysed using a one-way analysis of variance (ANOVA). Where a significant effect was observed, a post hoc Holm–Sidak pairwise comparison was applied. Condition factor at different days of deprivation was analysed with a non-parametric Kruskal–Wallis one-way ANOVA on ranks.

Differences between time points in the immunological studies were determined by one-way ANOVAs. When the data did not follow the Gaussian distribution, values were \log_{10} transformed before performing a parametric one-way ANOVA followed by Tukey's multiple comparison test. For non-parametric data, a Kruskal–Wallis ANOVA on ranks with Dunn's multiple comparison test was used. To

provide a pictographic representation of fish immune response following light and feed deprivation, a heatmap was constructed using PermutMatrix, version 1.9.3 (Caraux and Pinloche 2005). Activity values for each immune parameter were expressed as the relative response against the initial 1 day of deprivation sample. Thereafter, the values were normalised by expressing them relative to the maximum value of a data set. A 256-color-level spectrum with yellow representing negative values whilst blue corresponding to positive values was adopted.

The M_{O_2} data from each fish was pooled (within light and days of deprivation groups) to produce frequency distributions with $5 \text{ mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$ bins and representative peaks in distribution. The normality of these M_{O_2} distributions was tested individually using the Kolmogorov–Smirnov test. SMR was analysed with a non-parametric Kruskal–Wallis one-way ANOVA on ranks as the data was not normally distributed. The grams of O_2 consumed above SMR was analysed with one-way ANOVAs within deprivation treatments (1, 4, 8 and 12 days) and within light treatments (DD and LD). Where significant effects were found between days of deprivation in light treatments, a post hoc Holm–Sidak pairwise comparison was applied. A two-way ANOVA was also applied to both variables with light treatment and days of deprivation as factors. SGR and percentage body weight (%BW) lost were analysed using a one-way ANOVA. Where parametric analyses were applied, the data was first checked for equal variance and normality. Statistical analyses were performed on Sigmapstat, V 13.0 (Systat Software, Inc., London, UK). Significance was accepted at $P \leq 0.05$.

Results

Swimming performance with progressive feed and light deprivation

U_{crit} increased significantly from 3.35 to 4.46 BL s^{-1} ($F = 3522$) between 1 and 12 days of deprivation (Fig. 1a). The estimate of M_{O_2} at 0.0 BL s^{-1} decreased significantly from 123.71 to $85.06 \text{ mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$ ($F = 6586$) between 1 and 12 days of deprivation (Fig. 1b). Condition did not change significantly over 12 days of deprivation ($H = 4.48$).

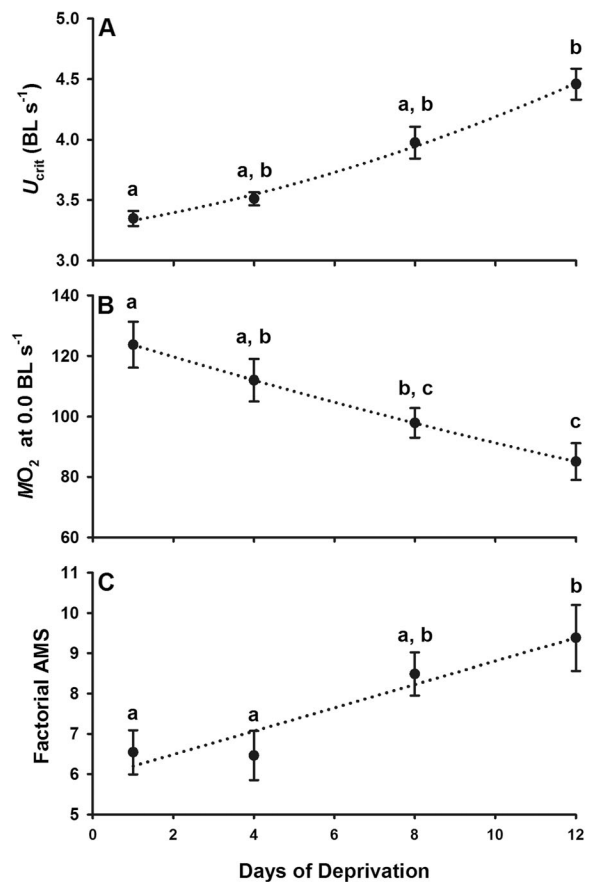


Fig. 1 The swimming performance of rainbow trout that have been deprived of feed and light for either 1, 4, 8 or 12 days. **a** Critical swimming speed (U_{crit}) in body lengths per second (BL s^{-1}), $y = 0.004x^2 + 0.052x + 3.28$, $R^2 = 0.99$. **b** Estimated mass-specific rate of oxygen consumption (M_{O_2} , $\text{mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$) at 0.0 BL s^{-1} from extrapolation, $y = 0.046x^2 - 4.10x + 127.73$, $R^2 = 0.99$. **c** Factorial aerobic metabolic scope (the 99th percentile of all M_{O_2} values for each individual, i.e. MMR divided by the 0.0 BL s^{-1} estimate, i.e. SMR), $y = 0.289x + 5.908$, $R^2 = 0.91$. Letters denote a significant difference between deprivation treatments. All values are shown \pm standard error

Factorial metabolic scope increased significantly between 4 and 12 days of deprivation ($F = 4.52$, Fig. 1c).

Immunological function with feed and light deprivation

ALP activity was significantly downregulated at days 8 and 12 of deprivation compared to day 1 in the serum, but there was no significant effect observed in the skin mucus (Fig. 2). Esterase and lysozyme activity was significantly upregulated at days 8 and 12 in the serum compared to day 1. No significant change was observed in skin mucus.

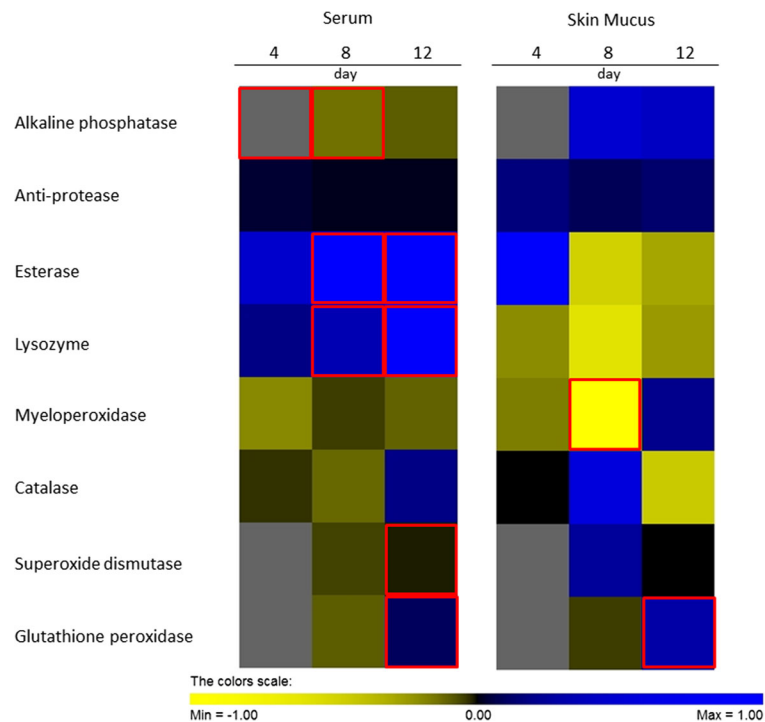


Fig. 2 Serum humoral and skin mucosal immune responses of rainbow trout exposed to short-term light and feed deprivation. The heatmap represents the relative response of several defence molecules in serum and skin mucus. Relative response values are expressed as ratios between the activity value at a specific sampling point and the initial control. Horizontal panel of the array displays the normalised relative response values of the defence

molecules at a specific time point (i.e. 4, 8 and 12 days post initiation). Spectral panels enclosed in red box signify that the response was significantly different ($P < 0.05$) from the initial control group. The color scale below indicates the mode of response relative to initial control: yellow represents negative whilst blue reveals positive response

Of the antioxidant enzymes investigated (i.e. myeloperoxidase, catalase, superoxide dismutase and glutathione peroxidase), only glutathione peroxidase was significantly upregulated in both the serum and skin mucus, both at day 12 (Fig. 2). Myeloperoxidase was only significantly upregulated at day 8 in skin mucus, and superoxide dismutase was only significantly down-regulated at day 12 in serum.

Oxygen consumption and activity with feed and light deprivation

For both the DD (Fig. 3a–d) and the LD treatments (Fig. 3e–h), oxygen consumption becomes increasingly unimodal as the days of deprivation increase. The modal peaks in the M_{O_2} distributions also decrease with the number of days of deprivation with the exception of days 8 and 12 in the DD treatment (Fig. 3c, d,

respectively) where the peak occurs in the same 75–80 mg O_2 kg^{-1} h^{-1} bin.

Weight loss over 12 days of deprivation, as measured by SGR and %BW loss, was significantly higher in the LD treatment than in the DD treatment. SGR was -0.43% $BW\ day^{-1}$ in the LD treatment and -0.35% $BW\ day^{-1}$ in the DD treatment ($F = 12.66$). %BW lost was 4.99% in the LD treatment and 3.33% in the DD treatment ($F = 13.06$).

SMR decreased significantly between 1 and 12 days of deprivation for both the DD and the LD treatments (Fig. 4a, $H = 19.57$ and $H = 27.35$, respectively). There was no significant difference in the SMR between the DD and LD treatments at any of 1, 4, 8 or 12 days of deprivation. A two-way ANOVA revealed that the light treatment had no significant effect on the relationship between SMR and days of deprivation ($F = 0.09$) and that there was no significant interaction between days of deprivation and the light regime ($F = 3.35$).

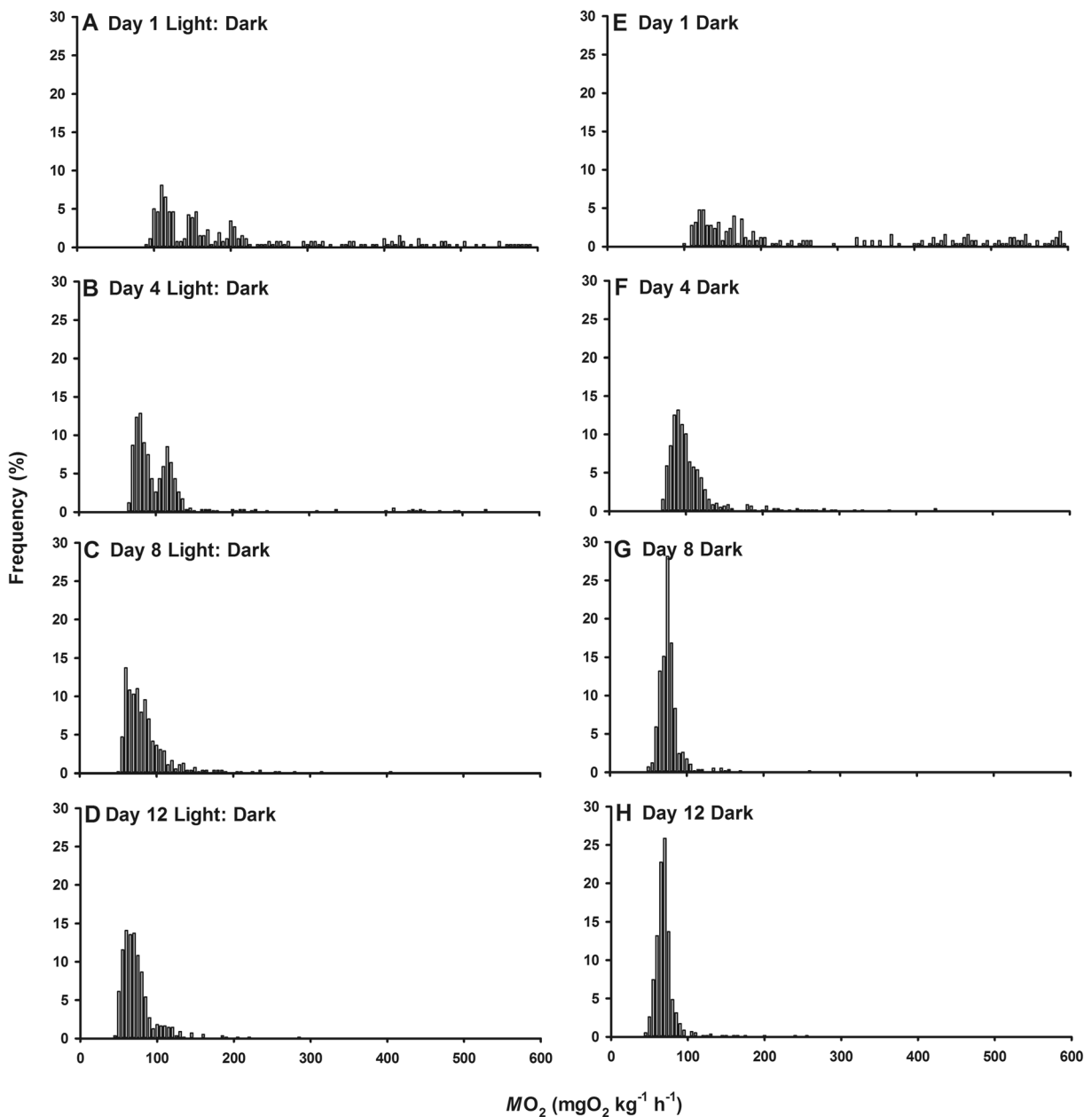
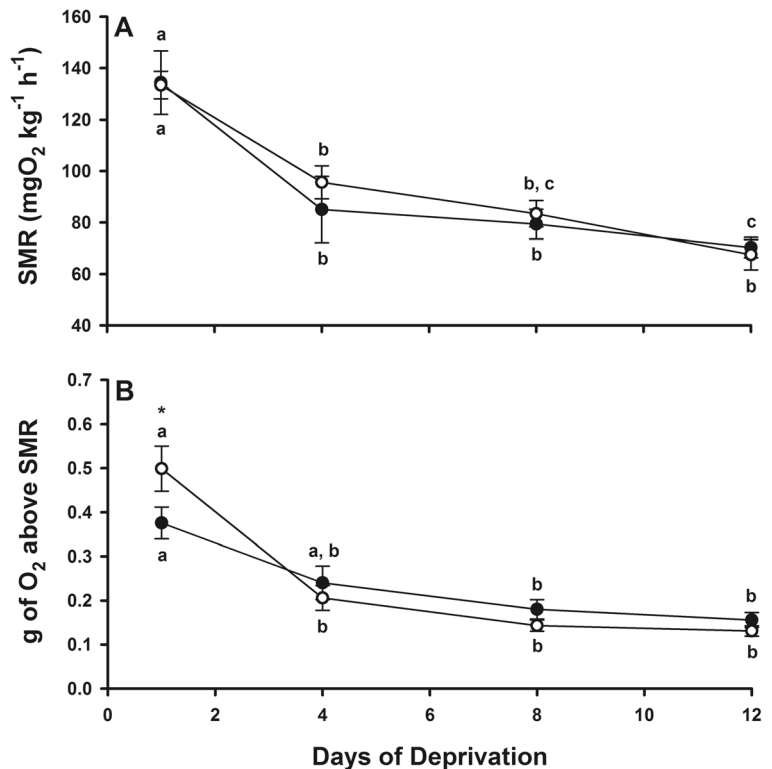


Fig. 3 Pooled frequency distribution of mass-specific oxygen consumption values (MO_2 , $mg\ O_2\ kg^{-1}\ h^{-1}$) for rainbow trout deprived of feed and kept in either a LD cycle (light–dark, **a–d**) or total darkness (dark, **e–h**) for 1, 4, 8 or 12 days

The grams of O_2 consumed above SMR also decreased significantly between 1 and 12 days of deprivation for both the DD and LD treatments (Fig. 4b, $F = 11.04$ and $F = 9.24$). At day 1 of deprivation, the fish in DD consumed significantly less oxygen above SMR on average than those in the LD treatment ($F = 4.70$). At days 4, 8 and 12, there

was no significant difference between the DD and LD treatments. A two-way ANOVA revealed that the light treatment had no significant effect on the relationship between grams of O_2 consumed above SMR and days of deprivation ($F = 0.17$). There was, however, a significant interaction effect between days of deprivation and the light regime ($F = 3.84$).

Fig. 4 **a** SMR and **b** total oxygen consumed above SMR (in grams, a proxy for energy expenditure above maintenance costs) in rainbow trout deprived of feed and kept in either total darkness (DD, filled circles) or 12:12 h LD (open circles) for a total of 12 days (values shown are at days 1, 4, 8 and 12). An asterisk denotes a significant difference between light regimes at a particular time point. Letters denote a significant difference between time points within the light treatment. All values are shown \pm standard error



Discussion

The hypothesis of the current study was based on the assumption that swimming performance would improve as the physiological costs of SDA are reduced and eventually ablated but then decrease as metabolic fuel stores are depleted. This was not the case as swimming performance continued to increase over the 12-day period (U_{crit} , Fig. 1a). The few examples of swimming performance in food-deprived fish have shown a considerable decrease in performance with food deprivation, though after longer periods. For example, Martínez et al. (2003) demonstrated that Atlantic cod *Gadus morhua* which had been food deprived for 16 weeks had 30% of the swimming endurance (i.e. time/distance swum at 1.8 BL s⁻¹) of fish that had been fed twice weekly for the same period. The same authors also reported a significant decrease in the glycogen content of both red and white muscles. In a later study, Martínez et al. (2004) also determined that short-distance sprint speeds were also significantly decreased in 12-week starved Atlantic cod compared to fed conspecifics. In both studies, there was an

obvious preference to retain red muscle oxidative capacity over white muscle glycolytic capacity which has also been seen previously in rainbow trout (Kiessling et al. 1990) and the crucian carp *Carassius carassius* (Patterson and Goldspink 1973). This suggests that swimming performance at high speeds, where white muscle fibres are recruited, would be reduced before performance at low speeds, though this is not seen between 1 and 12 days of deprivation in the current study. Lapointe et al. (2006) observed reduced swimming performance in food-deprived Atlantic cod, but with the addition of a significantly higher estimate of MMR, AMS and post-exhaustive M_{O_2} . Lapointe et al. (2006) observed an increase in AMS but did not observe the decrease in SMR observed in the current study (Fig. 1b). Rainbow trout appear to be able to improve performance at high speed without any reductions in performance across their entire swimming speed range.

Information on the direct effects of photoperiod on swimming performance is sparse. Juvenile largemouth bass *Micropterus salmoides* have a significantly higher U_{crit} when reared at shorter

photoperiods (9:15 h LD), but only at lower temperatures (Kolok 1991). In white crappie *Pomoxis annularis*, swimming performance was highest at a shorter photoperiod (8:16 h LD) than at all longer photoperiods, as well as DD, independent of temperature (Smiley and Parsons 1997). Although neither of these studies elucidated the exact mechanism, it appears that shorter photoperiods may increase swimming performance.

It is likely that the major mechanism driving the increase in swimming performance is that the short feed deprivation period reduces some of the physiological load that would otherwise reduce the aerobic scope for performance (Khan et al. 2014b). Following a few days of fasting, the energetic costs associated with specific dynamic action [i.e. digestion and assimilation of nutrients, SDA (Jobling 1983; Frisk et al. 2013; Li et al. 2013) and de novo protein synthesis for growth (Coulson et al. 1978; Houlihan et al. 1995; Smith and Houlihan 1995)] are nearly non-existent and that a larger proportion of AMS is available for swimming (Beauchamp et al. 1989; Jobling 1993; Khan et al. 2015; Auer et al. 2016). It is known that rainbow trout fed to satiation have significantly reduced U_{crit} values compared to those that have been deprived of their morning ration (Alsop and Wood 1997). It has also been shown that the duration of the SDA response in rainbow trout can range from 40 to 73 h depending on the individual, ration size and diet composition (LeGrow and Beamish 1986; Secor 2009). It is reasonable to assume that estimations of U_{crit} and swimming performance would increase over this period as less metabolic capacity is being allocated to protein synthesis and growth. A reduction in energetic costs associated with SDA might also explain the significant decrease in the estimate of M_{O_2} at 0.0 BL s^{-1} between 1 and 12 days of deprivation (Fig. 1b) as rates of protein turnover would decrease to a minimum. Fractional rates of protein synthesis in rainbow trout have been shown to decrease significantly after 14 days of feed deprivation [$\sim 70\%$ in white muscle and $\sim 49\%$ in red muscle (Loughna and Goldspink 1984)] which may account for some of the $\sim 30\%$ decrease in M_{O_2} at 0.0 BL s^{-1} between 1 and 12 days of deprivation (Fig. 1b). Considering that growth and rates of protein accretion are also positively related to photoperiod in fish (Boeuf and Le Bail 1999; Kissil et al. 2001), DD may also reduce the physiological load of protein synthesis.

Several defence molecules responded to feed and light deprivation, though the collective effect was minimal (Fig. 2). The humoral components were more affected than their mucosal counterparts as marked changes were observed more discernibly in serum than in the skin mucus. A decrease in ALP activity in the first 8 days of deprivation (Fig. 2) may pose an immunological threat to fish as it can lead to an increase in phosphorylated proteins (Xu et al. 2000; Molina et al. 2005), though it did return to basal levels after 12 days. Feed and light deprivation triggered significant elevation of the antibacterial defences, lysozyme and esterase. Lysozyme activity is thought to increase during exposure to longer photoperiods in rainbow trout and other fish (Pickering and Pottinger 1989; Ángeles Esteban et al. 2006; Uribe et al. 2011), but it is suppressed at 24:0 h LD, likely due to chronic stress (Leonardi and Klempau 2003). There was a significant increase in serum levels despite the lack of light, suggesting that short-term feed deprivation may play a significant role on its own. The varied response of antioxidant enzymes is consistent with measurements in Atlantic salmon and rainbow trout where antioxidant capacity was measured in response to vitamin supplementation (Andersen et al. 1998; Kiron et al. 2004). Given the minimal changes revealed by the selected biomarkers following deprivation, it is plausible that the imposed conditions did not have far-reaching impacts in the immunological status of fish. The changes observed in the present study may also be a general adaptive response to cope with changes in photoperiod, similar to the responses of Atlantic cod (Giannetto et al. 2014) and Nile tilapia *Oreochromis niloticus* (Lazado et al. 2016), when exposed to constant illumination.

Fish are able to conserve energy during food deprivation through biochemical, physiological and behavioural adaptations which could include reductions in voluntary activity. The current study has demonstrated that there is a significant reduction in energy use between 1 and 12 days of feed and light deprivation. Furthermore, there is a significant difference in the degree of energy conservation between rainbow trout that have been deprived of feed and light for 12 days and those that have been deprived of feed but kept on a LD cycle. This agrees with the hypothesis and is most evident from the significant difference in weight loss between the two light regimes. The simplest explanation for this is that voluntary/spontaneous activity levels were reduced in the fish that were kept in DD which

conserved energy stores and body mass. M_{O_2} frequency distributions also become unimodal more quickly in the fish exposed to total darkness compared to those who were exposed to a LD regime (day 4, Fig. 3b, f). The distributions on days 8 and 12 also appear to have less pronounced “tails” into higher M_{O_2} values in the fish exposed to DD, suggesting lower levels of spontaneous activity and energy consumption (Fig. 3c, d, g, h).

The absolute amount of oxygen consumed above SMR provides a measure of energy expenditure above the estimated minimum requirements, accounting for all voluntary and spontaneous activities (Fig. 4b). As expected, this value decreases significantly between 1 and 12 days of deprivation for both light regimes (Pandian and Vivekanandan 1976; van Dijk et al. 2002) and is what would be expected during confinement within a (large) respirometer (Steffensen 1989; Clark et al. 2013). SMR also decreases significantly over the 12 days of deprivation, as expected (Fig. 4a). On day 1 however, spontaneous activity is significantly lower in fish that were exposed to DD than those exposed to light, suggesting that darkness reduces activity quicker. The bimodal distribution of Fig. 3b may also be displaying a higher level of routine and spontaneous activity in fish exposed to light than the unimodal distribution of the fish exposed to DD after 4 days (Fig. 3f). Previous studies have shown that the highest proportional loss in body weight occurs in the first few days of food deprivation in fish (Einen et al. 1998), and the current data supports this. Changes in energy expenditure during this initial 2–3-day period may have important consequences as to how long the deprivation can be tolerated.

The current study has revealed that rainbow trout are not adversely affected by up to 12 days of feed and light deprivation and that their immunological states are not compromised. It also shows that weight loss is reduced as a result of reduced light exposure. In periods of controlled feed deprivation in aquaculture (e.g. prophylactic/disease treatment and preparation for slaughter), darkness may be an effective tool to limit weight and condition loss though reduced activity. Improved swimming performance over 12 days of deprivation is an interesting result and begs the question: When will U_{crit} decrease due to metabolic fuel limitation? Potentially, improved performance with short-term food deprivation may have adaptive benefits in terms of securing prey items when food is scarce.

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