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Additive effects of enhanced ambient ultraviolet B radiation and increased temperature on immune function, growth and physiological condition of juvenile (parr) Atlantic Salmon, *Salmo salar*

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ABSTRACT

Climate change models predict increased ultraviolet B (UVB) radiation levels due to stratospheric ozone depletion and global warming. In order to study the impact of these two environmental stressors acting simultaneously on the physiology of fish, Atlantic salmon parr were exposed, for 8 weeks in outdoor tanks, to different combinations of UVB radiation (depleted and enhanced) and temperature (standard rearing temperature of 14 °C or 19 °C). The immune function (plasma IgM, lysozyme activity and complement bacteriolytic activity), growth (body weight) and physiological condition (haematocrit and plasma protein concentration) of the fish were determined. Increased UVB level, regardless of water temperature, had a negative effect on immune function parameters, growth and physiological condition. Higher temperature increased plasma IgM concentration but had a negative effect on complement bacteriolytic activity under both spectral treatments. Increased temperature, irrespective of UVB level, increased fish growth but negatively affected haematocrit and plasma protein. Exposing the fish to enhanced UVB at elevated temperature increased plasma IgM concentration and slightly improved growth. However, complement activity and physiological condition parameters decreased more than when the fish were exposed to each stressor separately. The changes were mainly additive; no interactive or synergistic effects were observed. The negative impact of multiple stressors on immune function, together with predicted increases in pathogen load in warmer waters resulting from global climate change, suggest an increased risk to diseases in fishes.

1. Introduction

Global warming, caused by increasing concentrations of greenhouse gas in the upper atmosphere, is contributing to polar stratospheric ozone loss and delaying the recovery of the ozone layer [1]. Carbon dioxide, methane and other greenhouse gases warm the troposphere but cool the stratosphere [2] which increases the rate of ozone destroying chemical reactions. It is also now widely accepted that reductions in stratospheric ozone are connected to increases in ambient ultraviolet B (UVB, 290–320 nm) radiation [3,4]. Severe seasonal reductions in the thickness of the ozone layer are not restricted to Antarctic and Arctic regions [5,6]. As a result of air mass mixing UVB levels have also increased significantly at mid-latitudes in both the northern and southern hemispheres [7,8] including the Scandinavian region [9]. Recent (2005) observations revealed formation of polar stratospheric clouds over the Arctic, including Norway, and associated ozone losses of up to 30% reaching as far south as northern Italy (see: www.ozone-sec.ch.cam.ac.uk). Thus, ozone layer depletion and concomitant increases in UVB are worldwide phenomena that are inextricably linked to global climate change [10]. The strong link between global warming and ozone loss indicates that they cannot be treated in isolation [5].

UVB penetrates natural waters to greater depths than had previously been widely accepted [11,12]. Underwater measurements of UVB in Norwegian fjord systems indicate typical K_{d10} depths (depth at which 10% of surface impinging UVB penetrates)

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of 2–4 m at 310 nm [13]. Thus, in shallow rivers and streams with low dissolved organic carbon concentrations, some level of ambient UVB can penetrate to the bottom. When superimposed upon ozone depletion-related increases in UVB, extended daily exposures during the Nordic spring and summer (significant levels of UVB present from 05:00 h to 22:30 h) represent an additional factor affecting the susceptibility of aquatic organisms to UVB-induced damage in mid-latitude areas.

A growing number of studies indicate that UVB, even at current levels, is harmful to aquatic organisms and may reduce the productivity of marine ecosystems [14,15]. For example, exposing planktonic marine (including salmon) and freshwater fish eggs and their larvae to UVB results in increased mortality and may lead to poorer recruitment to adult populations [16,17]. Higher mortality in freshwater crustaceans that do not have effective photo-enzymatic repair mechanisms has been observed when they were exposed to UV radiation coupled with increased temperature [18]. Of five fish species studied three species, including salmonids Brook Trout (*Salvelinus fontinalis*) and Rainbow Trout (*Oncorhynchus mykiss*), were found to lack photo-enzymatic repair [19]. Thus, there exists the potential for effects of UVB and temperature on health measures in fish.

Exposure to stressors such as declining pH, heavy metal contamination, and habitat disturbance can have negative effects on wild salmon [20]. Added to these stressors is the threat of increasing levels of UVB radiation which has also been shown to negatively affect wild salmon populations [17]. Harmful effects of UVB radiation, at the organism level, include; increased stress [21] and reduced movement [22], changes in fatty acid profiles [23], skin lesions [24] and development of cataracts [25]. Further, several experimental short- and long-term exposure studies have reported negative effects of UVB irradiation on the immune system of fishes [26,27]. Earlier we reported that juvenile Atlantic salmon exposed to enhanced UVB grew more slowly, had reduced immunoglobulin level, lower haematocrit and decreased plasma protein concentration [28].

There are only a limited number of studies on biological impacts of multiple stressors on fishes. These studies demonstrate that the impacts have interactive, additive or synergistic effects on specific stress responses and, ultimately, can increase mortality [29,30]. Links between increase in UVB (stratospheric ozone loss), and the temperature shifts have been studied for example in frogs and *Daphnia* [31–33] but not in fish. In this context, we undertook a study to assess the simultaneous effects of increased temperature and increased UVB exposure on the immune system, physiological condition indices and growth in juvenile Atlantic Salmon.

2. Materials and methods

2.1. Experimental setup

The study was carried out at the Institute of Marine Research's (IMR) Austevoll Research Station (60°5′42″N, 5°13′8″E) in Norway. The Atlantic Salmon juveniles in the experiments were from the IMR's Matre Research Station. The fish were kept outdoors in round 4500 L, flow-through tanks. Three nylon cages (50 × 60 cm; 10 mm mesh size) were placed in a row in each tank for each of the spectral–temperature treatment combination. The fish in two of these cages were used for the assessment of growth, physiological condition and immune function. Each cage was stocked with 100 juvenile Atlantic Salmon (mean weight at start = 8.3 g). The fish were randomly divided into the cages to achieve even size distributions, and were fed with commercial salmon feed. The depth of the water column above the bottom of the net cages was 30 cm. The water in the tanks was sand-filtered freshwater from a lake. Oxygen in the water was monitored continuously and was always near full saturation.

2.2. Treatments

The fish were exposed to two spectral treatments differing in the dose of UVB radiation. These two spectral treatments were carried out simultaneously at two temperatures; at the normal rearing temperature of salmon in the area of the experimental site (14 °C) and at 5 °C above the normal rearing temperature (increased to 19 °C by a thermostatic heater) to explore potential additive/synergistic effects of different combinations of the two environmental stressors. The spectral treatments were: 1) UVB-depleted solar radiation: sunlight was screened though polyester plastic film (0.2 mm thick Mylar-D®, DuPont Teijin Films, Delaware, USA, 50% transmittance at 318 nm) [34], and, 2) solar radiation supplemented with UVB radiation from an overhead fluorescent tube lamp (TL40/12 RS, Philips Lighting, Rosendal, NL, emission maximum at 315 nm) placed 100 cm above the water surface. To remove UVC radiation the lamp was wrapped in cellulose triacetate film (CTA, 95 μm, Clarifoil Co., UK), and the film was changed every 18 h. The lamp was turned on at noon for 4 h. For the spectral output of the TL40/12 RS lamp, see Salo et al. [35]. The duration of the experiments was 54 d (July 17–Sept. 8, 2003).

2.3. Radiometry

Ambient radiation data was obtained from multi-channel radiometer (GUV-541, Biospherical Instruments, CA, USA) located in Bergen (University of Bergen) 22 km north of the experimental site. The diffuse attenuation coefficient (K_d) of the water used in the experiments was 18 m^{-1} at 310 nm, and was measured using a spectroradiometer (OL-754, Optronic Laboratories, Fl, USA) equipped with an underwater sensor (OL-470WP, Optronic Laboratories). The exposure experienced by the fish was determined by calculating irradiance at depths of 1 and 30 cm using this K_d. The irradiance output of the UV supplemental lamp was added to the ambient UVB levels to calculate mean daily irradiance of the enhanced UVB treatment. UVB supplemented irradiation simulated an ozone depletion of 21% based on calculation using the delta-Eddington approximation algorithm [36], ambient radiation data and the irradiance output of the supplemental lamps. The irradiance received in the UVB-depleted treatment was calculated from the ambient irradiance and the spectral transmission of the Mylar-D® material (measured with the OL-754 spectroradiometer). Average daily irradiances in the spectral treatments are given in Table 1.

Table 1
Average daily irradiances in the spectral treatments and under natural sunlight.

	Average daily irradiance (kJ/m^2)	
	305–320 nm	321–367 nm
Sunlight supplemented with UVB		
Ambient	36.0	360
At depth 1 cm	29.0	316
At bottom of cage	0.12	8.4
UVB-depleted sunlight		
Ambient	0.50	173
At depth 1 cm	0.44	153
At bottom of cage	0.00	4.5
Natural sunlight		
Ambient	30.7	355
At depth 1 cm	25.4	312
At bottom of cage	0.11	8.4

2.4. Sampling

The number of fish sampled from each treatment was $n = 50$ from each of the replicate cages (total $n = 100$ per treatment). The fish were first anesthetized with 0.01% tricaine (MS-222, Sigma), the weight and length were measured, and blood samples were taken into heparinised capillaries (75 μL, Hirschmann, Germany) after cutting off the tail. Capillaries were centrifuged 10,500 × g for 5 min to determine haematocrit, then plasma was separated from packed cells and frozen (−70 °C) until analyses.

2.5. Assessment of immune function, growth and physiological condition

The immune status of experimental fish was assessed with three immune function assays: plasma total immunoglobulin M (IgM) concentration, plasma lysozyme activity, and plasma complement bacteriolytic activity. The body weight of fish at the end of the study was used as a measure of growth. Blood haematocrit and plasma total protein concentration were measured and used as parameters reflecting the general physiological condition of fish.

2.6. Plasma total protein concentration, lysozyme activity and complement activity

The plasma total protein concentration was measured with a BioRad Protein Assay Kit (BioRad Inc., USA) using bovine serum albumin (BSA) as the standard. The lysozyme enzyme activity in plasma was determined with a turbidometric microplate assay [37] using a *Micrococcus lysodeicticus* (Sigma) suspension (1 mg mL^{-1} phosphate buffer, pH 6.2) as the substrate. The optical density of bacterial suspension in the wells was measured with a plate reader (Victor^2 1420 Multilabel Counter, Wallac Co, Finland) at 1 min intervals, for 30 min, at 450 nm. The complement total activity was determined using a previously published method [28,38]. In short, *Escherichia coli* KL12 pEGFLLucAmp, containing the reporter gene for luciferase, was grown in LB-medium with the antibiotic tetracyclin. Harvested bacteria were mixed in the wells of 96-well microplates (Microfluor 1 Black, Thermo Labsystems, USA) with plasma and chromatically purified anti-*E. coli* specific antibody prepared from the serum of *E. coli* KL12 pEGFLLucAmp-immunized Atlantic Salmon. After a 90-min incubation, luciferin (Sigma) in citrate buffer pH 5.0 was added and the luminescence was measured (Victor^2 1420 Multilabel Counter). The luminescence data was converted to viability of bacteria, and the volume of plasma killing 50% of bacteria (CV50) was calculated. The results were expressed as complement bacteriolytic (CB) activity Units/mL (CB50 U/mL = 1000 μL/CV50).

2.7. Quantification of plasma IgM

The concentration of IgM in plasma was determined with an enzyme-linked immunosorbent assay (ELISA) as described earlier [28]. Briefly, flat-bottomed 96-well microplates (Nunc MaxiSorp, Nunc, Denmark) were coated overnight with anti-salmon IgM specific CLF002 antibody (Cedarlane, Canada). After washing and saturation with BSA, plasma samples and chromatographically purified salmon IgM (used as a standard) were incubated in the wells. The trapped IgM was detected with biotin-conjugated CLF002 antibody. Alkaline phosphatase-conjugated avidin (Sigma) was added into wells after washing the plates. P-nitrophenylphosphate (Sigma) was used as the substrate and the optical density at 405 nm was read with a plate reader (Multiskan Plus, Flow Laboratories).

2.8. Statistical analysis

The values of haematocrit, plasma IgM and lysozyme activity were log-transformed to meet the requirements of normality and homogeneity of variance. The blood variables were grouped into two response sets for multivariate analysis [39]: immune function (plasma IgM concentration, lysozyme activity and complement bacteriolytic activity), and physiological condition (blood haematocrit and plasma protein concentration). The data were analyzed for effects in response sets using multivariate analysis of variance (MANOVA). The effects of treatments on growth were analyzed with ANOVA. A two-way ANOVA was used to analyze the separate and interactive effects of spectral and temperature treatments. In all analyses treatment (UVB or temperature) was used as the fixed factor, and cage in tank was the random factor hierarchically nested inside the treatment. SPSS Statistical software (ver. 16, SPSS Inc, USA) was used for all statistical analyses.

3. Results

Treatments (UVB level, temperature) had a statistically significant effect on immune function (MANOVA, Wilk's $\lambda = 0.00086$, df = 9; $F = 9.599$, $P = 0.011$), physiological condition (MANOVA, Wilk's $\lambda = 0.00295$, df = 6, $F = 17.411$, $P = 0.001$), and the growth of fish (body weight) (ANOVA, $F = 129.447$, df = 3, $P < 0.001$). Two-way ANOVA indicated that spectral and thermal treatments, separately applied to fish, had a significant effect on all parameters tested, except plasma lysozyme activity. Further, the interaction term of treatments was not statistically significant indicating that the effects of the treatments were independent (Table 2).

3.1. Effects on immune function

Spectral and temperature treatments had marked effects on fish immune function (Fig. 1). Exposure to enhanced UVB consistently decreased plasma IgM concentration at both temperatures. However, increased temperature alone nearly tripled plasma IgM concentration compared to that of fish kept at 14 °C in both spectral treatments. In the case of double stress, i.e. increased UVB and higher temperature, the IgM concentration was more than twice that of the group exposed to UVB-depleted sunlight at 14 °C. A

Table 2
Two-way ANOVA of responses to spectral (UVB-radiation level) and thermal (Temperature) treatments, and the interaction of the treatments (UVB * Temperature) in exposed Atlantic Salmon parr.

Parameter	UVB-radiation level			Temperature			UVB * Temperature		
	F	df	*P*	*F*	df	*P*	*F*	df	*P*
IgM	38.197	1	0.003	519.603	1	<0.001	0.108	1	0.759
Lysozyme	0.096	1	0.772	6.028	1	0.070	0.633	1	0.471
Complement	2102.986	1	<0.001	8.609	1	0.043	1.858	1	0.245
Weight	95.281	1	0.001	291.951	1	<0.001	1.108	1	0.352
Hct	17.002	1	0.015	129.835	1	0.001	5.722	1	0.075
Protein	27.125	1	0.006	14.520	1	0.019	3.132	1	0.151

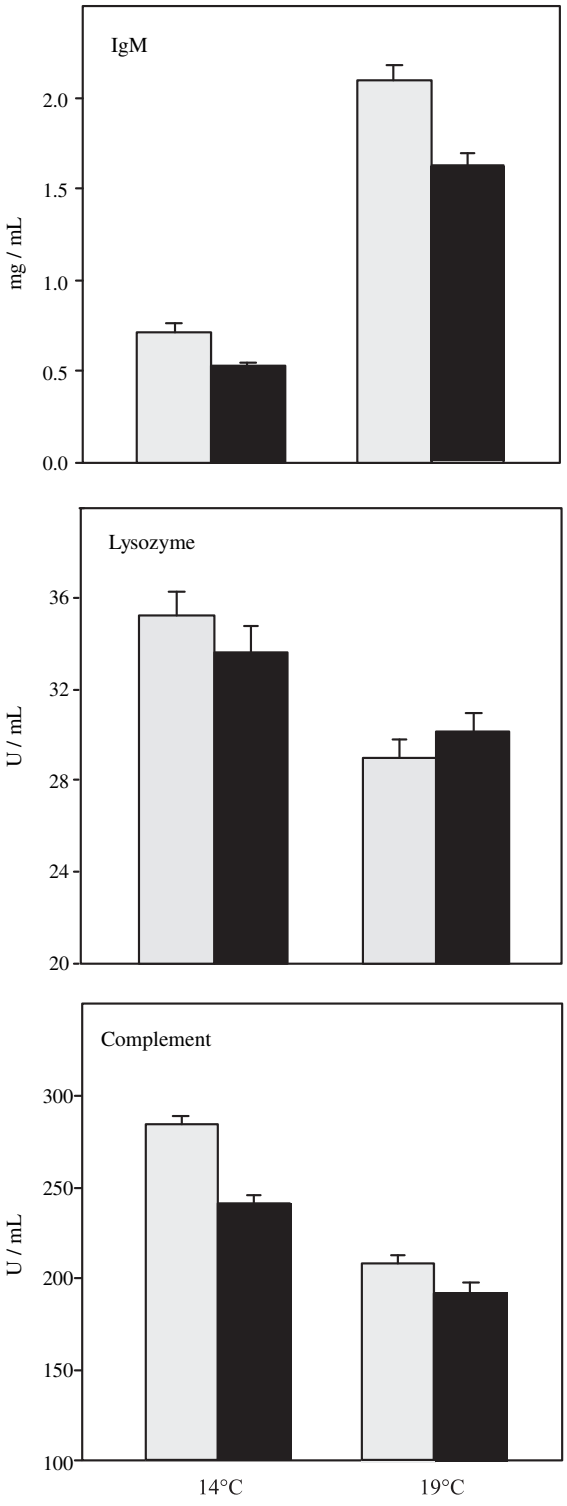

Fig. 1. Plasma IgM, lysozyme activity and complement bacteriolytic activity of Atlantic Salmon parr after exposure to spectral treatments: UVB-depleted sunlight (gray bar), or sunlight supplemented with UVB from a lamp (black bar) at normal rearing temperature (14 °C) and at increased temperature (19 °C). Each bar represents the mean ± SE (n = 100 fish).

decreasing trend was observed in lysozyme activity in responses to thermal treatments but the differences did not reach statistical significance. Complement bacteriolytic activity responded negatively to both stressors. Complement activity was highest in fish exposed to UVB-depleted sunlight at 14 °C and was significantly decreased in fish exposed to enhanced UVB at 19 °C.

3.2. Effects on growth and physiological condition

Fish growth (body weight) was significantly related to both spectral treatment and temperature (Fig. 2). Exposure to enhanced ambient UVB level resulted in reduced growth regardless of temperature, and the increase in temperature promoted growth in both spectral treatments. Comparison of extreme treatments (increased UVB at 19 °C vs. UVB-depleted sunlight at 14 °C) showed that growth inhibition due to increased UVB level was overshadowed by faster growth at higher temperature finally resulting in higher body weights.

Fish physiological condition parameters were affected by UVB exposure level and temperature (Fig. 2). Enhanced UVB significantly affected the haematocrit of fish kept at 14 °C but not in fish at 19 °C. Increased temperature reduced haematocrit regardless of UVB level. The haematocrit value in fish exposed to enhanced UVB at 19 °C was decreased compared to that of fish exposed to UVB-depleted sunlight at 14 °C. Plasma total protein concentration in the fish exposed to enhanced UVB was decreased, but only the difference between spectral treatment groups at 14 °C reached statistical significance. Increased temperature decreased protein concentration only at 14 °C. Plasma protein concentration decreased in fish exposed to enhanced UVB at 19 °C when compared to fish exposed to UVB-depleted solar radiation at 14 °C.

4. Discussion

Numerous physical and chemical stressors affect the physiology of fishes. For example, acute or chronic stress impairs growth, reproduction and resistance to infections [40,41]. Most studies on environmental stressors investigate the effect of exposure to a single agent although simultaneous exposure to multiple stressors is the norm under natural conditions. Our study examined the impact of two environmental stressors acting separately and in combination on the physiology of Atlantic Salmon parr. The stressors were enhanced UVB level and increased temperature, and the endpoints measured were the immune status (plasma IgM concentration, lysozyme and complement bacteriolytic activities), growth (body weight) and physiological condition (haematocrit and plasma protein).

4.1. Exposure simultaneously to two stressors

The effects of the stressors were not interactive or synergistic; the changes detected were additive. The final outcome from the two simultaneously applied treatments was close to the sum of effects of the stressors separately.

The response to thermal stress (19 °C) was an increase in plasma IgM concentration and growth while the other parameters decreased. When the treatments were applied simultaneously to fish, the effect of elevated temperature exceeded the effect of spectral treatment on IgM concentration and growth, but the increases in the parameters were smaller in the double stress situation than in thermal exposures alone. However, exposing fish simultaneously to both stressors resulted in a stronger negative impact on another immune function parameter, complement bacteriolytic activity. Plasma lysozyme activity was less affected by the two treatments. With respect to physiological condition indices (i.e. haematocrit and plasma protein), exposure to the double stress situation resulted in lower values than what was measured in the separate treatments.

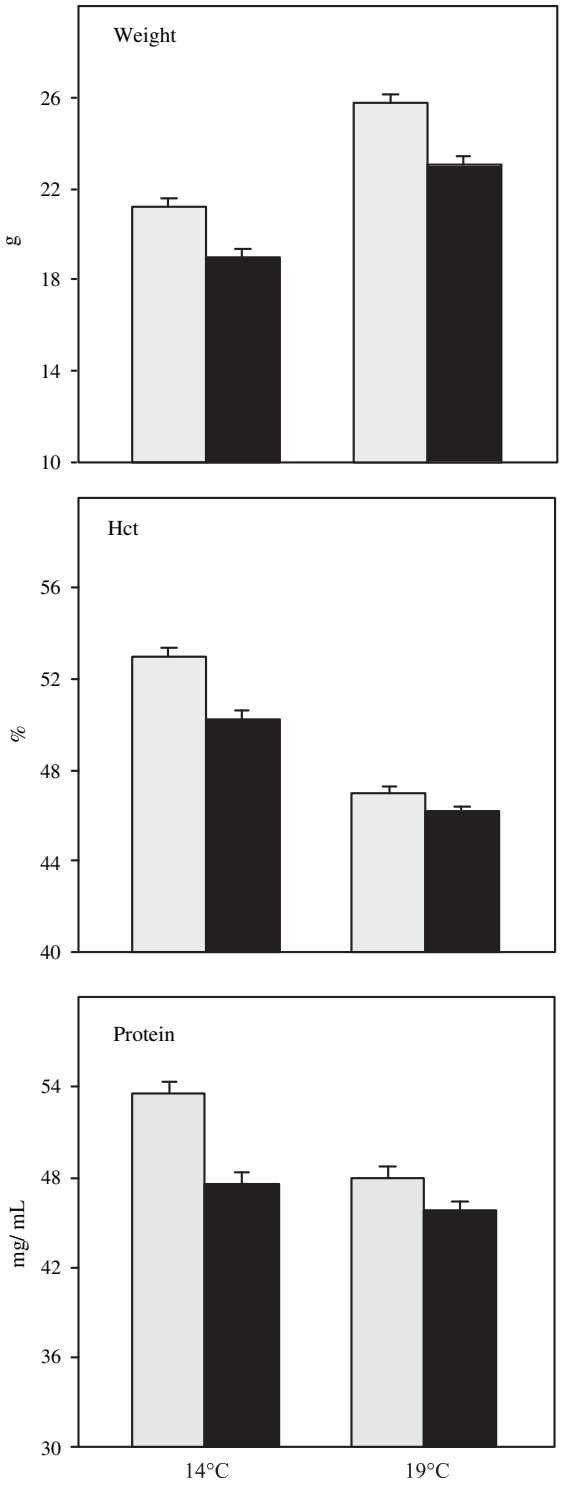

Fig. 2. Whole body weight, haematocrit and plasma total protein of Atlantic Salmon parr after exposure to spectral treatments: UVB-depleted sunlight (gray bar), or sunlight supplemented with UVB from a lamp (black bar) at normal rearing temperature (14 °C) and at increased temperature (19 °C). Each bar represents the mean ± SE (n = 100 fish).

The same combination of long-term treatments has not been applied to fish earlier. Prophete et al. [42] found that elevated temperature modulated the effect of nickel pollution on the cellularity of the spleen and innate immune functions in head kidney leucocytes, but not mitogen-induced proliferative responses of splenic lymphocytes in Japanese Medaka (*Oryzias latipes*). In other studies, increased temperature, combined with high pH, increased mortality of rainbow trout [43], and higher concentration of ammonia with simultaneous low pH significantly affected stress response in acute exposure of Brown Trout (*Salmo trutta*) and Rainbow Trout [44]. More relevant to the present study, constant sub lethal UVA radiation, combined with elevated temperature, had deleterious effects on survival and metabolism of Convict Cichlids (*Cichlasoma nigrofasciatum*) [45].

4.2. Effects of exposure to UVB

UVB radiation stresses juvenile and adult fish, and negatively affects the immune function in both short-term [21,27,46–48] and in long-term exposures [28,48,49]. In the present study, long-term exposure to UVB radiation alone decreased plasma IgM concentration and complement bacteriolytic activity. Decreased IgM production suggests disturbed lymphocyte functions in UVB exposed fish. Lysozyme level increases under acute stress [50] but decreases under more chronic stress [51], but in this study no consistent effect of enhanced UVB on lysozyme activity was found. Reduced growth and decreased plasma protein concentration were noted in fish exposed to UVB. Chronic stress suppresses the growth of fish by its negative impacts on appetite and by stimulating catabolism [52,53] or energy allocation to digestion [54]. A build-up of catabolic substrates was found in UVB exposed Atlantic Salmon [23], and suppressed feeding in Coho Salmon (*Oncorhynchus kisutch*) [22] suggests that fish exposed to UVR are more quiescent. In the present study the effects of increased UVB radiation on physiological parameters were negative. We have previously observed a reduction in haematocrit levels and in plasma protein in Rainbow Trout and European Carp (*Cyrinus carpio*) exposed to UVB [26]. Overall, the present results are in consistence with earlier findings on the effect of UVB on immune function, growth and condition in juvenile Atlantic Salmon [28].

4.3. Effects of increased temperature

Because fish are poikilotherms, ambient temperature has a pervasive effect on their physiological functions including immune function and growth. Temperature, along with photoperiod, causes seasonal changes in the immune function of fish affecting both innate and acquired immune responses; for reviews see [55,56]. In general, low temperatures inhibit immunological responsiveness in fishes [40,57], but higher temperatures may have either positive or negative effects on immune function [55,58]. Marked increases in temperature cause stress responses in fish resulting in physiological changes [41,59]. However, immune function in fishes is influenced by complex interactions with temperature, and it is therefore not easy to discern when temperature becomes stressful [60].

In the present study the plasma IgM concentration of fish kept at the elevated temperature was triple compared to that at the lower temperature, and the increases were similar in both spectral treatment groups. In Atlantic Cod (*Gadus morhua*) [61], Rainbow Trout [62] and Nile Tilapia (*Oreochromis niloticus*) [63] IgM levels increased at higher temperature. However, in Channel Catfish (*Ictalurus punctatus*) [64] there was no difference in plasma IgM. In European Carp, the number of immunoglobulin secreting cells in lymphoid organs remained the same at different temperatures studied [65]. The reason for the discrepancy could be differences in thermal adaptation between fish species [63]. Another soluble defense factor, plasma lysozyme activity, increased at higher temperature in Sockeye Salmon (*Oncorhynchus nerka*) [66], juvenile Atlantic Halibut (*Hippoglossus hippoglossus*) [67], and Nile Tilapia [68]. In this study, the changes in lysozyme activity did not increase at higher temperature,

and the measurements suggested a decreasing trend in both spectral treatments (see Table 2). Complement bacteriolytic activity in the plasma of fish kept at 19 °C was markedly decreased compared to that at 14 °C. Higher acclimation temperature increased complement lytic activity in Rainbow Trout plasma [69]. In Sockeye Salmon, no difference was noted in complement activity or activity decreased when fish were acclimated at 12 °C compared to 8 °C, depending on the age of fish [66].

During the 8-week experimental period in the present study fish nearly tripled their weight, but the weight of fish kept at 19 °C was only 10–20% higher than at 14 °C. The optimum growth temperature for Atlantic-Baltic salmon is reported to be 16–20 °C [54,70,71]. Haematocrit in Atlantic Salmon decreased with increased temperature, as has been noted earlier in Sockeye Salmon [66] and Atlantic Halibut [67]. Another general indicator of physiological condition, plasma protein concentration, decreased with increased temperature but only in fish exposed to UVB-depleted solar radiation.

The spectral and thermal conditions created in the present study were realistic with respect to changes in the environment predicted by models of increased UVB radiation and global warming (see references in Introduction). In an earlier study with Atlantic Salmon parr, at the same site using the same spectral treatments, we found no difference in any of the immune parameters between groups of fish exposed to natural sunlight and UVB-depleted sunlight [28]. Based on this, the results from the UVB-depleted group in the present study can be considered as a reasonable indicator of the effect on fish exposed to natural sunlight. UVB radiation negatively affects the immune system of fishes, and can affect disease resistance directly [47], or indirectly by inducing skin lesions and by exacerbating secondary bacterial and fungal infections [72,73]. Exposure to UVB radiation causes physiological stress manifested in changes in blood cortisol levels [21]. Although we did not measure cortisol in this study, it is well known that repeated acute disturbances, through the action of corticosteroids, result in cumulative physiological stress in fish [74]. Even slight but chronic increases in cortisol levels have been implicated in depression of immune function and disease resistance [75].

The interpretation of our results as well as predicting the well-being and health of fish under scenarios of environmental changes in temperature and UVB is complicated. The effects noted in fish exposed to stressors were mostly negative and additive, although some of the changes observed were contradictory (plasma IgM and growth). Changes in climate are expected to increase parasitism due to geographic shifts in parasite species distributions, and increase incidence, spread and intensity of diseases [76]. This would be even more probable with fish whose immune capacity is compromised because of increased UVB level and higher temperature. More direct studies on the significance of the present findings, in terms of lowered disease resistance, requires challenging fish with pathogens after exposure to combinations of increased ambient UVB level and increased temperature.

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