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No environmental effect on vaccine-induced reduced growth in Atlantic salmon *Salmo salar* smolts

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ABSTRACT: Oil-adjuvanted vaccines reduce long-term growth in farmed Atlantic salmon *Salmo salar*, possibly via an increase in metabolic rate due to the energetic demands of the immune system. We tested this hypothesis by comparing sham-vaccinated to vaccinated smolts (total n = 2096, ca. 80 g) under different scenarios of water temperature (12 vs. 17°C, n = 1048 per temperature) and oxygen (O_2) saturation (60, 70, 80, and 100%, n = 524 per O_2 saturation level) in order to manipulate metabolic rate and O_2 availability. We expected a more severe vaccination effect under conditions of high water temperature and low O_2 saturation. Groups were kept in duplicate tanks under controlled temperature and hypoxia conditions for 7 wk post-vaccination before being transferred to uncontrolled common-garden natural conditions for 5 mo in a sea-cage. Body mass and length were recorded at the initiation and end of the controlled and uncontrolled environmental conditions. Vaccination and low O_2 saturation at 17°C significantly reduced body mass (13 and 3% through vaccination and 9 and 20% through 60% O_2 saturation at the end of the tank and sea-cage periods, respectively). However, there was no interaction between vaccination, temperature, and O_2 saturation at the end of the tank or sea-cage period, lending no support to our hypothesis. A secondary observation was that emaciated 'loser' fish were mainly associated with the 17°C and low (mainly 60%) O_2 saturation treatment. In conclusion, although vaccination led to a reduction in body mass, this effect was not influenced by environmental conditions expected to alter metabolic rate.

KEY WORDS: Dissolved oxygen · Hypoxia · Immunity · Antibody · Temperature · Cataracts · Losers · Skeletal deformity

1. INTRODUCTION

Salmon farming in Norway relies on the protection provided by multi-component vaccines, and this has contributed both to the growth of the industry and its relatively low level of antibiotic use compared to other salmon farming regions (Love et al. 2020). However, oil-adjuvanted vaccines induce abdominal lesions and peritonitis (Midtlyng et al. 1996), lead to short-term reductions in feed intake (Sørum & Damsgård 2004), and reduce growth by between 9 and 23% post-vaccination (Midtlyng & Lilllehaug 1998, Fraser et al. 2014). Furthermore, tissue-specific inflammation and systemic autoimmunity has been suggested

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to be induced by multivalent oil-adjuvanted vaccines (Koppang et al. 2008, Haugarvoll et al. 2010), and an increased risk of skeletal deformities has been reported (Berg et al. 2006). The adjuvants are necessary in order to provide long-term protection due to slow release of the antigens within the peritoneal cavity, but the adjuvants are also related to the occurrence of many of the unwanted side effects (Midtlyng et al. 1996). Although improvements have been made in reducing the severity and frequency of vaccine side effects, they still occur (Brudeseth et al. 2013).

The immune system is energetically demanding, due to the metabolic requirement of immune cells, but also through indirect consequences such as tissue degradation or anorexia during inflammation (Lochmiller & Deerenberg 2000). As oil-adjuvanted salmon vaccines lead to a chronically active immune response that peaks at 6 mo post-vaccination (Mutoloki et al. 2004, 2006), one may suspect that the reduced growth is a result of an increased metabolic cost of the immune system. Indeed, several studies have found increases in standard metabolic rate following vaccination in fish. For example, rainbow trout *Oncorhynchus mykiss* vaccinated against *Aeromonas salmonicida* (Ackerman et al. 2000) or with a DNA vaccine (Skinner et al. 2010) have an increase in oxygen (O_2) consumption. In addition, Fraser et al. (2015) observed an increase in heart size in vaccinated vs. unvaccinated fish, which is suggestive of an increase in cardiac workload that one may expect with an increase in metabolic demand. Finally, an increase in abdominal lesions in salmon vaccinated at what is considered high (16°C) versus lower (10°C) water temperature has also been observed (Grini et al. 2011). Here, as factorial aerobic scope is known to be reduced with increasing environmental temperature in salmon (Hvas et al. 2017), one may speculate that higher than optimal temperatures may reduce the amount of energy available to the immune system that is expected to be energy-demanding (Lochmiller & Deerenberg 2000).

Our main objective was to investigate whether vaccine-induced reduced growth could be related to metabolic demands. Our hypothesis was that reduced growth is due to an increase in metabolic demand following vaccination. To this end, we manipulated water temperature (to alter metabolic rate) and O_2 saturation to produce various levels of metabolic limitation in vaccinated and unvaccinated salmon. The various environmental conditions, all combinations of 12 vs. 17°C, and 60, 70, 80, and 100% O_2 saturation, were maintained for 1 mo following vaccination. Water temperature is positively associated with metabolic rate in salmon (Hvas et al. 2017), and the ability to withstand hypoxia decreases exponentially between 6 and 18°C (Remen et al. 2013). As such, based on cyclic hypoxia studies, we expect ≤60% O_2 saturation to be physiologically challenging at 17°C, but not at 12°C (Remen et al. 2012, 2013). As a secondary objective, we also report on the long-term effects of the environmental conditions and vaccination on growth and production characteristics (antibody production, 'loser' fish (hereafter referred to as 'losers'), post-smolt maturation, cataracts, radiological deformities) during 5 mo of common garden rearing in a sea-cage.

2. MATERIALS AND METHODS

2.1. Fish stock and rearing conditions

Atlantic salmon *Salmo salar* from the Aquagen strain were reared from first feeding (14 March 2016) at the Institute of Marine Research (IMR) facilities at Matre Research Station. Throughout, fish were fed standard diets (Skretting) with an estimated 20% surplus based on estimated growth. The temperature and photoperiod throughout the experiment are summarized in Fig. 1. In brief, the temperatures and photoperiods used prior to the study are commonly used to rear underyearling (i.e. 0+) smolts. As such, a square wave photoperiod was used to initiate smoltification. Prior to vaccination, the fish were moved to 1 × 1 m tanks (n = 10) on 28 August 2016 (n = 2096 fish, mean weight 50 g, 10.5 kg m^{-3}). Between 9 and 12 September 2016, the fish were implanted with a passive integrated transponder (PIT) tag that allows for individual recognition and distributed among 16 tanks (1 × 1 m; n = 131 fish $tank^{-1}$). Between 3 and 10 October 2016, the experimental conditions were set according to Table 1. On 10 October 2016, approximately half of the fish (65–66 $tank^{-1}$) were vaccinated with 0.1 ml AQUAVAC® PD7 vet. (MSD Animal Health, vaccine against salmon pancreas disease virus, infectious pancreatic necrosis virus, *Aeromonas salmonicida* subsp. *salmonicida*, *Vibrio anguillarum* serotype O1 and O2a, *V. salmonicida*, and *Moritella viscosa*; further details can be found online at www.felleskatalogen.no), whilst the remaining fish received 0.1 ml of sterile saline via an intra-peritoneal injection (i.e. unvaccinated controls) according to standard operating procedures (initial body size of each group can be found in Fig. S1 in Supplement 1 at www.int-res.com/articles/suppl/q012p327_supp/, for all supplemental figures). The vaccinated and unvaccinated

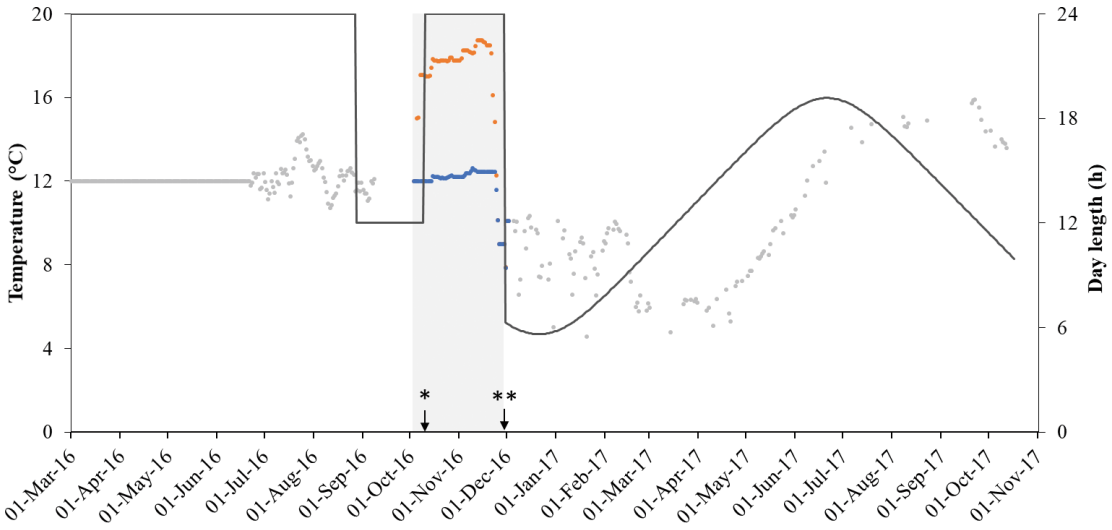

Fig. 1. Temperature (dots) and photoperiod (solid line) during the experiment. The grey zone represents the period of O_2 saturation (see Table 1) and temperature manipulation. Here, half of the fish were held at ~17°C (orange) and the other half at ~12°C (blue); otherwise, the fish were reared under a common temperature (grey dots). *Time of vaccination and **fish transferred to common-garden rearing within a sea-cage

Table 1. Daily overview during the initiation of the environmental conditions for Atlantic salmon reared under different controlled conditions of temperature and oxygen (O_2) saturation

Date	12°C groups					17°C groups				
	Temp. (°C)	Oxygen saturation (%) 60	70	80	100	Temp. (°C)	Oxygen saturation (%) 60	70	80	100
03.10.2016	12	100	100	100	100	12	100	100	100	100
04.10.2016	12	80	80	80	100	12	80	80	80	100
05.10.2016	12	70	70	80	100	15	70	70	80	100
06.10.2016	12	60	70	80	100	15	60	70	80	100
07.10.2016	12	60	70	80	100	17	60	70	80	100

fish were mixed in the same tanks (i.e. common garden). The water flow into the tanks was switched to seawater on 2 November 2016 for those fish reared at 17°C, and on 10 November 2016 for those fish reared at 12°C. The difference in seawater timing was related to the number of degree-days (the sum of the daily temperature) required for smoltification to peak. Here, 350 degree-days after the end of the winter signal are required to complete smoltification (i.e. Handeland et al. 2004). On 30 November 2016, all fish were transferred to a single sea-cage (5 × 5 × 7 m, stocking density of 1.2 kg m^{-3}) for common-garden rearing under natural temperatures and light (60° N). Data from the control fish reared at 100% O_2 saturation were previously reported in another study detailing the occurrence of out-of-season post-smolt sexual maturation (Fraser et al. 2019a).

On 18 November, an unexplained mortality event occurred in 1 tank with 100% O_2 from the 12°C group, wherein 14 fish died. Of these, 50% were unvaccinated, so mortality was not related to vaccination. A comparison of growth rates between this tank and the second tank belonging to this group showed no short- or long-term differences in mean body mass; therefore, these fish were included in all of the analyses (data not shown, but the models are given in Supplement 2 at www.int-res.com/articles/suppl/q012p327_supp/).

2.2. Sampling protocol

Body mass, fork length, and PIT tag number were collected from all fish at 3 time points: 10 October 2016 (vaccination), 30 November 2016 (transfer to sea-cage), and 26 April 2017 (end of seawinter, 28 wk post-vaccination). At each sampling time, fish were anaesthetised in 100 mg l^{-1} Finquel® (MS 222) prior to handling. Gills (stored in RNA later for gene expression) and blood serum (for antibody assessment, frozen at –80°C) were collected from 8–11 vaccinated and 9–12 unvaccinated, terminally anaesthetised fish from each of the 100, 80, 70, and 60% O_2 groups (4–6 fish $group^{-1}$ $tank^{-1}$) at 2, 4, and 6 wk post-vaccination, and blood serum was also collected in Week 28 (on 26 April 2017). These time points were chosen to provide good resolution in the development of smoltification and the antibody response. Here, all groups tested

(the 70% O_2 groups were not included) were considered seawater-adapted at 4 (17°C groups) and 6 (12°C groups) wk post-vaccination following gill gene expression analyses (Na^+ K^+ -ATPase isoforms α1a and α1b, i.e. Houde et al. 2019) by Pharmaq Analytic (Fig. S2). Cataracts (yes/no) were identified in April 2017 by visual observation of both eyes, and losers (yes/no) were identified subjectively in April 2017 based on external appearance (i.e. losers being notably small and thin, i.e. emaciated). In April 2017, a sub-sample of fish (n = 80) from the vaccinated and unvaccinated 60 and 100% O_2 saturation groups (n = 10 group^{-1}) were terminally anaesthetised and frozen for later radiological assessment of the vertebral column. Levels of sexual maturation, based on external morphology (see Fjelldal et al. 2018), were assessed throughout. Mature male post-smolts were identified by running milt, whereas females showed no sign of maturity based on the relative size of the gonopore. At the end of the study, all remaining fish were terminally anaesthetised and sexed by visual examination of the gonads.

The condition factor (CF) was calculated as CF = weight (g) / length^3 (cm) × 100. Specific growth rate (SGR,% d^{-1}) was calculated from the formula: SGR = (e^q − 1) × 100 (Houde & Scheckter 1981), where q = [In(W_2) − In(W_1)] (t_2 − t_1)$^{-1}$ (Bagenal & Tesch 1978) and where W_2 and W_1 were the live body weights in grams at times t_2 and t_1, respectively. The thermal growth coefficient (TGC) was also calculated as TGC = ($W_2^{1/3}$ − $W_1^{1/3}$) / (temperature [°C] × number of days) × 1000 (Cho 1992).

2.3. Radiology

Frozen fish were thawed and filleted to remove surrounding flesh around the vertebral column to increase the quality of the radiograph images. The vertebral columns were radiographed (Porta 100 HF; Eickemeyer Medizintechnik für Tierärzte) using a 35 × 43 cm image plate in a rigid cassette (Dürr Medical) with 40 kV and 10 mAs at a distance of 70 cm. The image plate was scanned (CR 35 VET; Dürr Medical) and the resulting image converted into a TIFF file (Vet-Exam Plus Software, version 4.14.0). Evaluation of vertebral deformities was done according to the classification of Witten et al. (2009).

2.4. Antibody assay

Enzyme-linked immunosorbent assay (ELISA) was used to determine the antibody titre in Atlantic salmon sera against one of the vaccine antigens, *Aeromonas salmonicida* subsp. *salmonicida*. Microtitre plates (96-well Nunc MaxiSorp, ThermoFischer Scientific) were coated overnight with *A. salmonicida* antigen (4.8 × 10^6 cells well^{-1}) and blocked for 1 h at 20°C with phosphate buffered saline (PBS) (Sigma Aldrich) with 1% bovine serum albumin (BSA). Serum was diluted with PBS with 1% BSA in initial dilutions of 1:100, 1:400, 1:800, or 1:1600, at Weeks 2, 4, 6, and 28, respectively. The samples were serially diluted by 2-fold dilution and incubated overnight between −2 and −8°C. Each plate included a positive and negative control serum (obtained from vaccinated and unvaccinated salmon). After incubation, microtitre plates were washed 3 times (250 µl well^{-1}) with PBS and Tween (polysorbate) 20 (PBS-Tw) (Merck Millipore) in an ELISA microplate washer (ELx405, BioTek). Rabbit-anti-salmon antibodies were used as secondary antibody (2°Ab) and, along with the conjugate (mouse-anti-rabbit HRP), were provided by Intervet International Boxmeer. The 2°Ab and the conjugate were diluted at 1:12 000 and 1:16 000, respectively. After incubation with the 2°Ab, the plates were washed 3 times using PBS-Tw. Plates were washed 6 times with PBS-Tw after incubation with the conjugate. The colour substrate 3,3′,5,5′-tetramethylbenzidine was diluted 1:60 with distilled water and ureum-peroxide buffer (Intervet International) before transferring 100 µl into each well. The reaction was stopped after 20 min by adding 50 µl of sulphuric acid (4 NH_2SO_4) into each well. Absorbance was read using a microplate reader (Sunrise, Tecan Group) at 450 nm. The raw data were imported into the analysis software CBA AbendVertical V1.21 (MSD, Proprietary Software) to calculate the antibody titres. The mean negative control value × 5 was used to set the threshold for calculating the antibody titres of the sampled sera, which were expressed in log^2, as the maximum dilution corresponding to this threshold.

2.5. Statistical analyses

The data were transferred to R version 3.6.0 (R Development Core Team, www.r-project.org). Significance was assigned at $p < 0.05$. All raw data can be found in Supplement 3 at www.int-res.com/articles/suppl/q012p327_supp/ (see tabs 'Growth', 'Prevalences', and 'Immunity') and the R script (Supplement 2) used to analyse the data. The data from 126 fish were removed from the analysis due to duplicate or triplicate PIT tag numbers (ranging from 1–27 vaccine^{-1} temperature^{-1} O_2 saturation^{-1}).

We compared models that either supported or rejected our hypothesis using the Bayesian information criterion (BIC). Two models were generated: (1) water temperature (12/17°C) × O_2 saturation (60/70/80/100%, categorical) × vaccination (yes/no) + sex (male/female); and (2) water temperature × O_2 saturation + vaccination + sex (male/female). The first model provides evidence that vaccine side-effects are dependent on metabolic demand, whereas the second model lends support to vaccine side-effects being independent of metabolic demands. Each time point was analysed separately, as we know time effects exist for the effects of water temperature on long-term growth (Grini et al. 2011), but there are no data on how O_2 saturation and water temperature interact, and a 4-way interaction (i.e. water temperature × O_2 saturation × vaccination × time) was rejected due to complexity. We also controlled for sex due to its influence on body size even in immature fish (Fraser et al. 2014). The model with the lowest BIC score was considered the 'true' model (Aho et al. 2014). Type II sums of squares were used for models without interactions, whereas main effects were calculated using type III sums of squares when interactions were present within the final model. Post hoc tests were done using least square means with a Tukey adjustment from the 'emmeans' library, whereby means for groups are adjusted for means of other factors within the model (Lenth 2016). The same model was used for SGR, but the body weight at the start of the investigated period was included as a main effect to control for differences in initial body size, as smaller fish tend to have higher growth rates (i.e. water temperature × O_2 saturation × vaccination + start mass + sex). Models for TGC are included in Supplement 2, but mirror the results of SGR and so are not presented.

To assess the prevalence of cataracts, we used the exact binomial test using the 'binom.test' command. To compare O_2 saturation, multiple comparisons were made (60 vs. 100%, 70 vs. 100%, 80 vs. 100%); therefore, a Bonferroni correction was made with significance assigned at $p < 0.017$.

To determine whether antibody response could be affected by environmental conditions, we used generalised least square models. Both temperature (Eggset et al. 1997), O_2 saturation (Kvamme et al. 2013), and their interaction (Niklasson et al. 2011) influence the immune response in fish, whereas antibody production initially increases before reaching a plateau over time (Eggset et al. 1997). Therefore, we compared an initial model of water temperature × O_2 saturation × time (week 2/4/6/28 post vaccination, categorical) with a second model of water temperature × time + O_2 saturation × time. The models were corrected with the 'weights=varPower()' command to correct for heteroskedasticity within the model residuals. For these models, only data from vaccinated fish were included, as unvaccinated fish from all groups showed low levels of antibody titres throughout (<6.6 titre; data not shown, but can be found in Supplement 3).

Due to an interest in the occurrence of losers, we included models to assess their relative performance compared to 'normal' fish, i.e. immature fish without cataracts. Here, as expected (e.g. Fjelldal et al. 2011, Imsland et al. 2014, Fraser et al. 2019a), exploratory statistics demonstrated that males that matured as post-smolts were heavier in November 2016, but smaller in April 2017, compared to immature males (Fig. S3A). Also, as expected (e.g. Bjerkås et al. 2001), fish with cataracts became smaller from November 2016 onwards compared to fish without cataracts (Fig. S3B). Therefore, these fish were not included when assessing the performance of losers. For losers, we used data from the 17°C and 60% O_2 saturation groups only, as this is where 87% (13 of 15) of the losers were observed. Two initial linear mixed effect (LME) models were produced to explore body mass and body condition: (1) loser (yes/no) × time (October 2016/November 2016/April 2017, categorical) + sex (male/female) + vaccination (yes/no); and (2) loser + time + sex + vaccination. The first model provided evidence that losers show transient growth effects over time, whereas the second model demonstrated that any loser effect was independent of time. In these last 2 models, fish ID was included as a random effect to account for repeated sampling of the same individuals. In addition, only fish for which data were available at all time points were included.

All model residuals were checked for normality following visual examination of q–q plots, and the model accuracy was checked using standardised residuals vs. fitted residual plots. If the model residuals failed to meet normality, the data were transformed using either natural log transformation (body mass in October 2016 and November 2016, body mass for losers, maturity, and cataract models), the 'weights=varPower()' to correct for heteroskedasticity (blood titres), or Tukey's ladder of powers (body mass in April 2017, SGR in April 2017) using the 'transformTukey' command from the 'rcompanion' library that finds the power transformation that makes the data fit the normal distribution as closely as possible. All models that included transformations gave results similar to models without transformations (see Supplement 2 for details). For models that assessed

growth over time, only those fish with data available from every time point were analysed.

3. RESULTS

3.1. Mortality

Total mortality was <6% in all groups at termination of the experiment (Fig. S4). Mortality prior to transfer to the sea-cage was low (<1% in any one group) and could not be analysed statistically. There was no general effect of vaccination or O_2 saturation, but the fish reared at 12°C had significantly lower mortality than those reared at 17°C.

3.2. Effects of vaccination on growth during controlled environmental conditions

Vaccinated fish were significantly smaller than their unvaccinated counterparts (Fig. 2A) due to reduced growth rates (Fig. 2B), irrespective of environmental treatment. Those fish kept on 60% O_2 saturation were significantly smaller than all other groups at 17°C, but not at 12°C (Fig. 2A). However, there was a generally positive association between growth rates and hypoxia that was more apparent at 17°C compared to 12°C (Fig. 2B). There was no interaction between vaccination and environmental conditions, as the models that included the interaction had consistently higher BIC scores than those without (November 2016 – body mass, −742 vs. −780 and SGR, 371 vs. 339 for the interaction and no interaction, respectively).

3.3. Effects of vaccination and historic environmental conditions on growth under natural uncontrolled conditions

Vaccinated fish were still significantly smaller following 5 mo of common garden conditions than unvaccinated fish (Fig. 2C), but they had significantly higher growth rates during this period (Fig. 2D). Fish previously exposed to hypoxia at 17°C had significantly lower growth rates than fish previously kept on 100% O_2 saturation (Fig. 2D), resulting in these fish being significantly smaller at the end of the trial (Fig. 2C). Fish previously reared at 12°C showed no hypoxia effect on long-term growth. However, there was no interaction between vaccination and historic environmental conditions, as the models that included the interaction had consistently higher BIC scores than those without (April 2017 body mass, 22 015 vs. 21 981 and SGR, 9639 vs. 9596 for the interaction and no interaction, respectively). The effects of vaccination and environmental conditions on growth were not explained by group differences in the levels of sexual maturation and/or cataracts (data not shown, but the models are provided in Supplement 2).

3.4. Production parameters

Sexually mature fish, losers, and those with cataracts were only found in groups that experienced 17°C (Fig. 3). There was no effect of vaccination on the prevalence of mature fish, but there was a non-significant trend for decreasing levels of sexual maturation with lower O_2 saturation in the 17°C fish (Fig. 3A). Cataract prevalence was significantly higher in unvaccinated compared to vaccinated fish (Fig. 3B). Losers were almost exclusively found among fish reared on 60% O_2 saturation, irrespective of the vaccination status (Fig. 3C). There was no effect of vaccination or O_2 saturation on skeletal deformities, as only 2 out of the 80 radiographed fish had any deformed vertebrae. Both of these fish were previously reared on 60% O_2 saturation at 17°C, 1 vaccinated (2 deformed vertebrae) and 1 unvaccinated (15 deformed vertebrae).

3.5. Antibody production

There was no interaction between temperature and O_2 saturation on antibody titres over time. However, those fish vaccinated at 17°C had significantly higher antibody titres at Weeks 2, 4, and 6 post-vaccination when under control environmental conditions, but not in April 2017 after 5 mo in natural uncontrolled conditions (28 wk post-vaccination), compared to those vaccinated at 12°C (Fig. 4A). In addition, the interaction between O_2 saturation and time was close to significant, as the fish vaccinated at 60% O_2 saturation had significantly lower antibody titres than those vaccinated at 100% O_2 saturation in April 2017, but not at Weeks 2, 4, and 6 post-vaccination (Fig. 4B).

3.6. Losers

Based on the subjective assessment, losers were found to be exclusively the smallest fish in April 2017 (no overlap with 'normal' fish, with the minimum value for 'normal' fish being 150 g and the maximum value for losers being 128 g), with a CF of <0.94 that

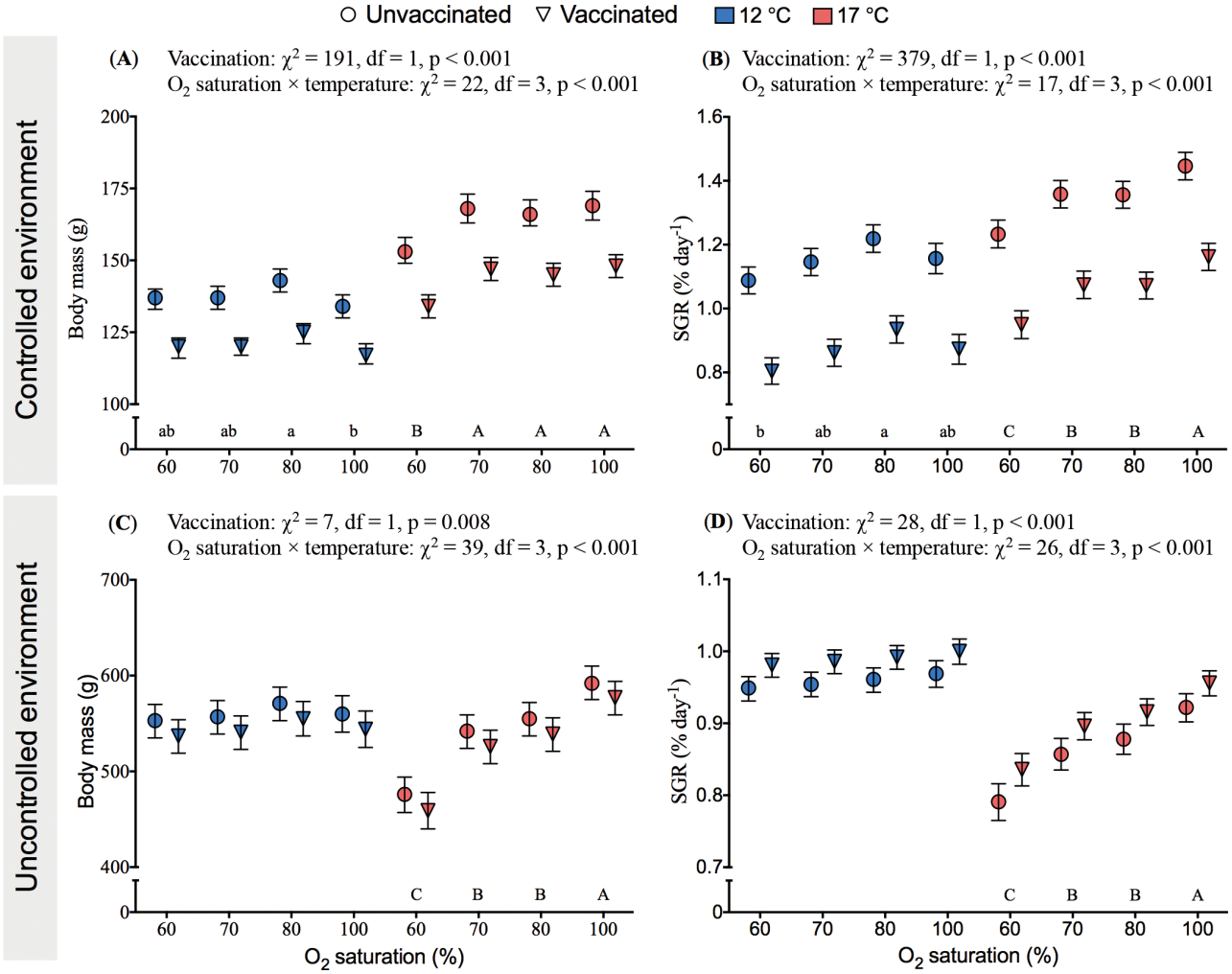

Fig. 2. Body mass and growth data over time for vaccinated and unvaccinated Atlantic salmon reared under different controlled conditions of temperature and oxygen (O_2) saturation between 10 October and 30 November 2016 before common-garden rearing in uncontrolled environmental conditions within a sea-cage. (A) Body mass on 30 November 2016, (B) specific growth rate (SGR) between 10 October and 30 November 2016, (C) body mass on 26 April 2017, and (D) SGR between 30 November 2016 and 26 April 2017. Data are means ± 95% CI (n = 67–89 group^{-1} time^{-1}). The results from linear mixed effect models are presented for each time point. Different lowercase letters indicate significant O_2 saturation effects at 12°C whereas uppercase letters indicate significant O_2 saturation effects at 17°C (post hoc, least square means, $p < 0.05$)

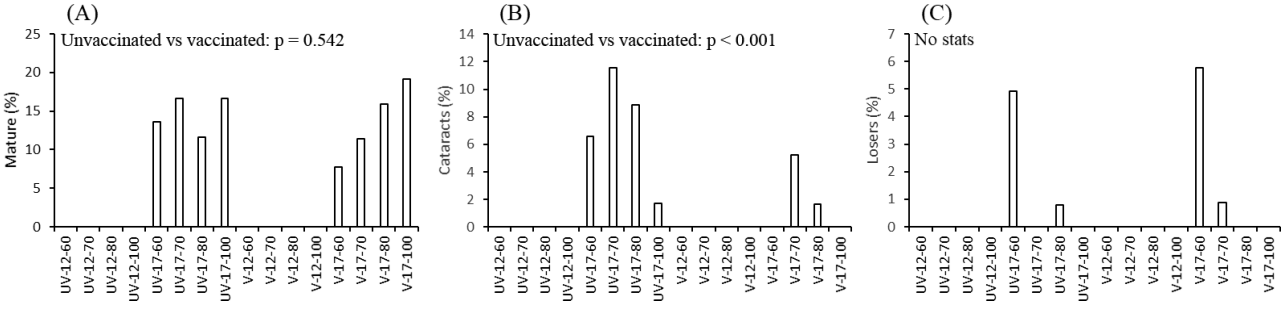

Fig. 3. Percentage of Atlantic salmon that (A) sexually matured as post-smolts, (B) had cataracts, and (C) were 'losers'. The statistics are from binomial tests of specific parameters. Data are (A) % of males only (n = 34–47 group^{-1}) and (B,C) % of the whole population (n = 96–122 group^{-1}). Groups on the *x*-axis are categorised by vaccination status (UV: unvaccinated, V: vaccinated)–temperature (12 or 17°C)–O_2 saturation (60, 70, 80, 100%)

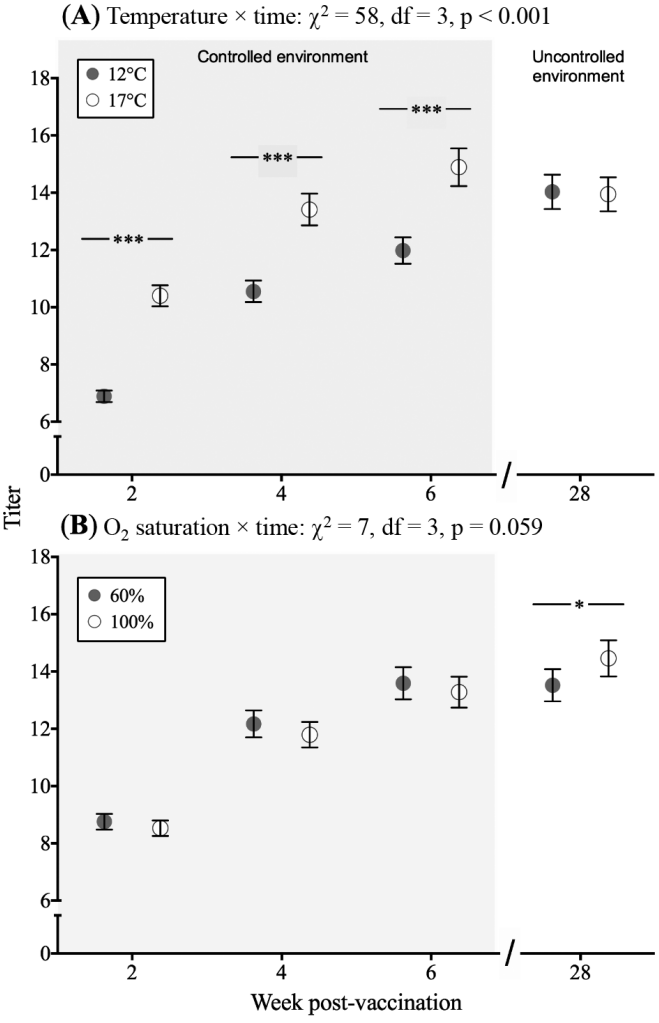

Fig. 4. Effect of (A) water temperature and (B) oxygen saturation at vaccination on antibody titres over time in vaccinated Atlantic salmon. The results from linear models are presented. Data are means ± 95% CI (n = 20 group^{-1} time^{-1}). Asterisks indicate a significant effect of temperature/O_2 saturation within a given time point (post hoc, least square means, *p < 0.05, ***p < 0.001)

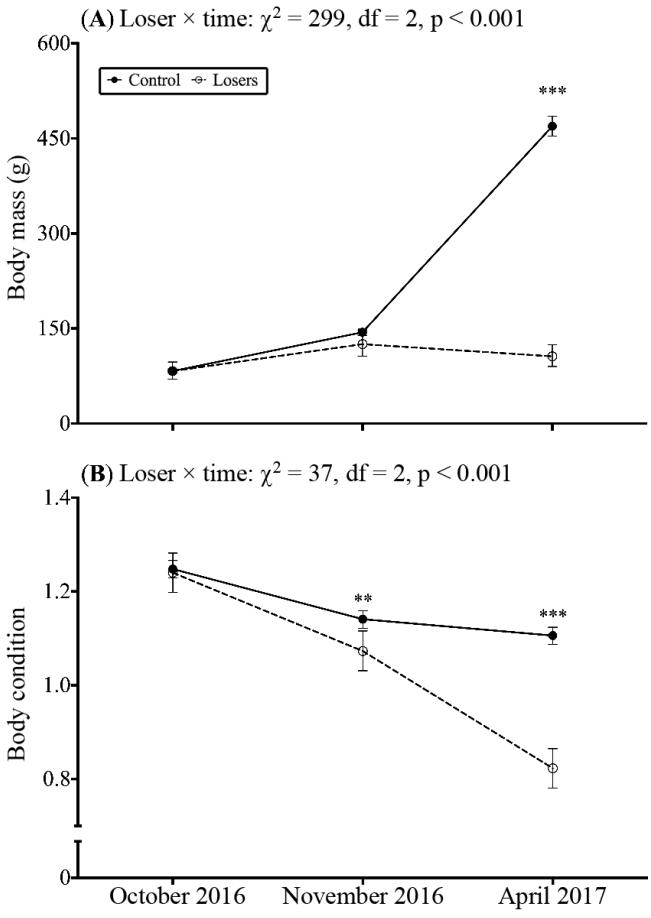

Fig. 5. Body mass and condition of Atlantic salmon reared at 17°C and 60% O_2 saturation for 7 wk followed by a further 28 wk in a sea-cage. (A) Body mass and (B) body condition for 'losers' and control individuals over time. Data include means ± 95% CI (n = 13–140 group^{-1} time^{-1}). Asterisks indicate a significant loser effect within a given time point (post hoc, least square means, **p < 0.01), ***p < 0.001

showed some overlap with normal fish (6 of the 1354 'normal' fish had a CF <0.94, but these 6 fish all weighed >290 g). In all instances, the models for body mass and body condition that included an interaction between losers and time had a lower BIC score than the model without the interaction (body mass, –158 vs. 35; body condition, –955 vs. –933 for models with and without the interaction, respectively), providing evidence that loser effects are transient over time. Subsequent analysis showed that losers were equal in body mass and condition compared to 'normal' fish in October 2016, equal in body mass but with a lower condition in November 2016, but had significantly lower mean body mass and condition in April 2017 (Fig. 5).

4. DISCUSSION

We investigated whether growth following vaccination was associated with environmental conditions expected to alter or limit metabolic rate and found no support for our hypothesis that vaccination leads to an increase in metabolic demand, resulting in reduced growth. However, we observed long-term effects on growth relating to short-term hypoxia treatment following co-treatment at 17°C, but not 12°C.

Environmental conditions were manipulated for 7 wk post vaccination to see whether they influenced vaccine-reduced growth. Based on cyclic hypoxia studies, we expected 60% O_2 saturation at 17°C to be the most challenging condition (Remen et al. 2012, 2013). Indeed, the fish reared at 17°C and 60% O_2 saturation showed a significant reduction in growth and body size at the end of the controlled environmental period

compared to those at 100% O_2 saturation, but the same hypoxia effect was not seen in 12°C fish. A level of around 39% O_2 saturation is expected to be physiologically challenging to salmon at 12°C (Remen et al. 2013). However, neither water temperature nor O_2 saturation interacted with vaccination, suggesting that direct costs to metabolism do not explain the reductions in growth observed following vaccination. Although theorised to be energetically costly, the direct metabolic cost of mounting an immune response is generally difficult to demonstrate, and studies in rainbow trout have shown inconsistent results. For example, vaccines without adjuvant have not led to significant increases in metabolic rate (Ackerman et al. 2000, Skinner et al. 2010, Zanuzzo et al. 2015). However, antigens combined with adjuvants have led to instances of increased metabolic rate (Skinner et al. 2010), although the effect is probably dependent on the type of adjuvant and antigens included in the formulation (Ackerman et al. 2000). In those instances where the standard metabolic rate did increase after exposure to adjuvanted vaccines, there was no subsequent reduction in growth (Ackerman et al. 2000, Skinner et al. 2010). Although we did not measure standard metabolic rate in the current study, in a parallel study using the same vaccine and fish stock, there was no effect of vaccination on routine metabolic rate in an experiment lasting 30 d post vaccination, although vaccinated fish grew more slowly (Torgersen et al. 2017). Taken together, we found no evidence to support the hypothesis that our vaccine reduced growth due to an increase in metabolic demand in salmon.

A number of mechanisms could have disguised the metabolic cost of vaccination. Firstly, fish were fed throughout the study, and feeding has a metabolic burden. Therefore, feed intake could be adjusted in response to vaccination. Although we are unable to address this in the current study, as vaccinated and unvaccinated fish shared the same tanks, previous studies have shown that short-term reductions in feed intake following vaccination in salmon can lead to a 20% reduction in growth 32 d post vaccination (Sørum & Damsgård 2004). The cause of reduced feeding may be related to vaccine-induced peritonitis reducing short-term feeding/appetite (Bjørge et al. 2011). Alternatively, vaccinated fish could have reduced the extent of the immune response under more demanding environmental conditions to conserve energy. Here, we found that antibody titres were responsive to water temperature and O_2 saturation, but the effects were not those expected if metabolism was restricting the antibody response. For example, the 17°C fish showed a more rapid antibody response than those vaccinated at 12°C, and the effect of O_2 saturation was not apparent during the period of controlled environmental conditions when the fish were actually experiencing hypoxia, it only occurred later once the fish were transferred to common-garden rearing with uncontrolled environmental conditions.

We found effects of both water temperature and O_2 saturation on the antibody response that is in line with previous studies. For example, salmon vaccinated against *Vibrio salmonicida* at 2°C showed a delayed or suppressed antibody response compared to salmon vaccinated at 10°C (Eggset et al. 1997), similar to our current finding of a quicker antibody response at 17 vs. 12°C. Regarding hypoxia, a study in Nile tilapia *Oreochromis niloticus* vaccinated against *V. anguillarum* found that 55% O_2 saturation reduced and delayed the antibody response compared to 85% O_2 saturation (Gallage et al. 2016), similar to the results in the current work. In addition, Atlantic salmon experiencing cyclic hypoxia, between 44 and 65% O_2 saturation, had a 38–56% reduction in head kidney leucocyte respiratory burst activity compared to those on normoxia (Burt et al. 2013). However, it should be noted that the lower antibody levels in the 60 vs. 100% O_2 saturation groups in this study were not apparent during the initial 6 wk period of controlled environmental conditions, but came later when the fish were all common-garden reared with uncontrolled environmental conditions. Further work is required to determine whether this is a genuine suppression of the immune system or a delay in the antibody peak.

The growth difference between vaccinated and unvaccinated fish occurred within the initial 7 wk period of controlled environmental conditions and remained evident at the end of the trial. However, the vaccinated fish displayed some 'catch-up' growth when reared for 5 mo under uncontrolled natural conditions. This may suggest that a short-term reduction in feed intake is the main cause of the growth disparity caused by vaccination (Sørum & Damsgård 2004). In contrast, those fish that experienced 60% O_2 saturation and 17°C showed a chronic reduction in growth rates that persisted even after 5 mo in common garden conditions, suggesting that a different mechanism is operating on growth compared to the vaccine effect, especially as there was no additive effect between O_2 saturation and vaccination. Of note, we cannot attribute the vaccine effect on growth to the occurrence of skeletal deformities. Previously, in some instances, vaccination has been found to lead to an increase in skeletal deformity prevalence (e.g. Berg et al. 2006) that is known to reduce growth

(Hansen et al. 2010). However, we found no vaccine effect on deformities after 120 d in seawater, similar to other studies (e.g. Fraser et a. 2014).

O_2 saturation during the period of controlled environmental conditions had long-term effects on growth in fish that initially experienced 17°C. Five months post hypoxia exposure, the 60, 70, and 80% O_2 saturation groups were 26, 10, and 7% smaller, respectively, than those that experienced 100% O_2 saturation. The reduction in growth during hypoxia is expected to be related to lower feed intake as a mechanism to lower metabolic demands under challenging conditions (Remen et al. 2012). Previously, salmon exposed to 60% O_2 at 16°C showed a 6% reduction in feed intake and growth rates during the hypoxia exposures, but showed compensatory growth after the cessation of cyclic hypoxia treatment (Remen et al. 2014). We report no evidence for compensatory growth 5 mo post hypoxia treatment. Indeed, the drop-off in growth between the 17°C and ≤80 and 100% O_2 saturation groups increased post hypoxia exposure. As there was no drop-off in growth in those fish that experienced low O_2 saturation at 12°C, hypoxia alone is unlikely to explain the current findings. Given that the metabolically challenging conditions of 17°C and 60–70% O_2 saturation were experienced during the parr–smolt transformation, a process that is essential for the long-term growth performance of the fish (Björnsson et al. 2011), it may be that this developmental process has been hindered in some aspect, although we found no treatment effect on gill markers of smoltification. Further work into the long-term effects of hypoxia and water temperature during the parr–smolt transformation would be of interest.

We observed a positive association between water temperature during the 7 wk of controlled environmental conditions and sexual maturation and cataracts, but no effect on skeletal deformities. The temperature effect on sexual maturation has been observed previously (Fjelldal et al. 2011, Imsland et al. 2014). The current 7 wk exposure to 17°C during smoltification resulted in fully mature males out-of-season in April 2017, as previously observed by Fjelldal et al. (2011). We also note a trend for reduced prevalence of sexual maturation in the 17°C/60% O_2 saturation group (Fig. 3A), suggesting that this temperature effect may be related to growth acceleration, but this requires further experimentation. Similarly, cataracts were more prevalent in the fastest-growing groups around sea transfer, unvaccinated fish kept at 17°C, which conforms with the current literature (Bjerkås et al. 2001) and would suggest growth rate during a critical developmental window is a risk factor for cataract development. Previously, salmon exposed to 16°C around smoltification have been found to have an increased prevalence of skeletal deformities (Grini et al. 2011, Fraser et al. 2019b). In the current work, we found that deformities were few and could not be linked to temperature or oxygen saturation around smoltification. The inconsistency may be explained by the length of the study. For example, in the study by Fraser et al. (2019b), temperature treatments around smoltification increased skeletal deformity prevalence at harvest size after 533 d in seawater, whereas no effects were observed at sea transfer or following 120 d in seawater.

Losers were mainly found among fish reared at 60% O_2 saturation at 17°C. To date, there is very little information on losers (Stien et al. 2013), although they are a concern for the industry with respect to reduced animal welfare (Vindas et al. 2016) and economic loss. We found that biometric data could not be used to identify losers in October, but a minor reduction in growth could be detected as early as November before the more dramatic decrease in performance following sea transfer. Further research is required to understand the contributing factors to this condition. However, as losers were most apparent under the most challenging environment, the 7 wk period of high water temperature and low O_2 saturation, one may suspect that the losers in the current study may be individuals that could not tolerate physiologically demanding environmental conditions. As the growth of losers was already impaired in November 2016, this could indicate a disruption of physiology during the parr–smolt transformation that may then have led to the more substantial negative effects on growth upon entering the seawater environment. Alternatively, it would also be of interest to screen for infectious agents, as fish may be at an increased risk of infection when environmental conditions are unfavourable, such as during high water temperature and periods of hypoxia (Bowden 2008). However, further work in this area is required.

To conclude, there was no interaction between environmental conditions expected to alter metabolic rate and vaccine-induced growth reduction. Therefore, the reduced growth observed following vaccination is unlikely to be related to alterations in basal metabolism.

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