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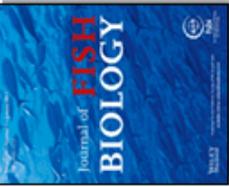
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Chronic stress of rainbow trout *Oncorhynchus mykiss* at high altitude: a field study

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The stress response of *Oncorhynchus mykiss* in high-altitude farms in central Mexico was investigated over two seasons: the cool (9.1–13.7° C) dry winter season, and the warmer (14.7–15.9° C), wetter summer season. Fish were subjected to an acute stress test followed by sampling of six physiological variables: blood cortisol, glucose, lactate, total antioxidant capacity, haemoglobin concentration and per cent packed cell volume ($V_{PC}\%$). Multivariate analyses revealed that lactate and total antioxidant capacity were significantly higher in the summer, when water temperatures were warmer and moderate hypoxia (4.9–5.3 mg l⁻¹) prevailed. In contrast, plasma cortisol was significantly higher in the winter (mean \pm s.e.: 76.7 \pm 4.0 ng ml⁻¹) when temperatures were cooler and dissolved oxygen levels higher (6.05–7.9 mg l⁻¹), than in the summer (22.7 \pm 3.8 ng ml⁻¹). Haemoglobin concentrations (mg dl⁻¹) were not significantly different between seasons, but $V_{PC}\%$ was significantly higher in the summer (50%) than in the winter (35%). These results suggest that in summer, effects of high altitude on farmed fish are exacerbated by stresses of high temperatures and hypoxia, resulting in higher blood lactate, increased total antioxidant capacity and elevated $V_{PC}\%$ levels.

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Key words: alpine; aquaculture; hypoxia; Mexico; salmonids; temperature.

INTRODUCTION

High-altitude environments are ideal for investigating the mechanisms of physiological adaptation. For fishes that live at high elevations, hypobaric hypoxia is a constant stressor that animals cannot evade by behaviour (Storz, 2010). It is well documented in humans and other mammals, that when they are exposed to high altitudes (1500–3500 m above sea level) the associated hypobaric hypoxia causes chronic stress and disturbs normal physiological functions (Brooks *et al.*, 1991; Zubieta-Calleja *et al.*, 2007). In humans, these include cardiovascular and ventilatory changes, elevated blood haematocrit and haemoglobin concentration (Meehan & Zavala, 1982; Storz, 2010). The much reduced partial pressures of oxygen (pO_2) and carbon dioxide (pCO_2) at high altitude decrease the oxygen carrying capacity of blood, which can be

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remedied by an elevation in haematocrit (Hall, 1946), initially brought about by the release of stored erythrocytes followed by an increase in erythropoiesis (Siri *et al.*, 1966). High-altitude environments are also characterized by low ambient temperatures relative to lowland environments at similar latitudes and are thus thought to be ideal for the production of species such as rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) (Turner *et al.*, 1983). Of the few studies that exist on the effects of high altitude on the physiology of *O. mykiss*, Georgiev (1972) and Ross *et al.* (1986) found that altitudes of 1500 and 4500 m were sufficient to elevate haematocrit and mean haemoglobin concentration. While elevated haematocrit and mean haemoglobin concentrations indicate that high altitude and associated reduced pO_2 has an effect on blood physiology, there have been no complementary investigations of the stress response at high altitude in fishes.

Responses to stress are often divided into two categories: acute and chronic. In the short-term, or in response to an acute challenge, the stress response is believed to be adaptive (Sapolsky *et al.*, 2000; Wingfield & Romero, 2001). Acute stress, defined as a transient change in metabolism, is commonly characterized by increased levels of epinephrine followed by elevated levels of plasma lactate, glucose and cortisol as well as changes in behaviour (Xu *et al.*, 2006) and immune factors (McEwen & Wingfield, 2003). Chronic stress, defined as either multiple, frequent exposure to stressors and long-term constant exposure to stressors, has received attention from comparative biology, because it often brings on disease, due to excess levels of stress mediators (Santos *et al.*, 2010). In fishes, evaluating the effects of acute stress is less difficult than assessing chronic stress as primary stress mediators, such as plasma cortisol, may not remain elevated in chronically stressed fishes, due to a negative feedback mechanism that results in the suppression of the hypothalamic–pituitary–interrenal (HPI) axis (Pickering & Stewart, 1984; Procarione *et al.*, 1999; Cyr & Romero, 2009; Santos *et al.*, 2010). One solution, suggested by Santos *et al.* (2010), to circumvent the problem of measuring chronic stress, is to impose an acute stress event on fishes that have been chronically stressed, to explore any differential stress response after exposure (Ruane *et al.*, 2002; Barton *et al.*, 2005; Di Marco *et al.*, 2008; van de Nieuwegiessen *et al.*, 2008). This is particularly appropriate for *O. mykiss* at high altitude in Mexico because in addition to the chronic moderate hypoxia at high altitude, *O. mykiss* are exposed to acute high summer temperatures that exacerbate hypoxia. This, in addition to over-crowding characteristic of intensive aquaculture (Iwama *et al.*, 2006), will probably result in increased energy demand, and compromised immune systems (Davis, 2006; Santos *et al.*, 2010).

The primary objective of the present field study was to determine the stress response by measuring blood stress indices concurrently with haemoglobin concentrations and per cent packed cell volume ($V_{PC}\%$) in *O. mykiss* at high-altitude (>2900 m) farms in Mexico. Seasons at high altitude in Mexico are characterized by a warm (14–16°C) wet summers (June to October) and cooler (9–13°C) dry winters (November to May). Based on seasonal temperatures, calculated average dissolved oxygen concentrations (DO_2) should typically range from 7.01 to 7.21 mg l⁻¹ in the summer, and 7.3 to 8.2 mg l⁻¹ in the winter. These DO_2 are much lower than at sea level at comparable temperatures, where DO_2 can be as high as 9.42–10.19 and 10.4–11.5 mg l⁻¹ in summer and winter, respectively. For *O. mykiss*, the lower DO_2 at high altitude should present a physiological challenge.

Measurements of blood lactate and glucose using hand-held instruments were used to relate field to laboratory values, and plasma cortisol and total antioxidant capacity

(enzymatic and non-enzymatic factors) were also determined to provide a clearer picture of the effects of multiple environmental factors on the stress response (Barton, 2002; Bonier *et al.*, 2009; Martinez-Porchas *et al.*, 2009; Pankhurst, 2010). Although not directly related to the stress response, and usually used to evaluate the effects of toxins on fish physiology (Livingstone, 2001), measurements of total antioxidant capacity are complementary to the more commonly measured stress hormones and enzymes. These measurements provide an indirect measure of the cell's ability to produce antioxidant defences to oxidative damage that may result from stress-related elevated metabolic rate (Hunt von Herbing & Turnbough, 2011). Together, the analyses should reveal whether *O. mykiss* are chronically stressed at high altitude in Mexico, as well as acutely stressed during the warmer summer months when temperature is high and DO_2 is low.

MATERIALS AND METHODS

STUDY SITE

The research site was located at an average altitude of 2939 m in the Corral de Piedra microbasin and Los Hoyos River catchment, which is part of the Trans-Mexican Volcanic Belt in central Mexico. This study was conducted on *O. mykiss* stocked and grown in farms located between 19° 10' 29" and 19° 14' 33" N; 99° 53' 27" and 100° 00' 13" W in the counties of Amanalco, Temascaltepec and Valle de Bravo. Sampling of fish occurred over a period of 2 weeks in each of the two seasons: June (summer, wet season) and November (winter, dry season) in 2010.

Over 50 fish farms are scattered throughout the Corral de Piedra microbasin and *O. mykiss* culture is carried out largely in a combination of earthen and concrete-lined ponds fed by springs derived from the neighbouring Los Hoyos River. Five farms [Dos Potrillos (D), Corral de Piedra (C), El Arroyo (E), Piedra Ancha (P) and Tizapa (T)] were sampled in this study comprising part of a fish farm co-operative (INTEGRAMEX). Specifics of the farms are listed in Hunt von Herbing *et al.* (2013).

ANIMALS

Feeding was withheld the night prior to sampling. Some variation in sampling time (± 1.5 h) was inevitable as at least two separate farms were sampled each day. *Oncorhynchus mykiss* were stocked for each of the farms as fry (3–5 cm total length, L_T) from a cohort of eggs obtained at a nearby hatchery (*i.e.* similar altitude), but the fish strain was unknown. All fish sampled were yearlings and sexually immature (3–12 months old). In each season (summer and winter), 15 fish were sampled per farm.

PHYSIOLOGICAL EXPERIMENTS

Time course

To characterize the time course of acute stress responses, 10 fish were individually netted, which briefly exposed them to air, and then placed in a temperature-regulated flow-through 54 l darkened confinement tank. Each fish was removed from the confinement tank at 5 min intervals for the first 20 min, then at 30 min and 60 min, when the last fish was removed and sampled. For each sample, a small non-lethal blood sample (1.0 ml) *via* caudal venipuncture was collected using 3 ml heparinized syringes with 22 gauge needle. This was repeated at each farm and a mean value was taken. Blood samples were immediately placed in water-ice slurry in a large cooler for field analysis prior to processing and storage. Fish standard lengths (L_S) ± 0.1 mm and mass (M_W) ± 0.1 g were recorded. After sampling and measurement, each fish was released

into a recovery tank until normal body orientation and gill ventilation was observed, at which point they were returned to the ponds. Blood sampling was rapid, taking <1 min and as a result no anaesthetic was used, because previous studies have shown that using anaesthetic may affect the stress response (Small, 2003; Velisek *et al.*, 2005). Less than 1% mortality occurred after sampling, but the small size of the fish precluded the taking of more than one blood sample, without increasing mortality.

Whole blood levels of lactate and glucose were measured using hand-held field meters described below and results were evaluated immediately to determine the optimal time (defined as the time when lactate and glucose concentrations reached an asymptote) for blood sampling. Baseline values for physiological variables were estimated from each farm as values at time zero, after fish had been netted, but not confined.

Acute stress response

Results from time course experiments indicated that 20–25 min, the time at which lactate and glucose first peaked in the blood after handling and confinement, was the optimal time for stress response sampling. The acute stress test followed the protocol of the time course experiments except that each fish was sampled only after 20–25 min of confinement. Samples for L_S , M_W , lactate (mM), glucose (mM), cortisol (ng ml⁻¹) and total antioxidant capacity (mM of Trolox; <http://pubchem.ncbi.nlm.nih.gov/compound/trolox>) were collected from 169 fish in two seasons: 102 fish in June and 67 fish in November. Levels of blood plasma metabolites (lactate, glucose, cortisol and total antioxidant capacity) were compared within and between seasons for each farm and among farms within seasons.

FIELD DIAGNOSTICS AND LABORATORY PHYSIOLOGICAL ANALYSES

Lactate and glucose levels were measured on whole blood by adding 10 µl to hand-held Lactate Pro LT-1710 portable lactate analyser (www.arkrayusa.com) and ACCU-CHEK glucose meter (www.roche-diagnostics.com). Both meters were calibrated with standards prior to analyses following manufacturer's guidelines. The Lactate Pro and ACCU-CHEK have previously been validated as reliable for field measurements on fishes (Cooke *et al.*, 2008). A sub-sample (60 µl) of whole blood from each fish was immediately injected into an i-STAT E3+ cartridge to measure haemoglobin concentration (g dl⁻¹) with an i-STAT1 point-of-care device (www.abbottpointofcare.com). The remaining whole blood was centrifuged using an LW Scientific portable centrifuge (www.lwscientific.com) at 3500 g for 5 min (following Cooke *et al.*, 2008). The $V_{PC}\%$ was measured as the proportion of packed red blood cells to the total volume of the sample. Plasma samples were separated by micropipette, transferred to 1.5 ml standard Eppendorf tubes and stored in a liquid nitrogen dry shipper (at a minimum of -80 °C) until laboratory analyses were conducted at the Marine Conservation and Aquatic Physiology Laboratory (MCAPL), University of North Texas (UNT), Denton, Texas, U.S.A. A microplate reader (Synergy 2, BioTek Instrument Inc.; www.biotek.com), with appropriate reagents and commercial assay kits, was used for assays of lactate (lactate assay kit; www.etonbio.com), glucose (Cayman's Glucose Assay; www.caymanchem.com), cortisol (Neogen Cortisol ELIZA kit; www.neogen.com) and total antioxidant capacity (Cayman's Antioxidant Assay). All samples were assayed in duplicate. All intra-assay and interassay coefficients of variability were under 10%.

WATER-QUALITY SAMPLING

Nine water-quality variables were measured in each of the five farms and in both seasons: water flow (l s⁻¹), temperature (°C), dissolved oxygen (mg l⁻¹), conductivity (mS cm⁻¹), nitrites (mg l⁻¹), nitrates (mg l⁻¹), total hardness (mg l⁻¹), pH and total alkalinity (meq l⁻¹). A total of 77 individual samples were collected from all five farms as well as the Los Hoyos River (H) (water source for all farms). For each farm, samples of water were

collected at the surface and at the bottom of the same ponds from which fish had been sampled for physiological variables. The samples were analysed on the same day as fish collected for the physiology samples. Nitrites, nitrates, total alkalinity and total hardness were measured with Mardel test strips (www.virbacvet.com), pH was measured using pHydrion test strip (www.MicroEssentialLab.com). Temperature, conductivity and dissolved oxygen were measured using an YSI Handheld (www.ysi.com) dissolved oxygen, conductivity, salinity and temperature meter. Input water flow rate in the ponds at each of the farms was calculated, using a simple, standardized method comprising a 25 l plastic bucket and a stopwatch.

STATISTICAL ANALYSIS

To assess the performance of field diagnostic tools for lactate and glucose, linear regression analysis was conducted between field and laboratory values. The laboratory values were assumed to be the gold standard enabling an evaluation of whether the slopes differed statistically from one and whether the intercepts differed significantly from zero ($P < 0.05$). For the time course experiments, regression analyses determined relationships of post-capture stress responses (lactate, glucose, cortisol and total antioxidant capacity) to time after handling and confinement. If relationships were not linear, a second-order polynomial or a second parameter exponential rise was applied, and the best fit to the data (defined by the highest r^2) was reported. A two-way ANOVA on ranked data followed by *post hoc* Tukey's pair-wise comparisons ($P < 0.05$) determined the effects of farm and season on L_S and M_W , lactate, glucose, cortisol and total antioxidant capacity, and as data proved to be non-normal, a Mann-Whitney U -test and χ^2 -test determined effects of season on haemoglobin concentration ($\text{Hb g}^{-1} \text{dl}^{-1}$) and $V_{PC}\%$. Due to instrument malfunction on two farms, effects of farm on haemoglobin concentration and $V_{PC}\%$ could not be determined. Principal component analyses (PCA) using STATGRAPHICS (www.statgraphics.com) determined if potentially predictive relationships existed among, farm and season and stress physiological (S) and water-quality (W) variables. Effects of body mass on each of the four S variables were removed by transforming data using regressions between blood variables and body mass. The principal components with the highest explained intra-farm variance were selected as S and W multivariate artificial indexes. Finally, linear regression analysis determined if a predictive relationship existed between S and W variables.

RESULTS

LENGTH AND MASS RELATIONSHIPS

L_S and M_W were not significantly different between seasons, but differences existed among farms such that farm D contained larger, heavier fish than the other four, which did not differ among each other (Table I).

ASSESSMENT OF FIELD DIAGNOSTIC TOOLS

For lactate and glucose, there were significant positive relationships between the field and laboratory-derived values (Fig. 1). The y -intercepts of laboratory and field lactate ($y = 0.53$, $t = 2.4$, $P < 0.05$) and glucose ($y = 0.53$, $t = 2.6$, $P = 0.01$) were both significantly different from zero. Paired t -tests showed that mean \pm s.e. laboratory lactate values (4.83 ± 0.25 mM) were significantly higher than field measurements (3.49 ± 0.17 mM) ($n = 65$, $t = 10.69$, $P < 0.001$), but mean \pm s.e. glucose values (5.50 ± 0.34 mM) did not differ from field measurements (5.45 ± 0.32 mM) ($n = 66$, $t = 0.42$, $P > 0.05$).

TABLE I. Summary results of two-way ANOVAs for *Oncorhynchus mykiss* of the effects of season and farm on standard length (L_S), body mass (M_W), cortisol, total antioxidant capacity, glucose and lactate levels, as they changed between seasons and among five farms in Mexico. Interactions of season and farm were not assessed for glucose due to missing values in Piedra Ancha farm (P) in summer

Variables	L_S (cm)		M_W (g)		Lactate (mM)		Glucose (mM)		Cortisol (ng ml ⁻¹)		Total antioxidant capacity (mM of Trolox)	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
	Season	2.79	>0.05	0.14	>0.05	20.56	<0.001	0.30	>0.05	93.48	<0.001	20.56
Farm	7.06	<0.001	3.93	<0.01	10.54	<0.001	15.10	<0.001	1.72	>0.05	10.54	<0.001
Season × farm	2.98	<0.05	2.91	<0.05	0.28	>0.05	N/A	N/A	10.10	<0.001	0.28	>0.05

N/A, not available.

PHYSIOLOGICAL EXPERIMENTS

Time course

Generally, both lactate and glucose increased linearly over the 60 min post-capture period, reflecting a stress response induced by the handling and confinement [Fig. 2(a), (b)]. Cortisol exhibited a relationship (best characterized by a two-order polynomial regression model) with time post-capture [Fig. 2(c)], but there was no relationship between total antioxidant capacity and time post-capture [Fig. 2(d)].

Acute stress response indicators: seasons and farms

Blood lactate concentrations were significantly higher in the summer with a mean \pm s.e. of 4.7 ± 0.2 mM compared with 3.3 ± 0.1 mM in the winter across all farms [Fig. 3(a)] and both were higher ($P < 0.05$) than the mean \pm s.e. baseline value of 3.0 ± 0.4 mM. Mean lactate values differed among farms, such that farm C was significantly ($P < 0.05$) higher than the four other farms (D, E, T and P), which did not differ from one another. For lactate, no significant interactions occurred between season and farm (Table I). In contrast, blood glucose concentrations did not differ between seasons (Table I), with a collective mean \pm s.e. of 5.0 ± 0.3 mM [Fig. 3(b)], which was higher ($P < 0.05$) than the baseline value of 3.2 ± 0.2 mM. Specific comparisons of blood glucose among farms and interactions of season and farm could not be determined due to missing values for farm P in summer. Plasma cortisol concentrations were three times lower in summer with a mean \pm s.e. of 22.7 ± 3.8 ng ml⁻¹ compared with winter (76.7 ± 4.0 ng ml⁻¹) [Table I and Fig. 3(c)] but both values were higher ($P < 0.05$) than the mean \pm s.e. baseline value of 18.7 ± 2.2 ng ml⁻¹ and no differences occurred among farms (Table I). There was a significant interaction for cortisol between season and farm (Table I) and *post hoc* Tukey tests showed that farms D, T and P had higher ($P < 0.05$) cortisol levels in the winter than the summer but farms C and E did not differ between seasons. Between seasons, total antioxidant capacity was higher in summer with a mean \pm s.e. of 2.1 ± 0.1 mM of Trolox compared with winter with 0.9 ± 0.1 mM of Trolox [Table I and Fig. 3(d)] and both were higher ($P < 0.05$) than the baseline value of

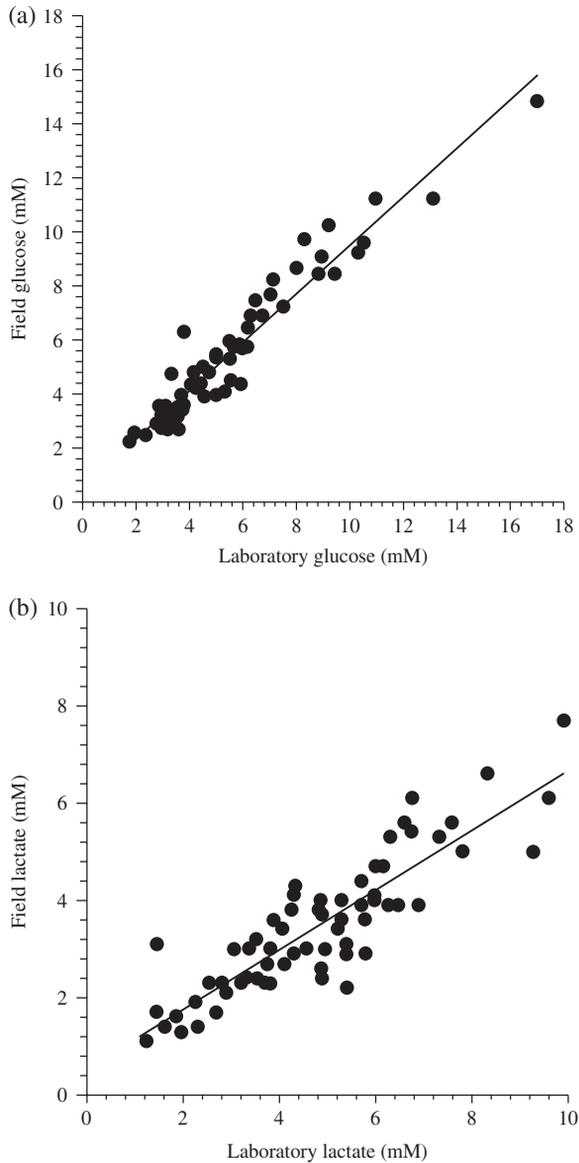


FIG. 1. Relationships between field and laboratory measurements in *Oncorhynchus mykiss*, of (a) plasma glucose and (b) lactate. Linear regression models are (a) $y = 0.53 + 0.90x$ ($n = 66$, $r^2 = 0.92$, $P < 0.001$) and (b) $y = 0.53 + 0.61x$ ($n = 65$, $r^2 = 0.77$, $P < 0.001$).

0.8 ± 0.1 mM of Trolox. Among farms, significant differences existed (Table I), such that farm E was higher ($P < 0.05$) than farms T and D, but farms C and P were not different from each other. No interactions between season and farm occurred (Table I).

Mean \pm s.e. haemoglobin concentration of 9.04 ± 0.30 g dl⁻¹ did not differ between seasons ($t = 2.03$, d.f. = 76, $n = 77$, $P > 0.05$) [Fig. 4(a)]. Among-farm comparisons

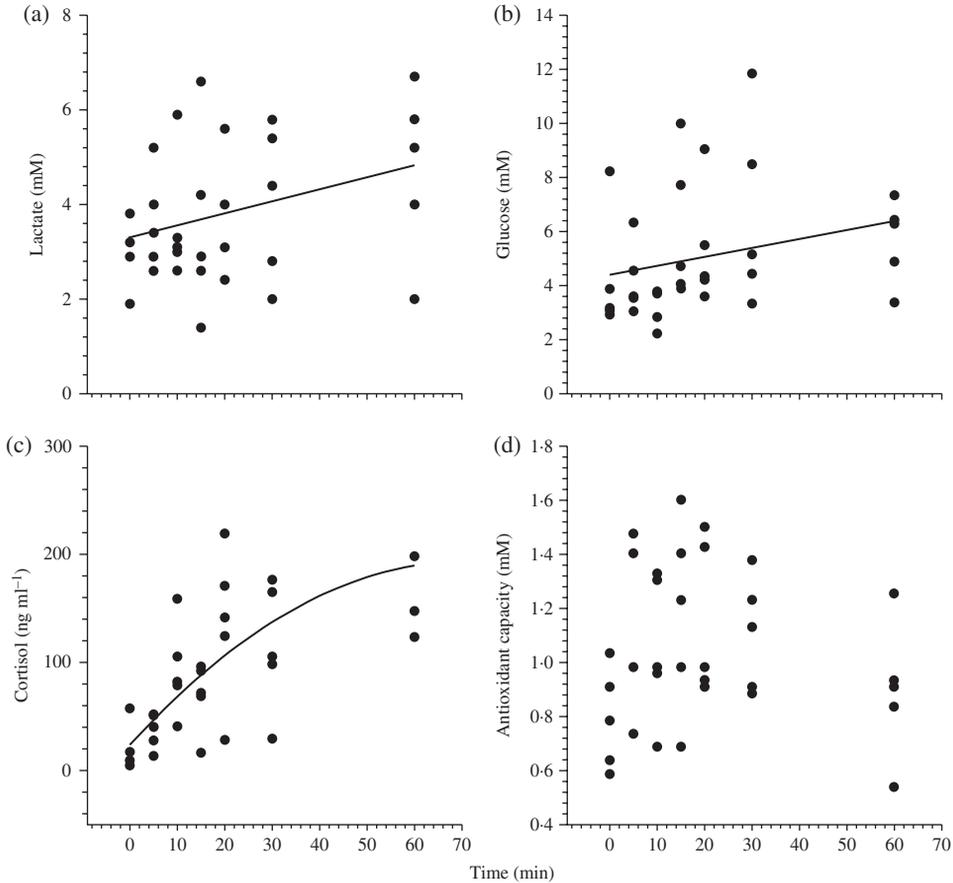


FIG. 2. Post-capture stress responses to time after netting and confinement of *Oncorhynchus mykiss* sampled from five high-altitude farms in Mexico: (a) lactate, $y = 3.31 + 0.03x$, $r^2 = 0.11$, $F = 3.90$, $P < 0.05$; (b) glucose, $y = 8.36 + 2.30x$, $r^2 = 0.08$, $F = 2.73$, $P < 0.05$; (c) cortisol, $y = 23.89 + 4.81x - 0.03x^2$, $r^2 = 0.50$, $F = 16.23$, $P < 0.001$; (d) antioxidant capacity, non-significant, $P > 0.05$; $n = 25$.

were not conducted due to missing values in both seasons. In the summer, mean \pm S.E. $V_{PC}\%$ was significantly higher and almost double ($53.3 \pm 16.8\%$) than that of the winter ($35.9 \pm 4.3\%$) ($\chi^2 = 33.10$, d.f. = 1, $n = 171$, $P < 0.05$) averaged across all farms [Fig. 4(b)].

WATER-QUALITY VARIABLES

The seasonal changes in temperature and DO_2 for the five farms are shown in Fig. 5. Temperature was significantly higher for all the farms during the summer with a mean \pm S.E. of $15.3 \pm 0.1^\circ\text{C}$ compared with $11.9 \pm 0.1^\circ\text{C}$ in the winter, but significant interactions occurred between farm and season (Table II). Dissolved oxygen concentrations were significantly lower during the summer with a mean \pm S.E. of $5.3 \pm 0.1\text{ mg l}^{-1}$ compared with $7.2 \pm 0.1\text{ mg l}^{-1}$ in the winter, and interactions occurred between farm and season (Table II). *Post*

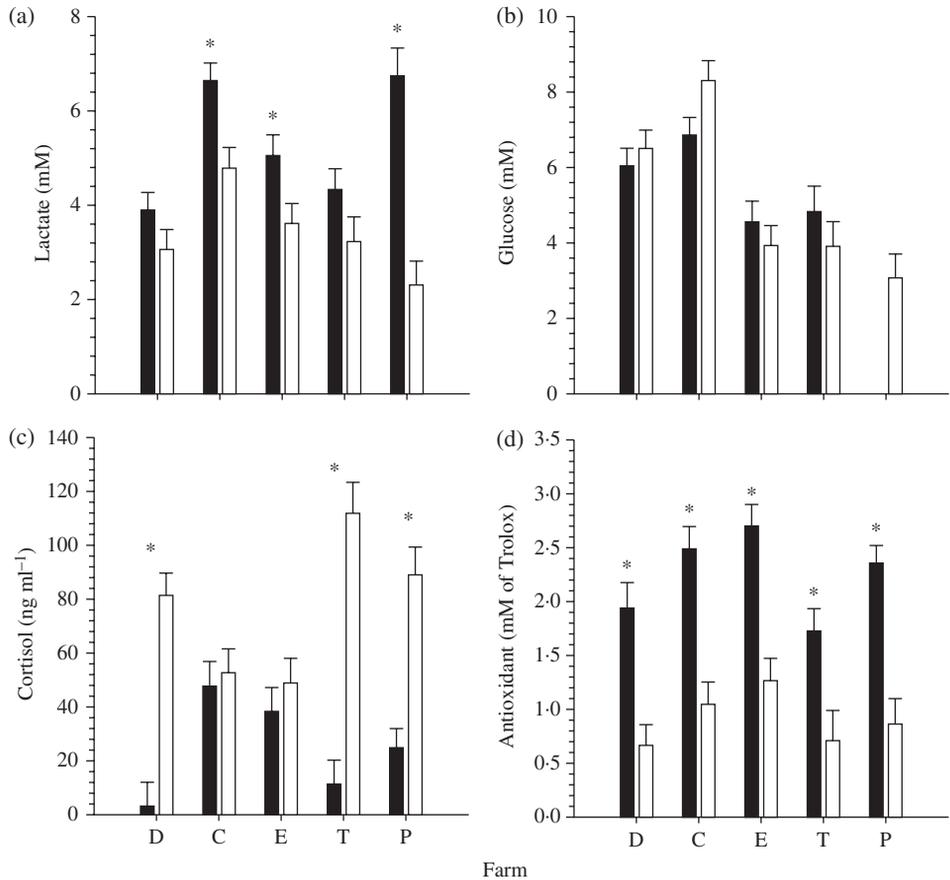


FIG. 3. Mean + S.E. ($n = 169$) (a) lactate, (b) glucose, (c) plasma cortisol and (d) antioxidant capacity (d) of *Oncorhynchus mykiss* sampled at five farms; Dos Potrillos (D), Corral de Piedra (C), El Arroyo (E), Tizapa (T) and Piedra Ancha (P), in summer (■) and winter (□). *, Farms showing a significant winter–summer difference ($P < 0.05$).

hoc Tukey tests showed that dissolved oxygen concentration differences among farms were not consistent between seasons, but reflected patterns in temperature (Fig. 5).

Seasonal measurements for seven water-quality variables and across farms are shown in Table III. Flow rates increased in the summer and wet season to double those in the winter and dry season. Conductivity ($\mu\text{S cm}^{-1}$) was significantly lower in winter compared with the summer (Tables II and III). Nitrites and pH showed no significant differences between seasons; hardness and alkalinity were significantly higher in summer than in winter, and nitrates were significantly higher in the winter than in the summer (Tables II and III). Interactions between season and farm showed interdependent seasonal effects on water quality, which were probably influenced by topographical location in the catchment and distance from the water source (Hoyos River).

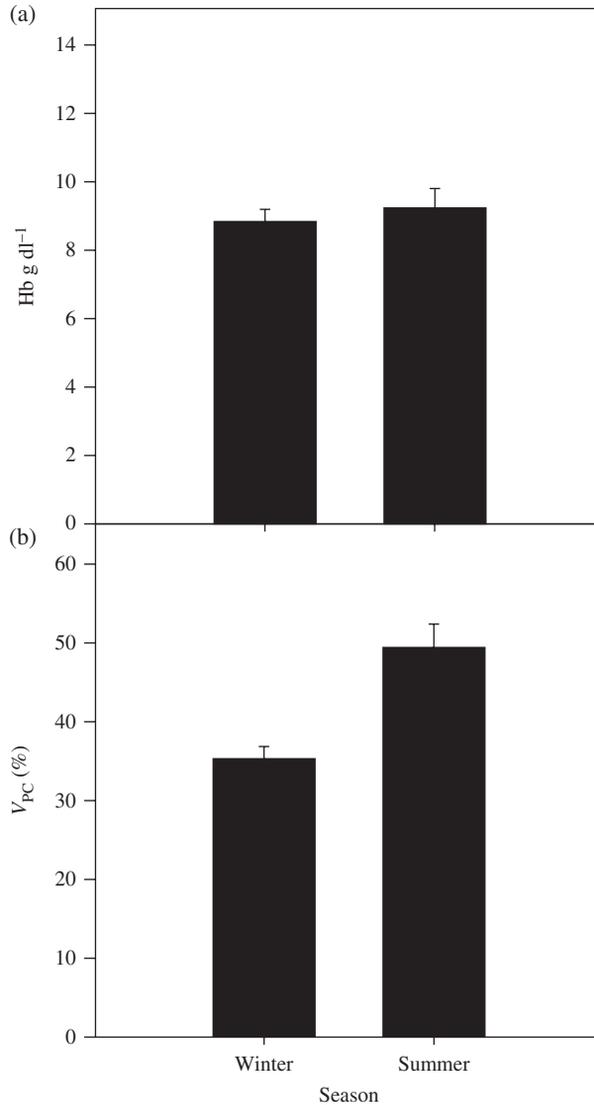


FIG. 4. Mean + s.e. (a) haemoglobin (Hb) concentration ($n = 77$) and (b) % packed cell volume ($V_{PC}\%$) ($n = 171$) over two seasons from five high-altitude *Oncorhynchus mykiss* farms in Mexico.

PRINCIPAL COMPONENT ANALYSIS

A scatter plot for the four S variables between seasons and among farms, which are mapped in the space spanned by PC1 v. PC2, is shown in Fig. 6. The two principal components accounted for 68% of the variability in the original data, with PC1 = 41% and PC2 = 27%. Each of the S variables had a very high positive load and weight with PC1. Results for PC1 supported significant distribution differences between seasons ($t = 9$, d.f. = 115, $P < 0.001$), while PC2 supported significant differences among farms ($F_{4,115} = 9$, $P < 0.001$). In the summer, cortisol exhibited low levels on PC1 and high

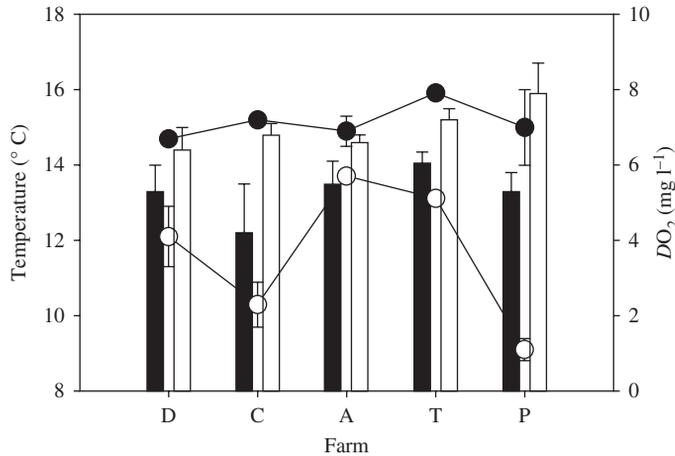


FIG. 5. (a) Mean \pm S.E. temperature in the summer (●) and winter (○) and (b) mean \pm S.E. dissolved oxygen concentrations (DO_2) in the summer (■) and winter (□) for five *Oncorhynchus mykiss* farms: Dos Potrillos (D), Corral de Piedra (C), El Arroyo (E), Tizapa (T) and Piedra Ancha (P), in Mexico ($n = 70$). Temperature and DO_2 were significantly different between summer and winter for each farm ($P < 0.05$).

levels on PC2 compared with winter. Lactate, glucose and total antioxidant capacity dominated in summer compared with winter.

A scatter plot for nine W variables between seasons, among farms and the Hoyos River, which are mapped in the space spanned by PC1 v. PC2, is shown in Fig. 7. PCA showed that the two principal components accounted for 62% of the variability, with PC1 = 44% and PC2 = 18%. Each variable had a very high positive load and weight with PC1. PC1 supported significant distribution differences in W variables between seasons ($t = 24$, d.f. = 71, $P < 0.001$), and PC2 among farms ($F_{5,71} = 9.29$, $P < 0.001$). In winter, DO_2 was higher than in summer, while in the summer, temperature, hardness, alkalinity and conductivity were higher than in the winter. PC2 showed that variance among farms was greater in winter compared with summer; and farms differed from the Hoyos River.

RELATIONSHIP BETWEEN PHYSIOLOGICAL STRESS INDICATORS AND WATER QUALITY

A significant correlation between S and W variables, expressed by the equation: $S = 0.52W - 0.078$ ($r = 0.82$, $P < 0.01$) is shown in Fig. 8. Winter had higher values of dissolved oxygen, nitrate and cortisol, and lower levels of lactate, glucose and antioxidants. In contrast, summer had higher temperatures, lactate, glucose and antioxidants, and lower levels of dissolved oxygen, nitrates and cortisol.

DISCUSSION

The results of this field study indicate that at high altitude, in Mexico at least, *O. mykiss* may exhibit the greatest response to acute stress during the winter, as well as being chronically stressed throughout the year by high-altitude associated hypoxia.

TABLE II. Summary of the results of two-way ANOVA of the effects of season and farm on water-quality variables, identifying significant differences between seasons and among five high-altitude farms in Mexico. Interaction of season and farm was not assessed for flow rates due to missing values

Variables	Flow (l s ⁻¹)		Temp. (°C)		DO ₂ (mg l ⁻¹)		Cond. (mS cm ⁻¹)		NO ₂ (mg l ⁻¹)		NO ₃ (mg l ⁻¹)		Hard. (mg l ⁻¹)		Alkal. (meq l ⁻¹)			
	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P		
Season	6.32	<0.05	492.64	<0.001	148.4	<0.001	80.3	<0.001	1.06	>0.05	91.16	<0.001	236.77	<0.001	0.06	>0.05	34.01	<0.001
Farm	12.95	<0.001	34.85	<0.001	8.75	<0.001	18.9	<0.001	2.77	<0.05	26.57	<0.001	4.18	0.003	20.16	<0.001	0.34	>0.05
Season × farm	N/A	N/A	28.06	<0.001	5.46	<0.001	6.7	<0.001	2.83	<0.05	26.14	<0.001	3.30	<0.05	10.71	<0.001	1.86	>0.05

Flow, flow rate; Temp., temperature; DO₂, dissolved oxygen; Cond., conductivity; NO₂, nitrite; NO₃, nitrate; Hard., hardness; Alkal., alkalinity; N/A, not available.

TABLE III. Mean \pm S.E. water-quality variables between seasons and among five high-altitude farms and Los Hoyos River in Mexico

Farm	Season	Flow (l s ⁻¹)	Cond. (μ S cm ⁻¹)	NO ₂ (mg l ⁻¹)	NO ₃ (mg l ⁻¹)	Hard. (mg l ⁻¹)	pH	Alkal. (meq l ⁻¹)
D (n=6)	Summer	2.6 \pm 0.0	68.4 \pm 0.8	BDL	BDL	120.0 \pm 0.0	6.8 \pm 0.1	73.3 \pm 16.3
D (n=6)	Winter	2.6 \pm 0.0	61.0 \pm 1.2	0.2 \pm 0.3	5.0 \pm 0.0	61.7 \pm 29.2	6.6 \pm 0.5	62.5 \pm 35.0
C (n=4)	Summer	7.3 \pm 4.1	61.0 \pm 0.0	0.4 \pm 0.3	0.2 \pm 0.2	120.0 \pm 0.0	6.8 \pm 0.5	80.0 \pm 0.0
C (n=6)	Winter	4.8 \pm 0.5	57.6 \pm 1.0	BDL	2.5 \pm 2.7	49.1 \pm 14.2	6.9 \pm 0.3	53.3 \pm 39.8
E (n=9)	Summer	2.5 \pm 0.5	70.3 \pm 6.0	0.2 \pm 0.3	0.5 \pm 0.2	120.0 \pm 0.0	6.8 \pm 0.1	97.7 \pm 53.3
E (n=6)	Winter	3.0 \pm 0.0	68.7 \pm 8.1	0.2 \pm 0.2	5.0 \pm 0.0	35.0 \pm 10.8	6.5 \pm 0.3	15.0 \pm 18.9
T (n=9)	Summer	5.2 \pm 0.9	65.2 \pm 1.5	BDL	BDL	104.4 \pm 18.4	6.7 \pm 0.1	75.5 \pm 13.3
T (n=2)	Winter	N/A	50.3 \pm 4.6	BDL	BDL	50.0 \pm 0.0	6.9 \pm 0.3	37.5 \pm 5.0
P (n=12)	Summer	2.8 \pm 0.4	64.6 \pm 3.7	0.4 \pm 0.1	0.3 \pm 0.1	120.8 \pm 2.9	6.2 \pm 0.2	79.5 \pm 1.4
P (n=4)	Winter	0.4 \pm 0.1	49.0 \pm 0.3	BDL	BDL	68.8 \pm 34.2	5.4 \pm 0.3	50.0 \pm 27.1
R (n=4)	Summer	20.0 \pm 0.1	66.9 \pm 1.6	BDL	BDL	85.0 \pm 0.1	6.2 \pm 0.1	80.0 \pm 0.0
R (n=2)	Winter	2.2 \pm 0.6	52.8 \pm 0.8	BDL	BDL	50.0 \pm 0.0	7.0 \pm 0.3	25.0 \pm 21.2

Flow, flow rate; Cond., conductivity; NO₂, Nitrite; NO₃, nitrate; Hard., hardness; Alkal, alkalinity; BDL, below detection limits; D, Dos Potrillos; C, Corral de Piedra; E, El Arroyo; P, Piedra Ancha; T, Tizapa; R, Los Hoyos River.

At 2900 m, DO₂ is naturally 30% less than that at sea level, but oxygen levels in the fish farms were even lower, ranging from 4.2 to 6.0 mg l⁻¹ in the summer to 6.4 to 7.9 mg l⁻¹ in the winter. In high-altitude Mexican fish farms, DO₂ never reached normoxic levels (9–10 mg l⁻¹) at sea level.

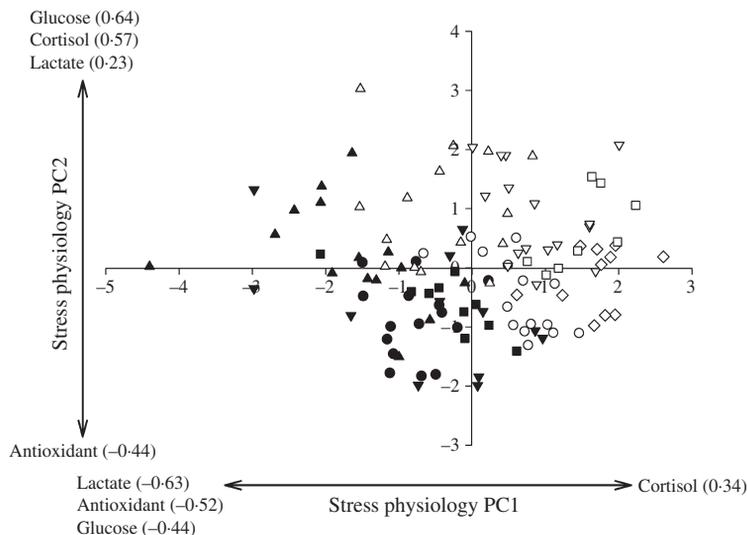


FIG. 6. Principal component analysis (PCA) of physiological variables for *Oncorhynchus mykiss*; loads are shown between parentheses, and increment directions of the stress physiology variables are shown by arrows. The symbols are data points describing season ($\blacktriangledown, \blacktriangle, \bullet, \blacksquare$ = summer; $\triangledown, \triangle, \circ, \square, \diamond$ = winter) and farms ($\blacktriangledown, \triangledown$ = Dos Potrillos; $\blacktriangle, \triangle$ = Corral de Piedra; \bullet, \circ = El Arroyo; \blacksquare, \square = Tizapa; \diamond = Piedra Ancha) ($n = 167$).

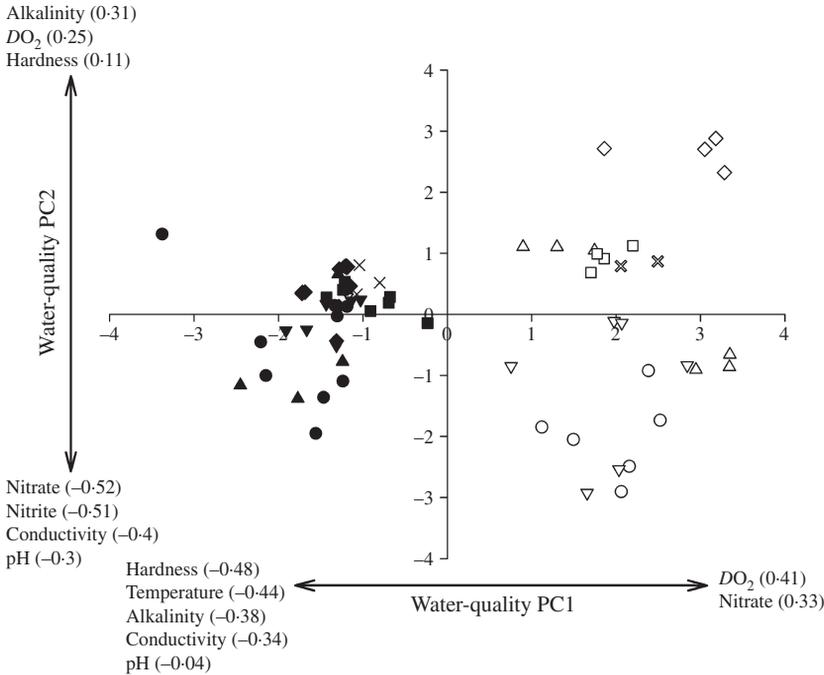


FIG. 7. Principal component analysis (PCA) of water-quality variables for *Oncorhynchus mykiss*; loads are shown between parentheses, and increment directions of the water-quality variables are shown by arrows. The symbols are data points describing season (∇ , \blacktriangle , \bullet , \blacksquare , \blacklozenge , \times = summer; ∇ , \triangle , \circ , \square , \diamond , \otimes = winter) and farms (∇ , ∇ = Dos Potrillos; \blacktriangle , \triangle = Corral de Piedra; \bullet , \circ = El Arroyo; \blacksquare , \square = Tizapa; \blacklozenge , \diamond = Piedra Ancha) ($n = 77$; \times , \otimes = Los Hoyos River).

FIELD DIAGNOSTIC TOOLS

Field diagnostic tools are commonly used in conservation physiology (Wikelski & Cooke, 2006; Cooke *et al.*, 2008) to determine stress levels in wild fish populations (Cooke *et al.*, 2008; Arlinghaus *et al.*, 2009). In Mexican fish farms, field values of whole blood lactate and glucose compared favourably with laboratory measurements, although field lactate values were consistently higher than laboratory values. This was supported by Clark *et al.* (2008), who found that the HemoCue (www.hemocue.com) haemoglobin analyser overestimated the haemoglobin concentrations in the blood. In contrast, previous diagnostic studies of *O. mykiss* and channel catfish *Ictalurus punctatus* (Rafinesque 1818) found that field values for lactate and glucose were consistently lower than established laboratory values (Wells & Pankhurst, 1999; Venn Beecham *et al.*, 2006); while Brown *et al.* (2008) found no differences in farm-raised Atlantic cod *Gadus morhua* L. 1758. In a recent study on the school shark *Galeorhinus galeus* (L. 1758), Awruch *et al.* (2011) found good correlations between field and laboratory values, and any differences were due to the use of whole blood in the field and plasma in the laboratory. Differences, therefore, may result from storage time of whole blood before centrifugation, affecting the concentration of plasma lactate when left in contact with red blood cells (Venn Beecham *et al.*, 2006; Clark *et al.*, 2011).

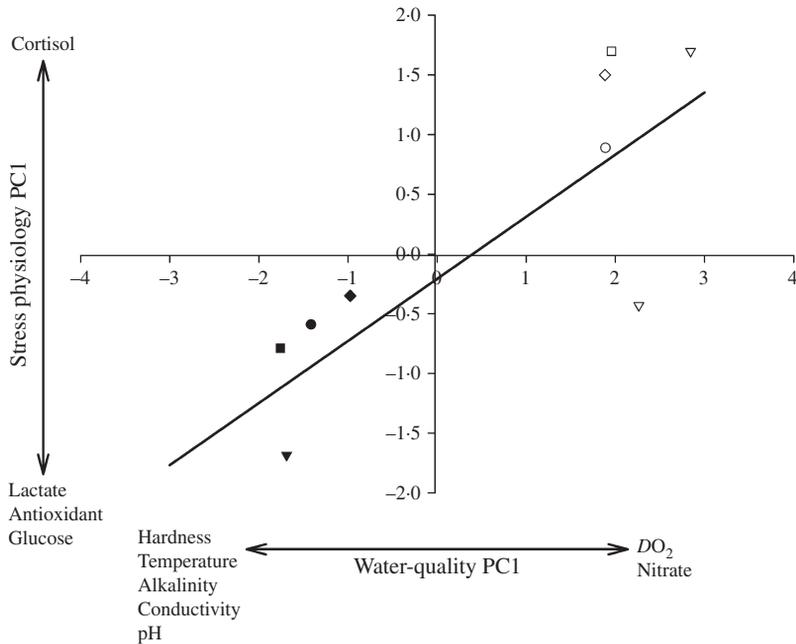


FIG. 8. Predictive relationship between physiology and water-quality indices for *Oncorhynchus mykiss*; increment directions of stress physiology and water-quality variables are shown by arrows. The symbols are data points describing season ▼, ●, ■, ◆ = summer; ▽, ○, □, ◇ = winter) and farms (▼, ▽ = Dos Potrillos; ●, ○ = El Arroyo; ■, □ = Tizapa; ◆, ◇ = Piedra Ancha).

RELATIONSHIPS AMONG PHYSIOLOGICAL STRESS INDICES, WATER QUALITY AND SEASONALITY

In this field study, results from stress tests (netting and confinement) indicate that in *O. mykiss* metabolic compromise was greater at times of higher temperatures and lower oxygen concentrations characteristic of summer in Mexico, which resulted in higher lactate values (5.2 ± 0.2 mM) compared with winter (3.5 ± 0.2 mM). Both seasonal values are several times greater than normoxic *O. mykiss* at rest (0.37 ± 0.27 – 0.83 ± 0.21 mM) (Dunn & Hochachka, 1986; Milligan & Girard, 1993), but are within ranges of fish exposed to hypoxia for 1–3 h, in which blood lactate increased from normoxic values of <1.0 to 6–7 mM (Dunn & Hochachka, 1986; Thomas *et al.*, 1992; Van Raaji *et al.*, 1996; Pankhurst, 2010), but did not reach 8.9 mM in *O. mykiss* exposed to deep hypoxia (2.55 ± 0.01 mg O₂ l⁻¹) (Omlin and Weber, 2010).

In summer, lactate can also rise because of increased swimming activity (Van Raaji *et al.*, 1996). Field lactate values after netting and confinement, however, did not reach those measured after exhaustive exercise (16.52 ± 2.74 mM) (Milligan & Girard, 1993), although they were higher than those of a seasonal study of *O. mykiss* at low altitude (40 m above sea level) [1.13 ± 0.11 (summer) and 2.54 ± 0.24 mM (winter)] (Koldjaer *et al.*, 2004). The above comparisons suggest that lactate values of *O. mykiss* at high altitude are a function of chronic exposure to moderate hypoxia, exacerbated by increased temperatures in the summer. Results from this field study

also showed a higher mean \pm S.E. haemoglobin concentration of 9.04 ± 0.30 g dl⁻¹ (or 5.61 ± 0.19 mM) and double the mean \pm S.E. $V_{PC}\%$ ($53.3 \pm 16.8\%$) compared with literature values of unstressed *O. mykiss* [0.89 ± 0.24 mM (haemoglobin) and $25.1 \pm 6.1\%$ ($V_{PC}\%$)] (Wells & Weber, 1991), which suggests that high-altitude *O. mykiss* in Mexico have acclimated to some extent and show the well-characterized stress response of salmonids (Turner *et al.*, 1983), which combines physiological changes to maintain oxygen supply to the tissues aided by increased haemoglobin concentration and $V_{PC}\%$ (Richards *et al.*, 2007).

Increase in blood glucose concentrations would also be expected with chronic exposure to hypoxia (Omlin & Weber, 2010). While field glucose levels were higher after acute stress tests, no seasonal differences were observed. Of the five farms sampled two, farms D and C, showed glycaemia after acute stress with summer mean \pm S.E. levels of 6.0 ± 0.3 and 7.5 ± 0.3 mM, respectively, and are reflective of glucose in *O. mykiss* after exposure to hypoxia, which increased from baseline levels of 4.9 – 6.1 mM (Omlin & Weber, 2010). While increases in plasma lactate and glucose are sometimes used as proxy measurements for activation of the HPI axis, they are less precise than cortisol (Wedemeyer *et al.*, 1990; Pottinger, 1998; Pankhurst, 2010).

Circulating levels of blood cortisol are primary indicators of both acute and chronic stress in fishes (Barton & Iwama, 1991; Wendelaar Bonga, 1997; Barton, 2002; Øverli *et al.*, 2005), and the pathways of cortisol secretion by the stimulation of the HPI axis are well documented (Morgan & Iwama, 1996; Reid *et al.*, 1996). Baker & Vynne (2014) cautioned that establishing relationships between cortisol and stress can be difficult because of several mitigating factors that could influence the stress response, such as sexual maturity (Sumpter *et al.*, 1987; Pottinger *et al.*, 1995), genetic pedigree (Pottinger & Carrick, 1999), water temperature, (Sumpter *et al.*, 1985) salinity adaptation (Shrimpton *et al.*, 1994) and seasonality (Pickering & Pottinger, 1987). In this study, mean \pm S.E. cortisol values after acute stress (netting and confinement) showed seasonal variation, and were lower during the summer (22.7 ± 3.8 ng ml⁻¹) than the winter (76.7 ± 4.0 ng ml⁻¹), but similar to mean values of netted *O. mykiss* held in 10001 tanks [10 – 20 (summer) and 40 – 80 ng ml⁻¹ (winter)] (Koldjaer *et al.*, 2004). While seasonal values after exposure to acute stress were much greater than resting levels of juvenile *O. mykiss*, consistently reported as ≤ 10 ng ml⁻¹ (Barton & Iwama, 1991; Gamperl *et al.*, 1994), the stressed values rarely exceeded levels (200 ng ml⁻¹) of fish chronically exposed to toxins (Barton & Iwama, 1991; Gamperl *et al.*, 1994) or levels measured in moderate hypoxia (55% oxygen concentration) when cortisol concentrations increased 10 times from 14.73 to 169.5 ng ml⁻¹ over 2 h (Lai *et al.*, 2006). In the field, the highest cortisol concentration recorded in the summer for an individual *O. mykiss* was 105 ng ml⁻¹, while in the winter it more than doubled to 231 ng ml⁻¹. Therefore, it is clear that both netting and confinement elevate the primary release of cortisol (Perry *et al.*, 1996; Koldjaer *et al.*, 2004), but cortisol levels may vary based on the type and duration of stress. For example, cortisol levels from 200 to 1400 ng ml⁻¹ were recorded in migrating juvenile Chinook salmon *Oncorhynchus tshawytscha* (Walbaum 1792) and continuously stressed juvenile *O. mykiss* had levels of 160 ng ml⁻¹. Further differences in corticosteroid response exist among strains or stocks within the same species and between wild and hatchery fish (Øverli *et al.*, 2005). In one study, hatchery *O. mykiss* were shown to set up dominance hierarchies within the ponds, with the most dominant fish having the highest cortisol values (LeBlanc

et al., 2011). Further, younger, smaller fish are more sensitive to chronic and acute stressors than older, larger fish (Barton, 2002). This was supported in the field study, which showed a significant relationship between cortisol concentrations (C) and length (L_S) [$C = 25.5 \log_{10}(M_W) - 22.4$; $n = 83$, $r^2 = 0.2$, $P < 0.001$]. In his review, Barton (2002) pointed out that whether fishes that display relatively high or low corticosteroid stress responses are actually 'more or less stressed' than others, or simply have different capacities to respond to stressors, is not clear. An additional explanation for lower mean values in the summer compared with winter at high altitude could be a result of hormonal habituation in the summer and individuals thought to be habituated to a stressor may actually be chronically stressed, despite a reduced stress response (Tache *et al.*, 1976; Marti & Amario, 1998; Cyr & Romero, 2009).

Total antioxidant capacity (antioxidant enzymes and non-enzyme components) was used, in this study, as an additional index of stress (Pedrajas *et al.*, 1995; Gorbi & Regoli, 2005) and it was significantly higher after acute stress in summer (2.1 ± 0.1 mM of Trolox) than the winter (0.9 ± 0.1 mM of Trolox). A balance is thought to exist between pro-oxidant production, *i.e.* increased reactive oxygen species (ROS) and antioxidant defence (Livingstone, 2001), with increases in antioxidant enzyme activities indicating ROS formation in the organism (Pinho *et al.*, 2005). The increased levels of antioxidant defences in anoxic and hypoxic conditions recorded in many different aquatic animals (Lushchak *et al.*, 2001) suggest that preparation for oxidative stress is a key adaptive biochemical mechanism for anoxia and hypoxia tolerance. Much ambiguity is still associated with understanding the antioxidant responses in fishes with changing DO_2 and to high altitude and chronic residence at high altitude (Jefferson *et al.*, 2004).

Finally, PCA showed that there were significant distribution differences in physiological and water-quality variables between seasons and among farms. Between seasons, PCA of physiological variables supported increased stress as measured by lactate in the summer, when temperature was highest and DO_2 was lowest. This suggests that acute stress may have more profound effects on lactate at warmer temperatures. The lower levels of cortisol in the summer than the winter suggested that either some attenuation of the stress response occurred in the summer due to chronic exposure to high temperature and low oxygen or that other mitigating factors, such as changes in water supply, led to differential seasonal responses. Among the farms, variance for water-quality variables was greater in the winter or dry season, than the summer or wet season. This may have reflected differences in farm-specific fish husbandry practices and water supply, which is significantly reduced during the drier winter season.

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