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The final countdown: Continuous physiological welfare evaluation of farmed fish during common aquaculture practices before and during harvest

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ABSTRACT

Welfare of farmed fish has become of increasing concern for consumers, producers, interest groups and authorities. To improve fish welfare, it is necessary to find indicators that can identify stressful situations early enough so that an intervention can take place before detrimental effects occur. By using heart rate bio-loggers in freely swimming rainbow trout (*Oncorhynchus mykiss*), combined with plasma cortisol levels and a range of haematological and blood chemistry parameters, we assessed the severity of stress responses induced by a range of common aquaculture practices before and during harvest. Following surgery, transportation and re-introduction with conspecifics in the sea cage, it took ~4 days for heart rate to stabilize and for a clear circadian rhythm in heart rate to emerge (*i.e.* average circadian fluctuation in heart rate of ~25 to 27 beats min^{-1}). The presence or absence of this circadian rhythm in heart rate could inform researchers in the aquaculture industry whether or not specific farming routines induce unnecessary and prolonged stress. The elevations in heart rate caused by common farming practises such as crowding and transportation (*e.g.* heart rate increased by ~8 and 9 beats min^{-1} above what would normally be expected for that time of day, respectively) corresponded well with increases in plasma cortisol levels. Stressful farming practises or events (indicated by elevated plasma cortisol levels) such as air exposure during brailing and aquatic hypoxia triggered a hypoxic bradycardia until fish were released back into oxygenated water whereupon heart rate significantly increased to repay the accumulated oxygen debt. Repeated stress induced by multiple farming practises (*i.e.* combined stressors of crowding, brailing and transportation) clearly had a cumulative and long-lasting effect as heart rate peaked at ~25 beats min^{-1} above what would normally be expected for that time of day. Heart rate also remained significantly elevated by ~9 beats min^{-1} the following morning, suggesting that if rainbow trout need to be subjected to multiple stressors during consequtive days, it is recommended that sufficient time for recovery is provided between stressors. This study demonstrates that heart rate monitoring can be useful to assess stress levels of freely swimming fish in sea cages. Moreover, the use of implantable bio-loggers opens up a broad range of possible applications that will allow researchers to investigate the effects of environmental and/or anthropogenic stressors on the welfare of fish under conditions more realistic to the aquaculture industry.

1. Introduction

Aquaculture is the fastest growing animal-based food producing sector in the world with a total annual production currently exceeding 76 million tonnes (FAO, 2017). The welfare of farmed fish, estimated to be between 37 and 120 billion individuals in 2012 (Mood and Brooke, 2012), has become of increasing concern for consumers, producers, interest groups and authorities (Ashley, 2007; EFSA, 2009a; Frewer et al., 2005; OIE, 2017).

The concept of animal welfare is complex and multifaceted. It has been defined as the ability of an animal to adapt to its environment and remain in good health, the ability of an animal to live a natural life and express its natural behaviour, and/or an animal's subjective mental state (Ashley, 2007; Broom, 1991a; Huntingford et al., 2006; Turnbull and Huntingford, 2012). Whilst physical health is universally accepted as a measure of welfare, whether fish experience mental suffering (*e.g.*

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from pain or fear) is a controversial issue (Ashley, 2007; Turnbull and Huntingford, 2012). This controversy is centred around whether fish have the necessary cognitive and behavioural complexity to experience feelings and emotions (Key, 2016; Rose et al., 2014; Sneddon, 2015). However, due to a growing body of evidence indicating that fish have such capabilities, current European legislation assumes that fish are sentient beings that may indeed have the capacity to suffer (Braithwaite, 2010; Council Directive 98/58/EC, 1998; Council of Europe, 2005; Turnbull and Huntingford, 2012; Van de Vis et al., 2012). Therefore, further research on the welfare of farmed fish is required to provide recommendations for best practices and future legislation (Ashley, 2007; EFSA, 2009b; Van de Vis et al., 2012).

Currently, behavioural and physiological stress responses of fish are commonly used as indicators of compromised welfare (Ashley, 2007; Broom, 1991b; Huntingford et al., 2006; Segner et al., 2012). The primary stress response of fish involves the release of catecholamines and activation of the hypothalamic-pituitary-interrenal axis, which results in the release of corticosteroids. This gives rise to a suite of secondary responses (*i.e.* heightened cardiorespiratory activity, splenic release of red blood cells and mobilization of energy stores), which serve adaptive functions for individual's experiencing demanding conditions in nature (Barton, 2002; Wendelaar-Bonga, 1997). However, prolonged and/or repeated stress imposed on fish may result in detrimental tertiary stress responses including impaired appetite, growth, swimming ability, immune responses, behavioural repertoire and reproductive ability (Ashley, 2007; Huntingford et al., 2006; Segner et al., 2012). Such tertiary stress responses have commonly been used as indicators for compromised welfare of fish in aquaculture. However, these are rather late indicators, which makes it difficult to determine the possible origin or cause of the stress response, as well as to allow for an intervention to take place before these detrimental effects have occurred (Huntingford et al., 2006; Turnbull and Huntingford, 2012; Van de Vis et al., 2012). Furthermore, it becomes increasingly difficult, if not impossible, to use behavioural measures (*i.e.* observations of altered behaviour, behavioural deficits, changes in attentional state, aggression levels and preferences/priorities of individuals) to investigate fish welfare when carried out on-site at aquaculture facilities due to factors such as poor water visibility and high stocking densities. Finding alternative welfare indicators that do not rely on visual observations of fish or on the occurrence of detrimental tertiary stress effects are particularly important during the final days before slaughter. This is because practices used immediately before and during harvest are not only critical from an animal welfare point of view, but that it also largely affects the quality of the final product (Ashley, 2007; Robb and Kestin, 2002).

An alternative approach for investigating fish welfare on-site is to use implantable bio-telemetry systems and bio-loggers, which allow the recording of a range of physiological variables (*e.g.* heart rate, blood flow, muscular movements and electroencephalography) in response to varying environmental and/or anthropogenic stressors in freely swimming fish (Axelsson et al., 2007; Clark et al., 2010; Cooke et al., 2004; Gräns et al., 2010; Hinch et al., 2002; Ropert-Coudert and Wilson, 2005). Heart rate and heart rate variability in response to various stimuli has been used as an indicator of stress, together with a wide range of other variables and activities such as swimming activity, metabolic rate, food intake, digestion and predator interactions (Altimiras and Larsen, 2000; Armstrong, 1986, 1998; Clark et al., 2010; Gräns et al., 2014; Höjesjö et al., 1999; Prystay et al., 2017). Recent innovations and miniaturization of bio-loggers (*i.e.* Star-Oddi DST milli-HRT loggers, STAR-ODDI, Gardabaer, Iceland) that can intermittently measure heart rate, body temperature and time of day over long periods of time (*i.e.* weeks to months) provide a particularly exciting avenue for on-site studies. This technology has the potential to allow the identification of 'normal' physiological patterns of freely swimming and undisturbed fish in aquaculture settings over long periods of time, as well as to identify and analyse deviations from this pattern due to environmental and/or anthropogenic stressors such as those experienced during harvest (Lines and Spence, 2012; Ropert-Coudert and Wilson, 2005).

The aim of the present study was to investigate whether *in vivo* monitoring of heart rate in freely swimming fish could be used as an early indicator of stress and as a tool for assessing the welfare of rainbow trout (*Oncorhynchus mykiss*) in sea cages during common aquaculture practices before and during harvest. Specifically, by using surgically implanted heart rate bio-loggers in focal fish, we monitored heart rate and temperature for an extended period of time prior to harvest in order to investigate recovery time from surgery and re-introduction with conspecifics in the sea cage, as well as to subsequently identify 'normal' circadian rhythm in heart rates. Using this information, combined with plasma cortisol levels and a range of haematological and blood chemistry parameters, we assessed the severity of the stress response induced by a range of farming practices that commonly occur during harvest (*e.g.* crowding, brailing, well-boat transport and CO_2 narcosis). This information is invaluable when developing specific recommendations for future legislation aiming to improve fish welfare, as well as to improve production and management systems.

2. Material and methods

2.1. Location, experimental animals and ethical statement

The experimental part of the study was conducted at the Brändö Lax AB facilities (60.444674°N, 21.057210°E), Brändö, Åland Islands, Finland between the 31st of August and 27th of September 2016. Female rainbow trout (*Oncorhynchus mykiss*, Walbaum 1972) with body masses ranging between 945 and 2952 g (mean ± s.e.m.: 1813 ± 36 g) were used. They were kept with ~5000 conspecifics (total biomass: ~9500 kg) in a circular sea cage (diameter: 40 m, depth: 4 m) anchored approximately 100 m away from the facilities in the Djurholms Sound. All experimental protocols were approved by Åland Provincial government project approval committee (decision 2/2016).

2.2. Programming and surgical implantation of DST milli-HRT bio-loggers

On the 31st of August, trout were lightly crowded in the sea cage and ~40 individuals were brailed into a smaller rectangular sea cage (width: 2 m, length: 6 m, depth: 1 m), which was subsequently towed to the Brändö Lax AB facilities by boat. These individuals were kept and fasted in this location for 1 week. On the 6th and 7th of September, 20 individuals with body masses ranging between 1024 and 2952 g (mean ± s.e.m.: 2082 ± 113 g) were randomly selected for the surgical implantation of DST milli-HRT bio-loggers (Logger version 8 DM/CRC16/4800, STAR-ODDI, Gardabaer, Iceland).

The DST milli-HRT bio-loggers (diameter: 13.0 mm, length: 39.5 mm, volume: 5 cm^3, mass in air: 11.8 g) monitor heart rate *via* a single channel electrocardiogram (ECG) amplifier that uses three measuring electrodes incorporated into the ceramic casing. Each logged heart rate value is derived from the mean RR-interval (*i.e.* time between two consecutive R waves in the ECG) from a burst of 600 measurements, which corresponds to a 6-s period when using a sampling frequency of 100 Hz. A temperature sensor is also located within the casing of the logger and has a resolution of 0.032 °C and an accuracy of ± 0.2 °C. Additionally, the sensor contains a real-time clock with an accuracy of ± 1 min $month^{-1}$. The loggers were programmed with the application software Mercury v 4.28 and the associated Communication Box (STAR-ODDI, Gardabaer, Iceland). Heart rate and temperature were measured every 10 min from the 6th of September (beginning at 6.00 am) until the 27th of September (ending at 6.00 am), where after measurements were taken every 2 min until 6.00 pm on the 27th of September. The aforementioned times, as well as all times reported below, are in Eastern European Summer Time (EEST or UTC + 2).

To surgically implant the logger, an individual trout was initially anaesthetized in a bin containing water from the Djurholms Sound with

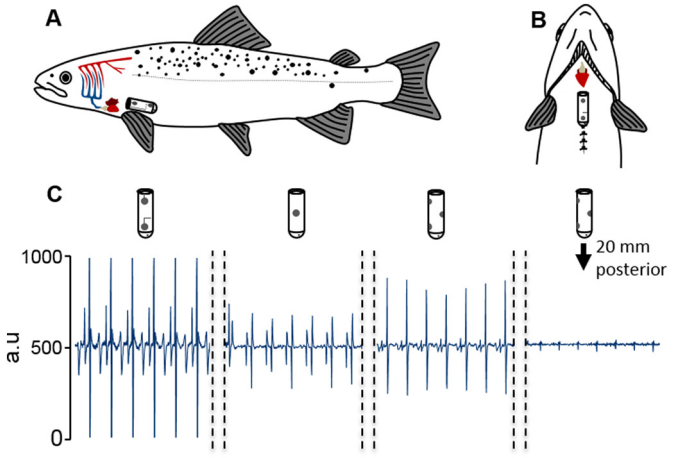

Fig. 1. Placement of the bio-logger inside the abdominal cavity relative to the heart of a rainbow trout (*Oncorhynchus mykiss*) is shown from (A) a lateral view and (B) a ventral view. (C) The four different logger positions tested and the effects on the raw ECG signal strength. The side of the logger facing the reader is the side facing the abdominal muscles of the fish and the far right panel of (C) demonstrates the ECG signal strength when the logger is positioned 20 mm posterior of the position demonstrated in (A) and (B). The ECG signal strength is optimized when the logger is positioned close to the heart and with the two in-built electrodes facing the musculature in the ventral midline (left panel of C).

150 mg L^{-1} ethyl-3-aminobenzoate methanesulphonic acid (MS222, Sigma-Aldrich Inc., St. Louis, Missouri, USA) buffered with 300 mg L^{-1} $NaHCO_3$. When ventilation ceased and the fish was deemed to reach surgical aneasthesia, it was placed on an operating table covered with soft, water-soaked foam. To maintain anaesthesia and a sufficient oxygen supply, the gills were continuously supplied with aerated water containing 75 mg L^{-1} MS222 buffered with 150 mg L^{-1} $NaHCO_3$ at 10 °C. The logger was implanted by first making a 25–30 mm mid-ventral incision ~40 mm posterior to the pectoral fins. The logger was then inserted with the flat end facing anteriorly and gently pushed towards the pericardium until only the rounded end of the logger was visible at the anterior end of the incision (Fig. 1A & B). This ensured that the logger was in close proximity to the pericardium. The logger was anchored with the side containing the two ECG electrodes facing ventrally towards the muscles of the abdomen, in-between the liver and the muscle tissue (Fig. 1A & B), by suturing the rounded end of the logger to the body wall using the channel provided in the ceramic casing. A 12 mm Passive Integrated Transponder tag (*i.e.* PIT-tag, Oregon RFID, Portland, Oregon, USA) was also inserted into the abdominal cavity. The wound was subsequently closed with interrupted stitches using 3-0 sterile monofilament non-absorbable Prolene suture material (Ethicon Inc., Somerville, New Jersey, USA). A mixture of Orabase® (a protective paste, ConvaTec, Bromma, Sweden), Pevaryl® (an antifungal agent, McNeil Sweden AB, Solna, Sweden) and Bacibact® (an antibacterial agent, Orion Corporation, Espoo, Finland) was applied on the surface of the wound while Baytril® vet (a broad-spectrum antibiotic, 10 mg kg^{-1}, Bayer Healthcare, Berlin, Germany) was injected intraperitoneally. Prior to returning the individual to the small rectangular sea cage for recovery, it was tagged with blue dots in the area between the pectoral fins using Alcian Blue dye injected with a pressure injector (AKRA Dermojet Polymedical, Barthou, France). This was performed to facilitate the identification of trout implanted with bio-loggers during the slaughter procedure so that they could be retrieved.

The optimal placement of the bio-logger with regard to signal quality and strength was determined in a pilot study. Three individual rainbow trout (568 ± 13 g) were used and the logger was secured in four different positions in each fish (Fig. 1C). The order of recording in a particular position was randomized among individuals and the logger recorded heart rate in each position for at least 10 min. Following insertion of the logger, the anaesthetised fish was placed with its ventral surface on the surgical table and the heart rate was recorded for at least 10 min. The sutures were then cut and the procedure was repeated with the logger in a different position until all four positions had been tested. The optimal positioning of the logger was subsequently determined visually from the individual ECG data buffer files. Clear differences were observed in the ECG signal with respect to the orientation and position of the logger (Fig. 1C). The strongest ECG signal in all three fish was when the two electrodes were orientated ventrally towards the abdominal muscles of the body wall (*i.e.* far left ECG segment in Fig. 1C). When the logger was withdrawn 20 mm away from the heart in a posterior direction, the amplitude of the ECG signal was markedly reduced (*i.e.* far right panel of Fig. 1C). Furthermore, it is important that researchers using these bio-loggers verify a number of ECG records from their experimental animals to ensure that the logger is using the QRS waveform interval to calculate heart rate.

2.3. Monitoring during recovery period and slaughter procedure

On the 9th of September (day 0), trout implanted with the bio-loggers together with the remaining uninstrumented trout were transported and transferred back into the sea cage, containing their ~5000 conspecifics, using a brail net. Trout were allowed to recover in the sea cage till the 25th of September (day 16) during which they were fed *ad libitum* once per day with commercial trout pellets (BioMar, Aarhus, Denmark) according to the normal feeding regime at the farm.

In accordance with pre-slaughter procedures employed at Brändö Lax AB, trout were fasted from the 20th of September (day 11) onwards (*i.e.* 1 week before transport to the slaughterhouse). On the 26th of September (day 17) beginning at 7.15 am, the trout were crowded at a low density and a randomly selected sub-sample of individuals (low density crowding group, $n = 20$) were individually netted carefully from the sea cage using a pole net, euthanized with a sharp cranial blow and sampled 2 ml of blood from the caudal vessels using heparinized syringes. This procedure took < 1 min and the blood samples were immediately placed on ice for further analyses.

At 8.15 am, the bottom of the sea cage was elevated in order to increase the density of trout. At 9.45, trout were transferred from the sea cage to the well-boat using a brail net. During this transfer, a sub-sample of individuals (high density crowding/brailing group, $n = 20$) were again randomly selected, euthanized and sampled for blood in the same manner as above.

Between 10.00 and 11.00 am, trout were transported around the Djurholm Sound in the well-boat to eventually arrive at the holding cage next to the Brändö Lax AB slaughter facilities. Following the transportation event, a sub-sample of individuals (well-boat transport group, $n = 20$) were randomly selected, euthanized and sampled for blood.

At 11.55 am, the remaining trout in the well-boat were transferred to the holding cage anchored by the slaughterhouse *via* a water chute and left undisturbed overnight. Before the slaughter procedures were initiated in the morning of the 27th of September (day 18), a sub-sample of individuals (pre-slaughter holding group, $n = 20$) were again randomly selected and carefully netted from the sea cage, euthanized and sampled for blood.

At 6.52 am, the slaughter began and trout were brailed from the holding cage to a chute leading to a tank in the slaughterhouse containing water saturated with CO_2. Trout were held in this tank until they could no longer maintain equilibrium and were deemed unconscious, whereupon they were mechanically lifted onto a grid designated as the 'gill cutting station'. Personnel then manually cut the gill arches and ventral aorta of each individual, and transferred them to an adjacent tank for exsanguination. The time taken for an individual leaving the sea cage until death was ~4–8 min. Directly following the CO_2 narcosis, a sub-sample of individuals (brailing and CO_2 narcosis group, $n = 20$) were randomly selected, euthanized and sampled for

blood. In addition, blood samples were also taken from all of the instrumented individuals when arriving at the 'cutting station' following CO_2 narcosis while retrieving the bio-loggers.

2.4. Data retrieval and analysis from DST milli-HRT bio-loggers

Data retrieval from the bio-loggers was performed using the application software Mercury v 4.28 and the associated Communication Box (STAR-ODDI, Gardabaer, Iceland). For validation purposes, all logged heart rate measurements were graded with a data verification quality index (QI), which ranged from 0 to 3, whereby QI_0 = Great, QI_1 = Good, QI_2 = Fair and QI_3 = Poor. To ensure the highest possible accuracy, only measurements graded with QI_0 were used in the present study (*e.g.* left panel of Fig. 1C). By only selecting measurements graded with QI_0, we used 34.4 ± 3.6% of the sampled data.

Heart rate of instrumented trout were analysed following the reintroduction to the sea cage in order to assess the recovery from the stress associated with surgery, transportation and reintroduction with conspecifics, as well as to determine the average circadian rhythm in heart rate. During the time between reintroduction to the sea cage and the initiation of harvesting procedures (16 days), daily mean heart rate, as well as the mean of the lowest 20% and highest 20% of daily heart rate values were calculated for each individual. These values were used for assessing the recovery from the abovementioned stressors following reintroduction to the sea cage and as a good approximation of heart rate during periods of rest and during periods of increased activity. This in turn could be used to determine the average circadian rhythm in heart rate of these individuals.

Heart rate of instrumented trout were also analysed during the two days associated with the slaughter procedures to evaluate the severity of, and recovery from, a range of different acute stressors. Specifically, mean heart rate of instrumented trout were determined at nine distinct 'events' during these two days, which corresponded to 1) directly before our arrival at the sea cage (*i.e.* undisturbed), 2) during low density crowding, 3) during high density crowding, 4) after brailing from the sea cage to the well-boat, 5) during transport in the well-boat, 6) when trout were held in the stationary well-boat, 7) shortly after the trout were released into the holding cage next to the slaughterhouse, 8) directly before the slaughter procedures were initiated, and 9) after brailing and CO_2 narcosis. The number of measurements used to calculate an individual's mean heart rate for each event (event 1, 2, 3, 5, 7 and 8 = 1–6 measurements, event 4, 6 and 9 = 1–3 measurements) varied depending on the length of the event, the sampling resolution and the number of heart rate values graded with QI_0.

To determine the mean heart rate response induced by each 'event', we compared the heart rate of individuals at each 'event' during the two days associated with slaughter with their 'normal' circadian rhythm in heart rate. This 'normal' circadian rhythm in heart rates were determined by calculating the mean heart rate for each individual every 10 min for 24 h based on heart rate data from days 14 to 16, as these days best represented the conditions the fish were exposed to during the slaughter procedures with respect to feeding status and water temperature.

2.5. Blood analyses

Blood samples were analysed for haematocrit (Hct, %), haemoglobin concentration ([Hb], $g\,dl^{-1}$) and the total number of red blood cells (RBC, 10^6 cells μl^{-1}). The Hct was determined as the fractional red cell volume after centrifugation of a subsample of blood in 80 μl heparinized microcapillary tubes at 10,000 rcf for 5 min in a Hct centrifuge (Haematokrit 210, Hettich, Tuttlingen, Germany). A handheld Hb 201^+ meter (Hemocue® AB, Ängelholm, Sweden) was used to determine [Hb] and values were corrected for fish blood (Clark et al., 2008). Mean corpuscular haemoglobin concentration (MCHC, $g\,dl^{-1}$) was subsequently calculated as [Hb]/Hct × 100. The RBC were counted at 400× magnification in five 0.2 mm^2 secondary squares within the large central square of a Neubauer improved haemocytometer (Sigma-Aldrich Inc., St. Louis, Missouri, USA) after 1:100 dilution with Natt-Herrick's solution and calculated as total count × 5000/10^6 (Huyben et al., 2017). Mean corpuscular volume (MCV, fl $cell^{-1}$) and mean corpuscular haemoglobin (MCH, pg $cell^{-1}$) were subsequently calculated as Hct/RBC × 10 and [Hb]/RBC × 10, respectively.

Following the haematological analyses, blood samples were centrifuged at 10000 rcf for 5 min in a microcentrifuge (Eppendorf® 5415D, Sigma-Aldrich Sweden AB, Stockholm, Sweden), and the plasma was collected and frozen at −80 °C for later analyses. Plasma cortisol concentration was determined using a radioimmunoassay as described by Young (Young, 1986) using a cortisol antibody (Code: S020; Lot: 1014–180182) purchased from Guildhay Ltd (Guildford, Surrey, UK) validated by Sundh et al. (Sundh et al., 2011). As a tracer, tritiated hydrocortisone-[1,2,6,7–^{3}H(N)] (NET 396; NEN Life Sciences Products, Boston, Massachusetts, USA) was used and cortisol standards were prepared from hydrocortisone (Sigma, St. Louis, MO, USA). The determination of the radioactivity was performed with a Wallac 1409 liquid scintillation counter. Intra- and inter-assay coefficients of variation (CV) for this cortisol RIA were 3.9% and 5.4%, respectively, and the detection limit was 0.7 ng ml^{-1} (Sundh et al., 2011). The concentration of electrolytes (*i.e.* K^+, Na^+, Cl^{-1} and Ca^{2+}) was determined from 100 μl plasma samples using an electrolyte analyzer based on ion selective electrode technology (Convergys® ISE comfort Electrolyte Analyzer, Convergent Technologies, Cölbe, Germany).

2.6. Statistical analyses

Statistical analyses were performed with SPSS Statistics 21 (IBM Corp., Armonk, NY, USA). All data subjected to statistical analyses were assessed to ensure that they did not violate the assumptions of the models used. In the cases where the assumptions were violated the particular transformations are described below. F- and P-values obtained from the statistical analyses are reported throughout the text and all *P*-values < .05 were considered statistically significant. Values of the dependent variables are reported as means ± s.e.m. unless otherwise stated.

To statistically analyse the heart rate data of instrumented trout during the time period between the reintroduction to the sea cage and the initiation of harvesting procedures, we used a linear mixed model with a first-order autoregressive (AR1) repeated covariance matrix, as the recordings that were close in time were also more dependent than temporally distant recordings. The model was run separately on fed (*i.e.* days 1–10) and fasted individuals (*i.e.* days 11–16), to avoid the potential confounding effects of feeding status on heart rate. In both models, individuals were used as the subject variable, days following reintroduction to sea cage as the repeated measure and fixed factor, body temperature as a covariate, and heart rate (*e.g.* mean of lowest 20%, mean, and mean of highest 20%) as the dependent variable. However, body temperature did not have a significant effect on heart rate in either fed or fasted trout (most likely due to the small differences in body temperature, ~1.6 °C, between the fed and fasted period), and was therefore removed from the model, which improved the fit of the model according to Akaike's Information Criterion (AIC). To statistically analyse the heart rate data of instrumented individuals during the nine 'events' that occurred during the harvesting days in comparison to the normal circadian rhythm, we used a similar linear mixed model as described above. However, in this model we used the time of day as the repeated measure and the two days (the days of harvesting *vs.* day of normal circadian rhythm), as well as the interaction between the two days at each time point, as fixed effects. For further comparisons between the repeated dependent variables in both linear mixed models, a Bonferroni confidence-interval adjustment was used.

To determine whether there were any statistically significant differences in body mass, body length, plasma cortisol, [Hb], Hct, RBC

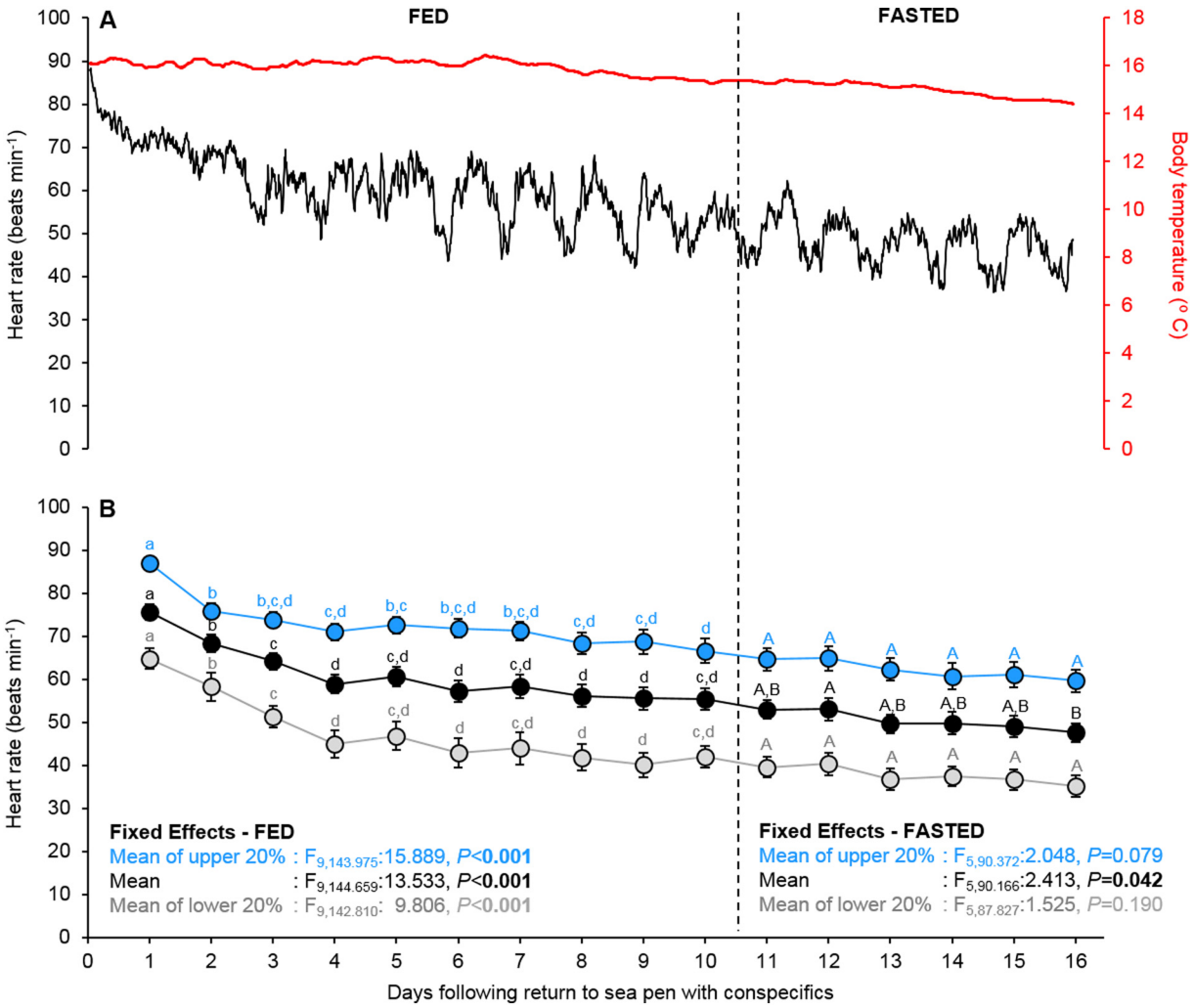

Fig. 2. (A) Hourly moving mean heart rate (black line) and body temperature (red line) of rainbow trout (n = 20) for 16 days after being reintroduced into the sea pen with conspecifics following the surgical implantation of the Star-Oddi DST milli-HRT bio-loggers. (B) Temporal changes in mean heart rate (black circles and line), and heart rate during periods of rest (mean of the lowest 20% of daily heart rate values, grey circles and line) and periods of increased activity (mean of the highest 20% of daily heart rate values, red circles and line) during the 16-day period. Linear mixed models were used to statistically analyse temporal changes in heart rate of trout during the fed and fasting periods (separated by black vertical dashed line) with different letters representing the significant differences in heart rate ($p < .05$, lower case: fed period, upper case: fasting period). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

count, MCHC, MCV, MCH, as well as $[Na^+]$, $[K^+]$, $[Ca^{2+}]$ and $[Cl^-]$ in the plasma of uninstrumented trout during five of the nine 'events', we used a one-way ANOVA with a Tukey's *post hoc* test. For variables that violated the assumption of normality, we applied either a 'square root' (*i.e.* plasma cortisol) or a natural logarithmic transformation (*i.e.* MCHC, plasma $[K^+]$ and $[Cl^-]$).

3. Results

3.1. Heart rate of freely swimming rainbow trout in the sea cage with conspecifics

Visual inspection of the hourly moving mean heart rate of the 20 rainbow trout in the sea cage reveals that > 3 days are required for a clear circadian rhythm in heart rate to emerge following the combined stressors of surgery, transportation and reintroduction with conspecifics in the sea cage (Fig. 2A). This is further supported by the statistical analyses of heart rate during the fed period (*i.e.* days 1–10), as the mean of the lowest 20% and the overall mean of daily heart rate significantly decreased for 4 days following reintroduction into the sea cage (Fig. 2B). After 4 days, these heart rate values stabilized for the rest of the fed period at 40–47 and 55–60 beats min^{-1}, respectively (Fig. 2B). The mean of the highest 20% of daily heart rate significantly decreased for the first day following reintroduction, and then tended to fluctuate between 67 and 76 beats min^{-1} for the remainder of the fed period. The average circadian fluctuation in heart rate (*i.e.* mean of highest 20% minus the lowest 20%) during the fed period (days 4–10) was ~27 beats min^{-1}.

During the fasting period (days 11–16), mean heart rate ranged between 48 and 53 beats min^{-1} and was relatively stable with the exception that heart rate was slightly, yet significantly, higher on day 12 when compared to day 16 (Fig. 2B). The means of the lowest and highest 20% of daily heart rate did not significantly differ across the days during the fasting period with values of 35–40 and 60–65 beats min^{-1}, respectively. The average circadian fluctuation in heart rate was ~25 beats min^{-1} during this period.

3.2. Stress responses of rainbow trout during slaughter

Visual inspection of the hourly moving mean heart rate of the 20 rainbow trout during the two days prior to slaughter revealed that although heart rate responses varied depending on the particular stressor,

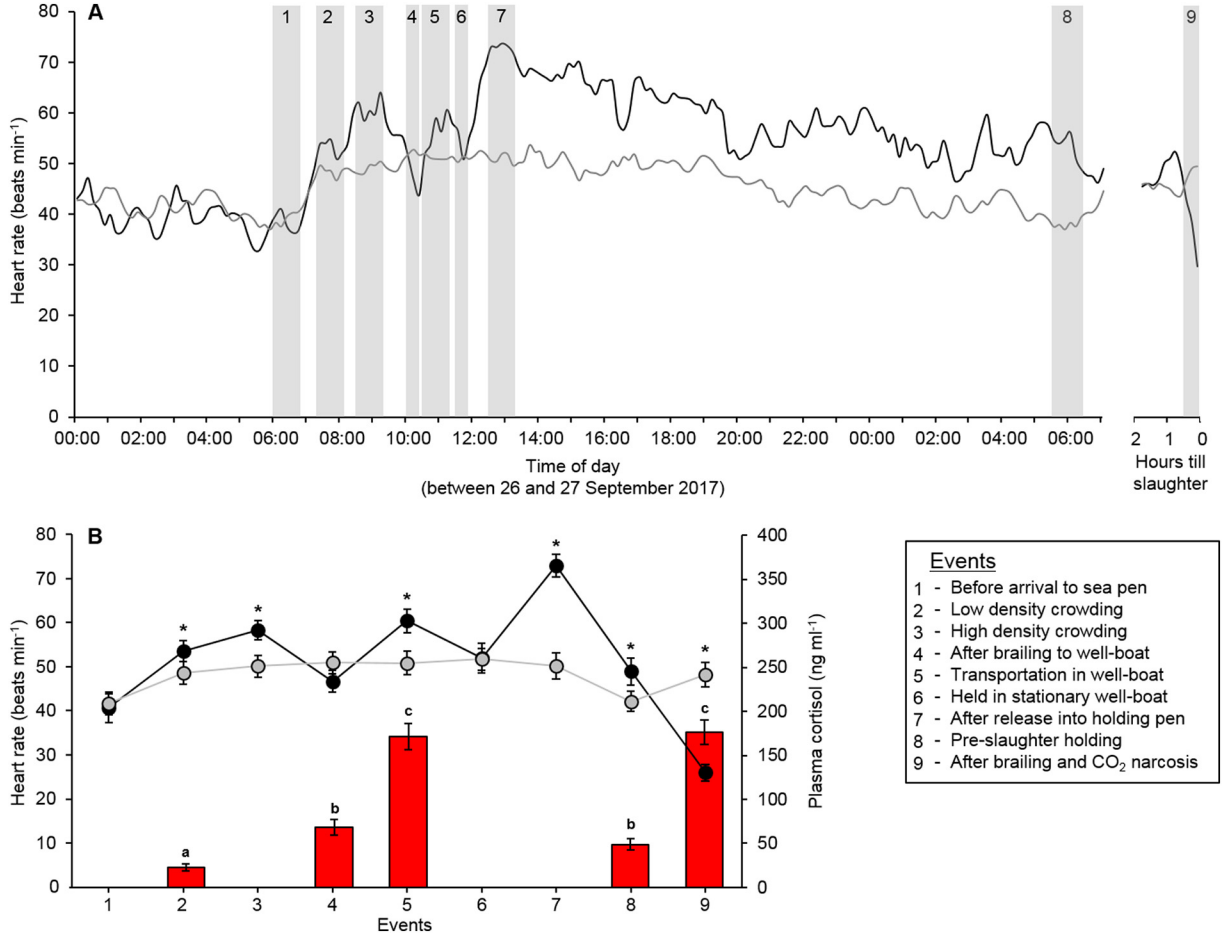

Fig. 3. (A) Hourly moving mean heart rate of rainbow trout (n = 20) during the final 2 days leading up to harvest (black line) compared to the hourly moving mean of the 'normal' circadian rhythm in heart rate previously observed in the same individuals (grey line, calculated from days 14–16 in Fig. 2, see materials and methods for more details). (B) The mean heart rate (black line and circles) during nine identified events (grey bars in panel A) compared to the mean of the 'normal' circadian rhythm in heart rate previously observed in the same individuals (grey lines and circles), as well as plasma cortisol from sub-samples of trout (red bars, n = 20 for each event) from 5 of the 9 identified events (*i.e.* event 2, 4, 5, 8 and 9). Statistical analyses were generated using a linear mixed model (heart rate) or a one-way ANOVA (plasma cortisol), and significant differences ($p < .05$) are represented by either an * (heart rate) or different letters (plasma cortisol). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

overall heart rate was typically elevated when compared to the 'normal' circadian rhythm in heart rate (*c.f.* black and grey lines in Fig. 3A). This was further supported by the statistical analyses of mean heart rate at the discrete 'events' during this period (Fig. 3B). Prior to the arrival of the well-boat at the sea cage, heart rate did not differ from the normal circadian rhythm (event 1, $P = .761$, Fig. 3B). However, the subsequent arrival of the boat and low density crowding in the sea cage resulted in a significant increase in heart rate (event 2, $P = .034$, Fig. 3B), which increased further with increased crowding density (event 3, $P = .003$, Fig. 3B). On average, the low and high density crowding resulted in mean heart rate elevations of ~6 and 8 beats min^{-1}, respectively. Heart rate then decreased following brailing from the sea cage into the well-boat, such that it did not differ significantly from the normal circadian rhythm (event 4, $P = .473$, Fig. 3B). Heart rate was significantly elevated during transportation in the well-boat from the sea cage to the slaughterhouse (event 5, $P = .002$, Fig. 3B), as mean heart rate was ~9 beats min^{-1} higher than normal. However, during the period when trout were held in a stationary well-boat, the dissolved oxygen concentration in the water decreased from 8.83 mg l^{-1} (81.1% oxygen saturation) to 5.58 mg l^{-1} (55.3% oxygen saturation), which was associated with a decreased heart rate that no longer differed when compared to normal (event 6, $P = .447$, Fig. 3B). After transfer to the holding cage next to the slaughterhouse, heart rate significantly increased again and peaked after ~1 h at a value that was ~25 beats min^{-1} higher than normal (event 7, $P < .001$, Fig. 3B). Heart rate was still significantly elevated by ~9 beats min^{-1} the following morning (event 8, $P = .003$, Fig. 3B). The combined effects of brailing and CO_2 narcosis significantly decreased heart rate by ~22 beats min^{-1} when compared to the normal circadian rhythm (event 9, $P < .001$, Fig. 3B).

Plasma cortisol from sub-samples of trout were determined from 5 of the 9 identified events (*i.e.* event 2, 4, 5, 8 and 9). The circulating levels of plasma cortisol measured following the initial low-density crowding was 23 ± 4 ng ml^{-1} (event 2, see Fig. 3B). Following the combined stressors of high density crowding and brailing, plasma cortisol had significantly increased three-fold (plasma cortisol at event 4 compared to event 2, $P < .001$, Fig. 3B). Plasma cortisol increased further following transportation in the well-boat to reach a level of 171 ± 15 ng ml^{-1} (event 5, $P < .001$, Fig. 3B). Following the overnight recovery in the holding cage next to the slaughterhouse, plasma cortisol had decreased significantly (event 8, $P < .001$, Fig. 3B), but still remained two-fold higher than the lowest levels measured the previous day ($P = .035$). Following the combined stressors of brailing and CO_2 narcosis, plasma cortisol again increased significantly (event 9, $P < .001$, Fig. 3B) to reach levels that were similar to the peak response observed during well-boat transportation the previous day (*i.e.* event 5).

No significant changes were observed for Hct, [Hb] and RBC count

Table 1
Body measurements, haematological parameters and plasma ion concentrations from sub-samples of rainbow trout at 5 events during the final 2 days leading up to harvest. Statistical analyses were generated using a one-way ANOVA for each variable and significant differences between groups are represented by different letters ($P < .05$).

Variable	ANOVA		Events				
	F	*p*	Low density crowding (event 2)	High density crowding/ brailing (events 3 and 4)	Well-boat transport (event 5)	Pre-slaughter (event 8)	Brailing and CO_2 narcosis (event 9)
Body measurements							
Mass (g)	$F_{4,95} = 0.294$	0.881	1785 ± 92	1847 ± 91	1611 ± 69	1770 ± 76	1768 ± 48
Length (mm)	$F_{4,95} = 0.149$	0.963	459 ± 7	459 ± 7	449 ± 6	460 ± 6	460 ± 4
Haematological parameters							
[Hb] (g dl^{-1})	$F_{4,93} = 14.456$	**< 0.001**	8.6 ± 0.3^{a}	9.3 ± 0.3^{a}	9.6 ± 0.4^{a}	8.7 ± 0.3^{a}	11.2 ± 0.2^{b}
Hct (%)	$F_{4,93} = 20.664$	**< 0.001**	35.7 ± 1.4^{a}	40.3 ± 1.4^{a}	40.8 ± 1.8^{a}	36.9 ± 1.4^{a}	51.9 ± 1.1^{b}
RBC count (10^6 cells μl^{-1})	$F_{4,94} = 9.722$	**< 0.001**	1.3 ± 0.1^{a}	1.3 ± 0.1^{a}	1.4 ± 0.1^{a}	1.3 ± 0.1^{a}	1.8 ± 0.1^{b}
MCHC (g Hb dl^{-1})	$F_{4,93} = 6.792$	**< 0.001**	24.2 ± 0.5^{a}	23.1 ± 0.3^{a}	23.7 ± 0.4^{a}	23.8 ± 0.5^{a}	21.7 ± 0.2^{b}
MCH (pg)	$F_{4,94} = 0.791$	0.534	69 ± 2	72 ± 2	69 ± 2	71 ± 4	65 ± 3
MCV (fl)	$F_{4,94} = 0.724$	0.578	288 ± 9	311 ± 11	292 ± 11	298 ± 14	298 ± 11
Plasma ion concentration							
Na^+ (mM)	$F_{4,93} = 3.973$	**0.005**	179.7 ± 1.6^{a}	188.3 ± 1.9^{b}	185.5 ± 2.0^{a,b}	179.6 ± 3.4^{a}	187.7 ± 1.6^{a,b}
K^+ (mM)	$F_{4,92} = 6.224$	**< 0.001**	4.0 ± 0.2^{a}	2.6 ± 0.3^{b}	3.8 ± 0.2^{a}	3.3 ± 0.2^{a,b}	3.3 ± 0.2^{a}
Ca^{2+} (mM)	$F_{4,93} = 7.382$	**< 0.001**	1.1 ± 0.1^{a}	1.2 ± 0.1^{a}	1.2 ± 0.1^{a}	1.1 ± 0.1^{a}	1.3 ± 0.1^{b}
Cl^- (mM)	$F_{4,93} = 3.723$	**0.007**	113.7 ± 0.4^{a,b}	114.9 ± 0.4^{a,b}	115.2 ± 0.6^{a}	112.5 ± 1.0^{a,b}	112.4 ± 0.8^{b}

in fish subjected to low density crowding, high density crowding and brailing, well-boat transport and pre-slaughter holding levels (events 2, 4, 5 and 8, respectively; Table 1). However, following brailing and CO_2 narcosis (event 9), Hct, [Hb] and RBC count increased significantly ($P < .001$ for all variables, Table 1). This was entirely due to the significant increase in RBC count, as MCV did not change ($P = .578$, Table 1). MCH also did not change ($P = .791$) while MCHC significantly decreased following brailing and CO_2 narcosis ($P < .001$, Table 1). After the combined stressors of high density crowding and brailing (event 3 and 4) plasma [Na^+] was significantly elevated ($P = .038$) and plasma [K^+] was significantly reduced ($P < .001$) when compared to the values measured during low density crowding (event 2; Table 1). Lastly, [Ca^{2+}] significantly increased following brailing and CO_2 narcosis (event 9, $P < .001$, Table 1).

4. Discussion

This is the first study to monitor the heart rate and body temperature of freely swimming fish in an aquaculture setting. Once the rainbow trout had recovered from the combined stressors of surgery, transportation and reintroduction with conspecifics in the sea cage (~4 days), a strong circadian rhythm in heart rate emerged with a higher heart rate (~25 beats per min^{-1}), during the day than at night. Similar circadian rhythm in heart rates have also been observed previously in brown trout (*Salmo trutta*) freely swimming in the wild, and most likely reflect daily changes in locomotor activity (Holliday et al., 1974; Priede, 1978; Priede and Young, 1977; Young et al., 1972). Although a disruption in the normal behavioural pattern of the trout may in part explain why the circadian rhythm in heart rate is absent during the first days, another plausible factor is that stressed animals may not have the same capacity to control their heart rate (*i.e.* heart rate variability) as a healthy unstressed animal. Heart rate variability (*i.e.* the variation in the time interval between heart beats), which is controlled by the activity of the autonomic nervous system, has previously been used as a welfare indicator to determine the level of recovery after anaesthesia and surgery in fish (Campbell et al., 2004; Gräns et al., 2014). However, in order to collect the long-term recordings required for this study, it was necessary to conserve battery and storage space in the bio-loggers. Therefore, a continuous ECG could not be obtained, which prevented the assesment of heart rate variability. Nonetheless, since a clear circadian rhythm in heart rate did not emerge up until fish had recovered from the combination of the abovementioned stressors, the presence or absence of a circadian rhythm in heart rate could potentially be used as a welfare indicator and a proxy for restored heart rate variability for fish in aquaculture. Although further investigations are required concerning the effects of environmental and anthropogenic stressors on the circadian rhythm in heart rates of fish, the continuous monitoring of heart rate could allow the identification of farming routines and/or particular locations of sea cages (*e.g.* cages in areas of high boat traffic, noise, unfriendly environmental conditions, *etc.*) that induce unnecessary and prolonged stress (*e.g.* absence of circadian rhythm in heart rate). Furthermore, when using heart rate to assess the severity of different stressors during farming practices, it is clear that the strong diurnal heart rate variation needs to be taken into consideration, as these normally occurring rhythms may otherwise bias the interpretation of results.

Previous studies have demonstrated that heart rate in various fish species is often correlated with metabolic rate (Armstrong, 1986; Campbell et al., 2004; Clark et al., 2005; Eliason et al., 2011), which provides the opportunity to explore the relative energetic consequences of the different stressors, such as those examined here during various common aquaculture practices and harvest (Cooke et al., 2016). Crowding of rainbow trout in the sea cage resulted in significant tachycardia. Similar responses have previously been demonstrated in freely swimming adult coho salmon (*Oncorhynchus kisutch*) in response to corralling, with heart rate doubling from ~30 to 60 beats min^{-1} at a water temperature of ~9 °C (Donaldson et al., 2010). Although the maximum heart rate of trout in the present study was similar following high density crowding, the magnitude of the increase was much smaller (~8 beats min^{-1}) when accounting for the natural circadian fluctuations in heart rate. The effects of this increase above the 'normal' circadian rhythm in heart rate on factors such as recovery duration or excess post-exercise heart beats (Raby et al., 2015) cannot be concluded from the data presented but warrants further investigation. In other salmonid species, it has been suggested that the increase in heart rate in response to various aquaculture and commercial fisheries related stressors may be a relatively poor predictor of the severity of a given stressor, as heart rate approximately doubled from routine values with all stressors examined so far (Cooke et al., 2001; Donaldson et al., 2010; Prystay et al., 2017; Raby et al., 2015). However, at least with respect to the stress induced by crowding in the present study, our data suggest that the tachycardic response in rainbow trout increased with an

increasing crowding intensity. This discrepancy may be due to the fact that our focal animals were surrounded by ~5000 conspecifics during crowding (*i.e.* safety in numbers) and so the stress response to crowding in the net cage may be more gradual, while isolation and corralling of a smaller number of individuals in a raceway may result in a more acute and maximal response (Donaldson et al., 2010). Regardless of the underlying causes, the gradual heart rate response to crowding observed here suggests that bio-logging of heart rate may be a useful tool to gauge crowding stress in farmed fish.

Heart rate was substantially lower immediately after brailing, as well as during the period when they were held in the stationary well-boat, such that heart rate was not different from the normal circadian rhythm in heart rate. Based on heart rate alone, it may seem that these events did not induce a stress response. However, the significantly elevated plasma cortisol level following brailing reveals that this was not the case. In fact, the decrease in heart rate during these stressful events was not unexpected, as aquatic hypoxia (*i.e.* period in stationary well-boat) and air exposure (*i.e.* during brailing) are known to trigger a hypoxic bradycardia resulting from an increase in inhibitory cholinergic (*i.e.* vagal) tone on the heart through stimulation of muscarinic receptors on the cardiac pacemaker cells (Olson, 1998; Randall, 1982; Sandblom and Axelsson, 2011; Wood and Shelton, 1980; Wood et al., 1979). This idea is consistent with the finding that during the transportation in the well-boat when water oxygen levels were high, heart rate increased and reached levels similar to those observed during the high density crowding. This tachycardia was likely due to a combined effect of high density crowding in the well-boat and a delayed increase in heart rate to repay an oxygen debt following the preceding air exposure during brailing (Prystay et al., 2017). Indeed, a delayed increase in heart rate was also observed when trout were transferred from the well-boat to the holding cages next to the slaughterhouse. Despite being left undisturbed, the heart rate continued to increase and peaked ~1 h after release at a level that was higher than what was observed with any of the preceding individual stressors. While a delay in the tachycardic response following various stressors has previously been reported in a number of studies (Donaldson et al., 2010; Ekström et al., 2016; Prystay et al., 2017; Raby et al., 2015), the underlying mechanisms responsible for this delay and how it is affected by abiotic and biotic factors is unknown and warrants investigation.

Even after > 16 h of recovery from the combination of stressors induced by the common farming practises during the transport of the fish from the sea cages to the slaughterhouse; heart rate and plasma cortisol levels were still significantly elevated. Based on the findings discussed earlier, this is not surprising as it took ~4 days to recover heart rate and establish a clear circadian rhythm in heart rate after the stress experienced during surgery, transportation and reintroduction with conspecifics in the sea cage. Moreover, previous studies have demonstrated that heart rate recovery duration can be quite prolonged in fish, even following a single stress inducing event such as exhaustive exercise (up to 16 h), corralling (7.6 and 11.5 h depending on the duration of corralling) and a simulated angling event (up to 16 h) (Anderson et al., 1998; Donaldson et al., 2010). Also, plasma cortisol levels of rainbow trout exposed to a single acute stressor, such as netting and confinement, can generally take up to 24 h to recover to pre-stressed levels depending on the applied stressor (Huyben et al., 2017; Pickering and Pottinger, 1989; Procarione et al., 1999). Thus, since physiological stress in an animal is known to affect the quality of the marketed product (Ashley, 2007; Robb and Kestin, 2002), it may be beneficial to allow for relatively long recovery times (*i.e.* several days based on heart rate recovery rather than one day for plasma cortisol recovery) after the fish has been transported to the slaughter facilities before initiating slaughter procedures.

The final procedures prior to slaughter involved the combined stressors of brailing from the holding cages next to the slaughterhouse and narcosis by immersion in CO_2 saturated water. Similar to previous studies, plasma cortisol levels were substantially elevated during these procedures (Gräns et al., 2016; Sandblom et al., 2013; Seth et al., 2013), while heart rate plummeted. The latter response likely resulted from cardiovascular collapse due to a severe acidosis (Seth et al., 2013). In addition, Hct and [Hb] increased following CO_2 narcosis, which was due to a ~40% increase in circulating red blood cells. This was likely due to an increased sympathetic nervous activity causing splenic contraction and the release of red blood cells (Pearson and Stevens, 1991; Wendelaar-Bonga, 1997). A decrease in MCHC following acute stress is often attributed to an increased level of circulating catecholamines causing red blood cell swelling (Nikinmaa, 1983). However, in the present study, no significant changes were observed in MCV, and so the decrease in MCHC may be attributed to a large proportion of immature cells among the red blood cells released from the spleen, which are known to contain less haemoglobin (Härdig and Höglund, 1983; Huyben et al., 2017).

5. Conclusions

The present study demonstrates the usefulness of bio-loggers when investigating the effects of environmental and/or anthropogenic stressors on the welfare of fish. Implantable bio-loggers allow researchers to continuously collect data over time in the same individual as opposed to the 'snapshot' provided by more traditional measures (*e.g.* stress hormones). Thus, by using bio-loggers, potentially less experimental animals are necessary since researchers avoid the large individual variation associated with repeated sampling on different individuals, as well as the accumulative risk of sampling errors with repeated sampling. Furthermore, bio-loggers can continuously collect data from 'undisturbed' fish, which allows researchers to identify 'normal' physiological patterns of freely swimming fish in an aquaculture setting over long periods of time. The present study revealed that upon recovery from a combination of stressors, a clear circadian rhythm in heart rate of fish emerged, which we suggest could serve as a useful welfare indicator. The elevations in heart rate due to stressors caused by common farming practises such as crowding and transportation corresponded well with increased plasma cortisol levels, suggesting that heart rate is a useful tool for assessing stress levels of freely swimming fish in sea cages under normoxic conditions. However, heart rate is not a suitable predictor of stress during situations triggering a hypoxic bradycardia (*i.e.* air exposure during brailing and aquatic hypoxia) and therefore we recommend that researchers also monitor dissolved oxygen levels in sea cages when monitoring heart rate over long periods of time to aid in explaining any potential discrepancies. Significant changes in plasma ion concentrations and haemological parameters were only apparent after one or two of the handling events and thus these parameters may also not be suitable as reliable indicators of fish welfare. Repeated stress induced by multiple farming practises clearly had a cumulative and long-lasting effect as determined from heart rate and plasma cortisol levels. Thus, when fish are subjected to multiple stressors in series during one or several days, it is strongly recommended that sufficient time for recovery is provided between stressors. The development and refinement of implantable bio-loggers for fish opens up a broad range of possible applications allowing researchers to investigate the effects of environmental and/or anthropogenic stressors on the welfare of fish in scenarios more realistic to the aquaculture industry.

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