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Effects of repeated anaesthesia on gill and general health of Atlantic salmon, *Salmo salar*

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Fish are the second most widely utilized vertebrate group used for scientific procedures in the United Kingdom, but the development and application of 3Rs (the principles of replacement, reduction, and refinement) in aquaculture disease research lags behind methodologies in place for mammalian studies. With a need for individual monitoring and non-lethal sampling, the effect of repeat anaesthesia on experimental fish needs to be better understood. This study analyses the effect of repeat anaesthesia with MS-222, metomidate and AQUI-S upon the gill and general health of post-smolt Atlantic salmon *Salmo salar*. A single, lethal dose of anaesthetic was compared with seven anaesthetizing time points over 28 days, terminating in a lethal dose. No anaesthetic showed significant differences in accumulation in the muscle tissue, or changes in plasma glucose after repeated or single dosing. Fish repeatedly anaesthetized with MS-222 or AQUI-S exhibited upregulation of osmoregulatory genes in the gill and AQUI-S-treated individuals showed, histologically, epithelial lifting from the lamellae capillary irrespective of whether they had a single or repeated dose history. No significant changes were seen in inflammatory or stress genes in the head kidney of fish repeatedly anaesthetized with AQUI-S or metomidate, however MS-222 treatment resulted in upregulation of *tnfα3*. Repeated anaesthesia with MS-222 and metomidate gave a significant decrease and increase in peripheral blood neutrophils, respectively. This study concludes that no increase in cumulative stress or inflammation is induced by the repeated anaesthetization of *S. salar* with any of the tested anaesthetics, however gill osmotic regulation and blood parameters may be affected.

KEYWORDS

anaesthesia, AQUI-S, Atlantic salmon, metomidate, MS-222, non-lethal stress

1 | INTRODUCTION

Fish are the second most widely used vertebrate group, after mice, for scientific procedures in the UK; a total of 287,000 procedures were carried out on fish in 2016, representing 14% of scientific procedures for that year (Home Office, 2017). While a significant number of these procedures (66%) correspond to studies involving the model species zebrafish *Danio rerio* (Hamilton 1822), a large proportion utilize food-fish species, such as the salmonids (Home Office, 2017), due to their importance in the growing aquaculture industry (FAO, 2016).

In terms of implementing the 3Rs principles of replacement, reduction, and refinement (Russell *et al*., 1959), aquaculture research methodologies are currently less developed than systems used for mammalian studies (Sneddon *et al*., 2017). However, recent work has shown a new focus on reduction and refinement for salmonid pathogen challenges in the framework of non-destructive individual monitoring (Braceland *et al*., 2015; Chance *et al*., 2018; Collet *et al*., 2015; Monte *et al*., 2016; Urquhart *et al*., 2016), with the scope that the approach allows to obtain more informative and robust data from the same number of individuals or that fewer fish can be used to obtain similar quality data, (Hall *et al*., 2018). Individual monitoring relies on the ability to obtain non-lethal samples, such as small amounts of blood or mucus, which in practice would be difficult and cause excessive stress to the fish, without the use of anaesthesia. Therefore,

repeat anaesthesia is a key component of the individual monitoring approach. Anaesthetics are also commonly used in other areas of fish research and aquaculture industry, such as during transportation, fieldwork and disease control. Therefore, any contribution to the understanding of how repeat anaesthesia may affect fish health is relevant to multiple fields (Balamurugan *et al*., 2016; Nilsen *et al*., 2017; Scott, 2013).

While there are well-established guidelines in place to manage repeat anaesthesia of animals in the mammalian research sphere (Flecknell, 2016), currently, only a limited number of references reporting on teleost fish repeated anaesthetization are available and only few conducting analysis on any general effects. Available articles on the subject focus on ornamental or freshwater species, juvenile life stages, have lengthy intervals between anaesthesia or include no immunological observations in their analysis (Basrur *et al*., 2010; Braley & Anderson, 1992; de Lima Silva *et al*., 2012; Hoskonen & Pirhonen, 2004, 2006; Kildea *et al*., 2004; Mitjana *et al*., 2014; Mylonas *et al*., 2005; Palmer & Mensinger, 2004; Posner *et al*., 2013; Smith *et al*., 1999; Soto, 1995).

In this study, the anaesthetics of interest were MS-222, metomidate (AquaCalm; www. syndel.ca) and isoeugenol (AQUI-S; www.aqui-s.com). MS-222, was introduced as an effective, powder-based anaesthetic in 1967 and has since become the most extensively used chemical for sedation, anaesthesia and euthanasia in fish research, aquaculture and ornamental fish rearing (Bourne, 1984; Popovic *et al*., 2012; Readman *et al*., 2013). A sulphonated analogue of benzocaine, MS-222 blocks the generation of action potentials through voltage-dependent Na^+ channels (Ramlochansingh *et al*., 2014). The use of MS-222 in food-fish is licensed in the United States and Norway under the provision of 21 day withdrawal time, 5 day withdrawal period in Canada and 70° days (°D; a development value calculated by multiplying the average temperature by number of days incubated) in the United Kingdom (FDA 2007; Kiessling *et al*., 2009; Popovic *et al*., 2012).

Metomidate [DL-1-(1-phenylethyl)-5-(methoxycarbonyl) imidazole hydrochloride] is a powder-based, nonbarbiturate, hypnotic anaesthetic which is used in research but not currently approved for use with food fish (Iversen *et al*., 2003). This anaesthetic was chosen due to its ability to act directly on inter-renal cells, blocking the stress response of cortisol synthesis (Olsen *et al*., 1995), production of which is shown to inhibit the immune response (Barton & Iwama, 1991; Iversen *et al*., 2005; Pickering & Pottinger, 1988). Owing to the nature of repeated sampling, a cortisol-blocking anaesthetic may be more favourable in terms of avoiding or reducing extraneous effects when analysing the natural host immune responses to pathogen challenges. It should be noted that while metomidate acts to block cortisol production, fish may still be experiencing stress, therefore the upmost care in animal handling and sampling methodology should be carried out while using this anaesthetic. Although metomidate is not currently licensed for use in food fish it can be used for research.

Isoeugenol is the active ingredient (50%) found in the gel-based anaesthetic AQUI-S. Isoeugenol is similar to clove oil (eugenol), which anaesthetizes by inhibiting Na^+, K^+ and Ca^{2+} channels, as well as N-methyl-D-aspartate (NMDA) receptors (Lee *et al*., 2005; Li *et al*., 2007; Park *et al*., 2006; Wie *et al*., 1997). First developed in New Zealand in 1996, AQUI-S is increasing in popularity and licensed internationally; currently AQUI-S can be used with a zero-day withdrawal time for food fish in Australia, Chile, Costa Rica, Honduras, South Korea and New Zealand (AQUI-S, 2018) and as a stress-reduction aid for transport and husbandry practices in Norway (Kolarevic & Terjesen, 2014).

This study compared the possible effects of seven repeat anaesthetizations utilizing MS-222, metomidate or AQUI-S over a 28 day period, simulating the sampling intervals suggested by Collet *et al*. (2015) for individual monitoring of Atlantic salmon *Salmo salar* L. 1758, with fish which experienced a single overdose of their respective anaesthetic. The approach taken was to emulate procedures around sampling at each time point, encompassing catching of fish, removal from and replacement in water, with the anaesthetic as a variable. The variables investigated were: the accumulation of anaesthetic residues in skeletal muscle tissue as determined by liquid chromatography tandem mass spectrometry (LC-MS/MS); peripheral blood leukocyte populations derived from blood smears; plasma glucose concentration measured by spectrophotometric assay; gill condition was evaluated through histopathology; selected inflammatory, osmotic regulatory and stress-related genes in the gills and head kidney were analysed with quantitative (q) PCR. To the authors' best knowledge, this is the first study to look at the possible effects of repeated anaesthesia in post-smolt *S. salar*, with a focus on gill health and immunological parameters.

2 | MATERIALS AND METHODS

2.1 | Fish

This study was carried out in strict accordance with the UK Animals (Scientific Procedures) Act 1986 (ASPA) under project licence 70/7897. The Marine Scotland Science Ethical Review Committee approved the protocols and all housing and procedures were carried out in a contained biosecure aquarium facility at Marine Scotland, Aberdeen (MSS). Freshwater *S. salar* parr were sourced from a commercial aquaculture company and carried through smoltification at the MSS aquarium. A total of 120 individuals (mean ± SD mass = 219 ± 42 g) were randomly distributed into twelve 1 m diameter (350 l) tanks (n = 10). Throughout the experiment, fish were held at 13°C, in full strength seawater (*ca.* 35 ppt) in a flow-through system with approximately 7.3 mg l^{-1} oxygen and ambient, natural photoperiod light conditions (57.15° N). Fish were acclimatised in experimental tanks for 1 week and hand-fed daily throughout with Nutra 50 (Skretting; www.skretting.com), except for a period of 24 h when food was withheld prior to terminal sampling.

2.2 | Anaesthetic exposures

Three tanks were allocated to each repeated exposure treatment for the three anaesthetics. Concentrations used for MS-222, metomidate and AQUI-S were 80, 12 and 17 mg l^{-1} respectively, selected due to previous effective protocols and standardized use at MSS or manufacture's recommendations (AQUI-S, 2018). AQUI-S is a viscous gel-like

substance, therefore the required volume needed was calculated using the AQUI-S calculator, available on the manufacturers website (AQUI-S, 2018). In order to disperse AQUI-S evenly, it was first emulsified in 1 l of seawater and inverted until the solution turned an opaque white colour.

Anaesthetising points started at day 0 and continued every 4 days (Collet *et al*., 2015) until day 28 when all fish were terminally sampled. Throughout the experiment, to mitigate any stress experienced by the fish during the anaesthetizing process, an in-tank anaesthesia methodology was applied as described previously by Collet *et al*. (2015). Briefly, tanks were slowly drained to 120 l, liquid stock solutions of each anaesthetic were poured into their respective tanks in a figure of eight to ensure even dispersal throughout the tank and fish remained submerged until reaching Stage III, Plane 1 deep narcosis anaesthesia, as described by Burka *et al*. (1997). Induction times were recorded for each tank at each anaesthetizing time point. Once the required level of anaesthesia had been reached, fish were individually netted out and placed on a post-mortem table for precisely 1 min to simulate the collection of non-lethal samples. While exposed to the air, fish were placed on damp tissue material instead of the bare surface of the table to help protect from any irritation or injury. Once the minute had elapsed, fish were temporarily (*ca*. 2 min) held in an opaque, 240 l aerated recovery container filled with refreshed seawater from the same source as the housing tanks, at the same salinity and temperature, while their previous housing tanks were drained of water containing anaesthetic and refreshed with seawater of 35 ppt salinity. Fish remained anaesthetized while being netted and returned to housing tanks once these had been refilled to 120 l; tanks continued to refill to full capacity of 350 l.

2.3 | Terminal sampling

The experiment was terminated at day 28 with a lethal dose of the respective anaesthetic for each treatment group, including the thus far undisturbed control tanks for each anaesthetic (n = 10). In-tank anaesthesia methodology used throughout the experiment was applied, with the same concentrations of anaesthetic as previously described, but exposure times were increased as needed to ensure death was reached. Death was ensured through severing of the spinal cord.

Blood collection from all fish was performed by caudal vein puncture with a heparinised BD Vacutainer (Becton, Dickenson & Company; www.bd.com) and stored on ice until further processing during the same day. For histology, the second gill arch from the left gill chamber was removed into in an histology cassette and fixed in 60 ml 10% neutral buffered formalin (NBF). For gene expression, a small clipping (*ca*. 4 mm wide) of gill tissue from the right gill chamber, ranging from raker to distal tip from the second gill arch was taken and head-kidney tissue was also collected; these clippings were stored in Eppendorf tubes containing 1.5 ml of RNA*later* (Sigma Aldrich; www.sigmaaldrich.com) and stored at −80°C until RNA extraction for gene expression analysis. A square (1 × 1 cm) of skeletal muscle without skin, was removed, placed in a 2 ml cryogenic vial (Camlab; www.camlab.co.uk) and placed immediately in liquid nitrogen and subsequently stored at −80°C until use in LC-MS/MS.

2.4 | Histological processing

The second left gill arch was fixed for 24 h in 10% neutral buffered formalin (NBF). Samples were decalcified for 2 h (Surgipath Decalcifier II, Leica Biosystems; www.leicabiosystems.com) before being routinely processed for light microscopy in a Shandon Excelsior tissue processor (Thermo Fisher Scientific; www.thermofisher.com). Gill arches were embedded horizontally in paraffin wax and 3 μm thickness sagittal sections of the distal surface were cut with a microtome, float mounted onto x-tra adhesive slides (Leica Biosystems) and stained with haematoxylin and eosin (h & e).

2.5 | Histological examination

Gill histology sections from 10 randomised individuals from each repeat anaesthesia group and the 10 fish of each single lethal-dose anaesthetic group were examined and imaged with an upright Zeiss Axio Imager M2 microscope (www.zeiss.com). Assessment of slides was blinded to avoid experimental bias. One filament from three sections of the gill (upper limb, arch and bottom limb) was examined from raker to tip and scored for epithelial lifting from the lamellae capillary, hyperplasia, lamellar fusing, filament fusing and presence of necrotic tissue.

2.6 | Determination of metomidate, MS-222 and AQUI-S accumulation in muscle tissue by solid-phase extraction (SPE) and LC-MS/MS

Metomidate, MS-222, isoeugenol and their respective internal standards (MS-222, metomidate and propofol) were dissolved in methanol at a concentration of 1 mg ml^{-1} and stored at −20°C (MS-222 is tricaine mesylate and 1.6 mg dissolved in 1 ml of methanol gives a final concentration of 1 mg ml^{-1}). Tissue samples were weighed and the appropriate internal standard added (MS-222 20 μg, metomidate 4 μg and propofol 10 μg). Samples were then homogenised for 30 s in 50:50 0.1 M sodium acetate, pH 4.0 methanol using an Ultra Turrax tissue homogeniser (IKA; www.ika.com). Calibration standards were prepared by adding metomidate, MS-222 and isoeugenol to blank homogenised muscle tissue in the concentration range 0.06–20 μg and spiking with the internal standard. All samples and standards were then vortex mixed for 30 s and centrifuged at 3,169 *g* for 10 min. The supernatants were applied to BondElut Plexa cartridges (60 mg, 3 ml; Crawford Scientific; www.crawfordscientific.com) that had been conditioned with 1 ml of methanol and equilibrated with 1 ml 0.1 M sodium acetate buffer, pH 4.0. After washing with 2 × 1 ml volume of 0.1 M sodium acetate, pH 4.0 buffer, the cartridges were eluted with 1 ml 95:5, methanol:ammonium hydroxide. All solid phase extraction (SPE) steps were carried out under gravity.

For metomidate and MS-222, 60 μl of the SPE eluate was mixed with 40 μl of 0.1% formic acid in water and 5 μl injected onto the chromatograph. For isoeugenol, 100 μl of 0.1 M sodium hydroxide and 100 μl of 20 mg ml^{-1} dansyl chloride (in acetone) were added to the SPE eluate and incubated for 10 min at 60°C. One hundred μl was then mixed with 100 μl of 0.1% formic acid, centrifuged at 36,733 *g* for 5 min at 4°C and 5 μl injected onto the chromatograph.

Chromatography was performed on a Thermo Surveyor (Thermo Fisher Scientific) system. Metomidate and MS-222 were resolved using a 150 × 2.1 mm ACE 3 μ C18-PFP column (Hichrom Ltd, UK) under isocratic conditions with a mobile phase consisting of 35/65/0.1, water/methanol/formic acid. Isoeugenol and propofol were resolved using a 150.0 × 2.1 mm Hichrom HIRPB column (Hichrom Ltd; www.hichrom.com) under isocratic conditions with a mobile phase consisting of 10:90:0.1, water:methanol:formic acid. Both flow rates were 200 μl min^{-1} and the samples were maintained at 4°C in the autosampler.

A Thermo TSQ Quantum triple quadrupole mass spectrometer (Thermo Fisher Scientific) was used in positive electrospray ionisation mode for detection. Quantification was performed using single reaction monitoring (SRM) scan mode using the following transitions: metomidate m/z 231.0–95.0, MS-222 m/z 166.0–138.0, isoeugenol (dansylated) m/z 398.0–171.1 and propofol (dansylated) m/z 412.0–171.1. Flow-injection analysis was used to optimise the MS/MS conditions for metomidate, MS-222 and isoeugenol respectively as follows: spray voltage 4,000, 4,000, 4,500 V; sheath gas pressure 40, 60, 60; auxiliary gas pressure 0, 25, 0; capillary temperature 375°C, skimmer offset −10, −10, −12 V; collision pressure 1.7, 1.7, 1.4 mTorr (0.13 Pa); collision energy 24, 15, 22 V. The collision energy for propofol was 22 V.

Instrument control and peak integration and quantification were performed using Thermo Xcalibur 2.0.7 SP1 (Thermo Fisher Scientific). Metomidate and MS-222 calibration curves best fit a quadratic function with equal weighting whereas weighted least-squares linear regression with a weighting factor of x^{-1} was used to quantify isoeugenol concentration in unknown samples by comparing peak-area ratios (analyte:IS) with those obtained from a multi-level calibration curve.

2.7 | RNA extraction & cDNA synthesis

Once gill and head-kidney samples were defrosted, excess RNAlater (Sigma Aldrich) residues were removed from the tissues by compressing with sterilized tissue paper. Total RNA was extracted with TRI Reagent (Sigma Aldrich) as per the manufacturer's instructions, with the addition of GlycoBlue (Invitrogen; www.invitrogen.com; 100 μl 50 ml^{-1}) in 2-propanol to ease identification of pellet formation. Pellets were left for 5 min to air dry (to remove any excess 75% ethanol) in a sterile laminar flow cabinet. Pellets were then dissolved with 32 μl of oligo dTs, (Eurofins; www.eurofins.com) at 70°C for 5 min, then placed on ice for 5 min. Complementary (c) DNA synthesis was achieved with a mastermix of 1 μl reverse transcriptase, 8 μl of 5x reaction buffer (both Bioscript; Bioline; www.bioline.com), 1.6 μl 25 mM deoxynucleotide triphosphate (dNTP; Bioline), mixed with 29.4 μl RNA/oligo dT to make a 40 μl reaction. Solutions were then vortexed for approximately 15 s to ensure thorough dispersal of reagents, then incubated with the following programme: 42°C for 1 h, 45°C for 30 min, 50°C for 30 min and reaction terminated at 90°C for 5 min. Reactions were removed from the thermocycler and 400 μl molecular grade water added to each.

2.8 | Primer design and q-PCR analysis

Primer pairs were designed to cross an intron-exon boundary to prevent the amplification of genomic DNA. Genomic and RNA sequences for target genes were sourced from National Centre for Biotechnology Information (NCBI, 2017). Exon–intron alignments were visualised with Spidey (NCBI, 2017) and potential regions were assessed with Primer3Plus 2.4.2 (Untergasser *et al*., 2012). Primer suitability was assessed with OligoCalc (Kibbe, 2007). All primers used were ordered from Eurofins Genomics (Eurofins) and primer pair sequences are listed in Table 1. Each primer product was validated by melt-curve analysis and gel electrophoresis imaging to ensure amplification from genomic DNA was not present.

QPCR analysis was completed on a LightCycler 480 (Roche Applied Science; www.roche.com). Four μl of pre-diluted cDNA was added in each 20 μl reaction, using SYBR Green I Nucleic Acid Gel Stain (Invitrogen) and IMMOLASE DNA polymerase (Bioline). Each programme run began with 1 cycle of denaturation (95°C for 10 min), followed by amplification (95°C for 30 s, 63°C for 30 s, 72°C for 30 s) for 40 cycles, then completing with melt–curve analysis (from 75 to 95°C). Programme profiles differed in the annealing temperatures of different primers (Table 1). Primer efficiency was tested with three pooled RNA serial dilution standards. The expression of each gene was normalised against the housekeeping gene elongation factor alpha (*elfα*) and expressed as arbitrary unit where the expression in single exposure is 1.

2.9 | Peripheral leukocyte blood smear analysis

Blood-sample tubes were vortexed for approximately 15 s to ensure even distribution of cells throughout the sample. A volume of 8 μl was used to create individual blood smears which were stained using a modified Giemsa Differential Quik Stain Kit (Polysciences, Inc; www.polysciences.com) following manufacturer's protocols, with the exception of avoiding the suggested xylene and applying Histo-Clear (National Diagnostics; www.nationaldiagnostics.com) as a safer option for cleaning agent. DPX (Sigma Aldrich) was used as synthetic resin to mount the cover slips. Each blood smear was divided into quarters and the first 50 leukocytes per quarter were differentially counted (lymphocytes, thrombocytes, monocytes, neutrophils) (Ranzani-Paiva *et al*., 2003; Ros *et al*., 2006) under a compound light microscope.

2.10 | Plasma-glucose assay

After making a blood smear, remaining whole blood in the heparinised vacutainers was centrifuged at 1,073 *g* for 5 min. The separated plasma was aliquoted into sterilised 2 ml Eppendorf tubes and stored at −80°C until needed. The glucose (GO) assay kit (Sigma Aldrich) was scaled down to a 96-well plate to obtain plasma-glucose concentrations from all fish sampled; plates were read at 540 nm with a SpectraMax Plus 96 Spectrophotometer (Molecular Devices; www.moleculardevices.com).

TABLE 1 Primer sequences from *Salmo salar* used in qPCR, and their annealing temperature and efficiency

	Oligonucleotides (5′–3′)		Annealing	
Gene (Accession number)	Forward	Reverse	Temperature (°C)	Efficiency
elfα (AF498320)	CAAGGATATCCGTCGTGGCA	ACAGCGAAACGACCAAGAGG	63	1.988
tnfα1 (AY929385)	ACTGGCAACGATGCAGGACAA	GCGGTAAGATTAGGATTGTATTCACCCTCT	65	1.992
tnfα3 (EF079662)	CACGGCAAGAAACAAGATCCCA	GATCCACTGGGGTTGTATTCACCTTCTA	65	1.911
il-1β (AY617117)	GCTGGAGAGTGCTGTGGAAGAAC	CGTAGACAGGTTCAAATGCACTTTGTG	63	1.977
p53 (BT058777.1)	CTGTGCAATCAGGAGTTTCG	ACGTTCTCCACCAGATCAGC	57	1.974
cox2 (AGKD04000045)	GATCGCTGGAAGGGTGGCTG	GCCAGCTCTGTCTCTCCTGTGAGGT	66	1.994
Sparc (BT043718.1)	CCTCCAGGGCGATGTACTTG	AGCACTGCACCACTCGTTTCT	59	2.027
Na–KA-TPase *α1a* (XM_014194628.1)	CTGAGAGGATCCTGGACAGC	CAAAGCCTTCTGGAAACTGG	54	1.988
Na–KA-TPase *α1b* (AY692144.1)	ACAGCCTCTGGATGACGAGT	ACATTAGGCCAACGAAGCAC	57	2.013
Na–KA-TPase *α1c* (AY319389.1)	TAATGTGCCTGAGGGTCTCC	TGGTTCTCTGTGGTGTCAGC	59	2.028
Na–KA-TPase *α3* (CA059214)	TCCACGATCTTCCTCTGTTCA	TTGTGGGTATCAGGCTCAACT	59	1.976
Na–KA-TPase *β1* (BT059032.1)	CAGCTCCAATGAAAGCATCC	AAGCAGCTTGCCGTAGTAGG	63	2.006
nkcc (AJ417890.1)	CTGTCCAATGACCAGACACG	CGTTCTTGAAGCCAATCACC	57	1.990
cftr1 (AF155237.1)	CACTGTGGATGGACTGATGC	ATTTGACGGTCAGTCCTTGG	57	1.991
cftr2 (AF161070.1)	CAGCCTGTACCTGGAGAAGG	CCAAACAGGATGTTGTCACG	57	1.996
hsp90α1a (XM_014205881.1)	TCTGGTCATCTTGCTGTTCG	TCATCATCTCCCTCCAGGAC	57	2.048
hsp90α2 (KC150879.1)	TCCTCAGATGCTCTGGACAA	TGTTGATGAGGTCAGCCTTG	57	1.956
hsp90β1 (KC150882.1)	CCTCAGGGAGTTGATTTCCA	ATGCCAATTCCAGTGTCGAT	59	1.930
hsp90β2 (KC150883.1)	TTGATCTCCAATGCCTCTGA	ATGCCAATTCCAGTGTCGAT	55	2.017

2.11 | Statistics

Induction times to Stage III Plane 1 anaesthesia durations were analysed with repeated-measures ANOVA using SPSS for Windows 24 (IBM; www.ibm.com). All other statistical analyses were completed in R (R Core Team, 2016; www.r-project.org). Histopathological score data were compared with an ordinal regression with section included as a fixed factor, utilizing the statistical packages MASS (Venables & Ripley, 2002) and lme4 (Bates *et al*., 2015) in R. Accumulation of anaesthetic residue in muscle tissue and gene expression data were log transformed to normalise and compared by ANOVA. Plasma glucose concentrations and leukocyte population counts were analysed with van der Waerden normal scores test, with the van der Waerden *post hoc* test applied where appropriate, utilizing the PMCMR package (Pohlert, 2014) in R.

3 | RESULTS

3.1 | Anaesthetic induction times

The total mean (±SD) duration required for fish to reach Stage III, Plane 1 for each anaesthetic were: metomidate = 2 min 5 s ± 17 s; MS-222 = 3 min 50 s ± 42 s; AQUI-S = 21 min 2 s ± 3 min. Repeated measures ANOVA found no significant differences in induction times between non-lethal sampling points for each anaesthetic (Figure 1). No fish were lost in relation to any of the anaesthetizing timepoints in any of the treatment groups, with successful recovery for all animals after each non-sampling simulation.

3.2 | Histological examination

No frequent or statistically relevant levels of inflammation, hyperplasia, lamellar fusion, filament fusion, or necrotic tissue were seen in any of the samples inspected for any of the anaesthetic treatments (data not shown). Epithelial capillary lifting from the lamellae was seen in all AQUI-S treated gills (Table 2 and Figure 2). Fish that had been repeatedly anaesthetized with AQUI-S had a mean (±SD) score of 0.6 ± 0.2, whereas fish which had a single overdose of

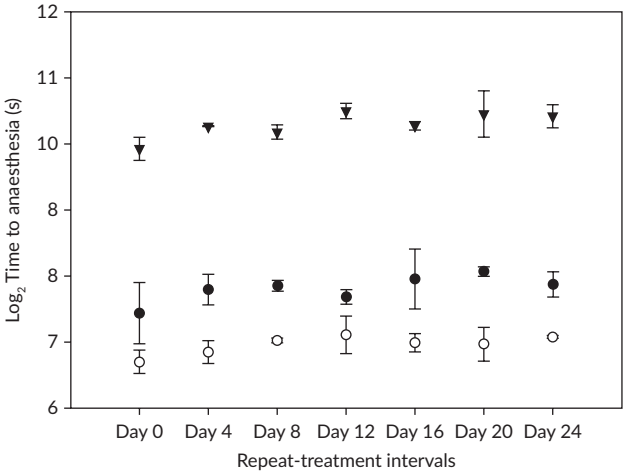

FIGURE 1 Log_2 mean (±SD, n = 3) repeat-treatment induction times of *Salmo salar* () to Stage III, Plane 1 anaesthesia with MS-222 (80 mg l^{-1}), metomidate (12.5 mg l^{-1}) and AQUI-S (17 mg l^{-1}). $P > 0.05$ for each anaesthetic, as assessed by repeated measures ANOVA (—●—) MS-222, (—○—) Metomidate, and (—▼—) AQUI-S

TABLE 2 Number of *Salmo salar* with gills exhibiting epithelial lifting after single (n = 10) or repeated (n = 10) anaesthetization with AQUI-S

	Single treatment			Repeat treatment		
Epithelial lifting*	**Upper limb**	**Arch**	**Bottom limb**	**Upper limb**	**Arch**	**Bottom limb**
Light	8	7	5	4	3	6
Moderate	0	0	1	1	1	0
Severe	0	0	3	0	0	0

One filament from three sections of each gill (upper limb, arch, bottom limb) were followed from raker to tip and allocated a score for severity of epithelial lifting.
*Light, low level detachment, slight line of epithelial cell can be seen; Moderate, clear and defined separation of epithelial layer from lamellae capillary; Severe, epithelial layer has little to no remaining contact with lamellae capillary.

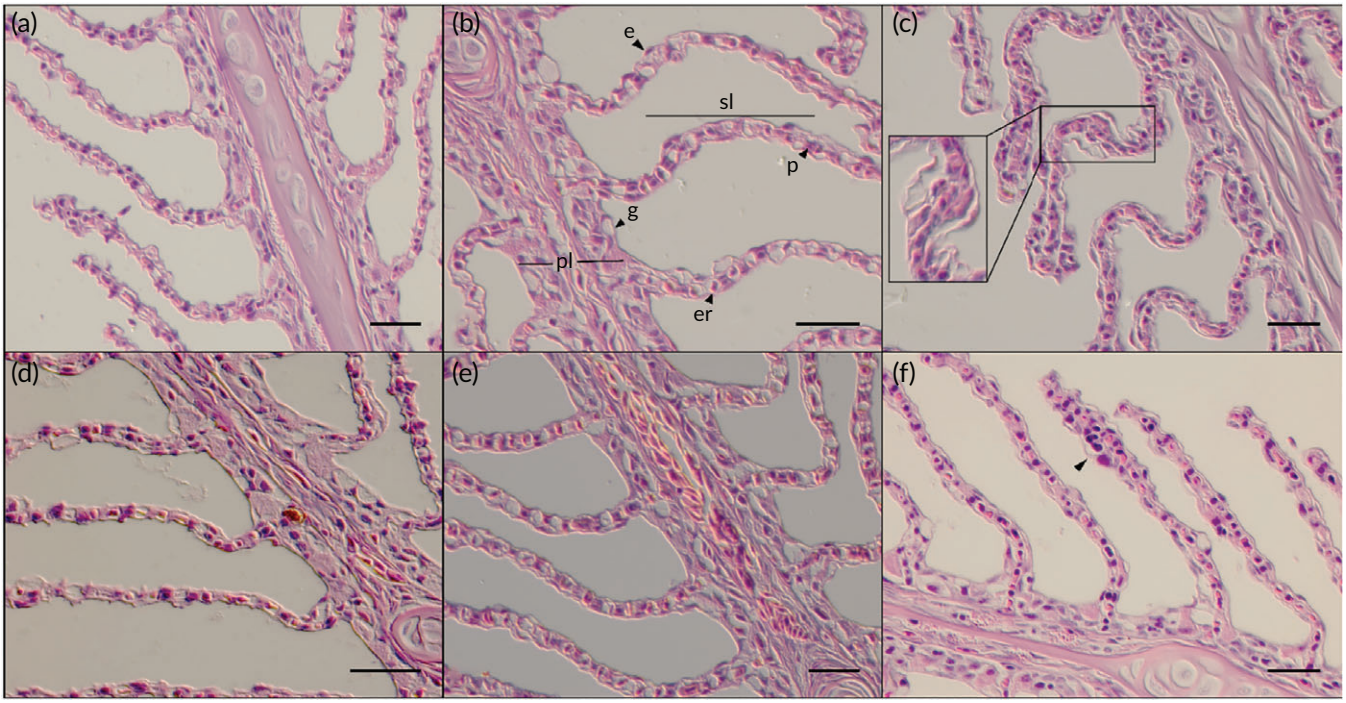

FIGURE 2 Differential-interference contrast images of *Salmo salar* gill after dosing with three anaesthetic agents: (a) single and (d) repeat exposure to MS-222 (80 mg l^{-1}); (b) single and (e) repeat exposure to metomidate (12 mg l^{-1}); (c) single and (f) repeat exposure to AQUI-S (17 mg l^{-1}). e, epithelial cell; sl, secondary lamella; p, pillar cell; g, goblet cell; pl, primary lamellae; er, erythrocyte. □, Area showing evidence of epithelial lifting; S.I. ▶, foci of mild subepithelial infiltration. Scale bar 100 μm. Magnification ×400

AQUI-S scored a mean of 1.2 ± 0.2, which was a statistically significant difference ($P < 0.05$; Figure 3). No significant differences in the distribution of epithelial lifting scores between the three areas of assessment were seen.

3.3 | Accumulation of residues in muscle tissue

There were notable differences between the levels of detected residues between the different anaesthetics used in this study after a single dose, particularly when evaluating the differences in concentrations required for an appropriate level of sedation; MS-222 (80 mg l^{-1}) 45 mg g^{-1} ± 13, metomidate (12.5 mg l^{-1}) 4 μg g^{-1} ± 1, AQUI-S (17 mg l^{-1}) 112 μg g^{-1} ± 36 (Figure 4). No significant differences ($P > 0.05$) were found between detected skeletal muscle residues from fish that had received a single dose compared with those that saw multiple anaesthetizing timepoints for any of the anaesthetics tested (Figure 4).

3.4 | Gene expression in gill tissue

The abundantly expressed isoform of pro-inflammatory cytokines *il-1β* (Il-1β1; Husain *et al.*, 2012) and *tnfα* (Tnfα1; Hong *et al.*, 2013) were chosen as immune markers. Inflammatory cytokines Il-1β1 and *tnfα1* showed no significant differences in expression levels in fish that received a single anaesthetic dose compared with fish that had been repeatedly anaesthetized prior to lethal overdosing with any of the anaesthetics tested (Figure 5). Tumour suppression protein p53, cyclooxygenase 2 (*cox 2*) and collagen related gene secreted protein, acidic, rich in cysteine (*sparc*) also showed no significant differences in expression levels for single dose exposure *v.* repeated exposure of fish for any of the anaesthetics (Figure 5).

Metomidate treated fish had no significant differences in expression of any osmoregulatory marker genes, namely Na–K-ATPase *α1a*, *α1b*, *α1c*, *α3*, *β1*, Na-K-Cl cotransporter (*nkcc*) and cystic fibrosis transmembrane conductance regulator 1 and

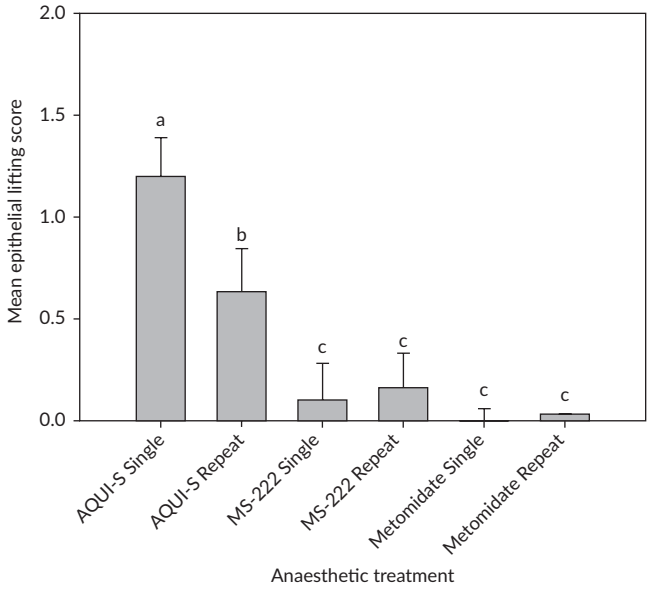

FIGURE 3 Mean epithelial lifting score (Table 1) for *Salmo salar* gills assessed by histology after repeated or single dose exposures of fish to the anaesthetics MS-222 (80 mg l^{-1}), metomidate (12.5 mg l^{-1}) and AQUI-S (17 mg l^{-1}). Different letters denote significant differences ($P < 0.05$) between groups assessed by ordinal regression analysis

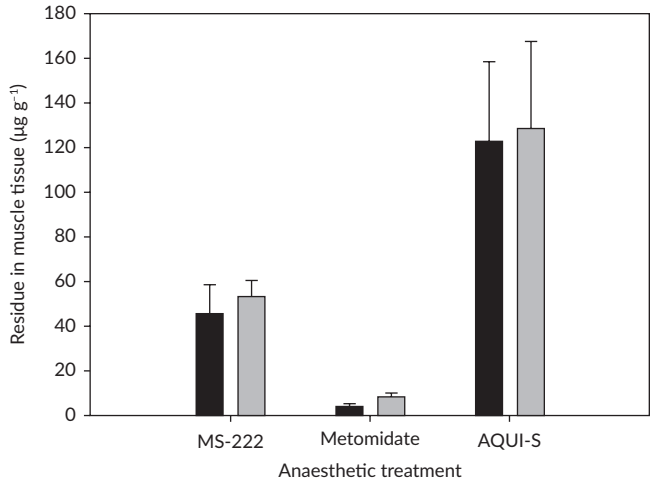

FIGURE 4 Mean (+SE) accumulation of anaesthetics MS-222, metomidate and AQUI-S in muscle tissue of *Salmo salar*, as detected by liquid chromatography mass spectrometry. Repeated exposure groups were anaesthetized to Stage III Plane 1 at seven timepoints every four days (Figure 1), followed by a lethal dose of anaesthetic at day 28. Single exposure groups were left undisturbed throughout the experiment, until day 28 and then treated with a lethal dose of anaesthetic. No significant differences were found between single and repeated doses of respective anaesthetics (■) Single, and (▨) Repeated

2 (*cftr1* & *2*) when comparing single dose *v.* repeated dosing (Figure 5). Fish that had been repeatedly anaesthetized with AQUI-S showed upregulation in all osmoregulatory marker genes when compared to single dose fish (Figure 5), with the exception of Na–K-ATPase *α3* ($P > 0.05$) and *cftr1* ($P > 0.05$). MS-222 also induced significant upregulation of Na–K-ATPase *β1* ($P < 0.05$) and *cftr2* ($P < 0.01$) when used repeatedly to anaesthetize fish compared to single dosing (Figure 5).

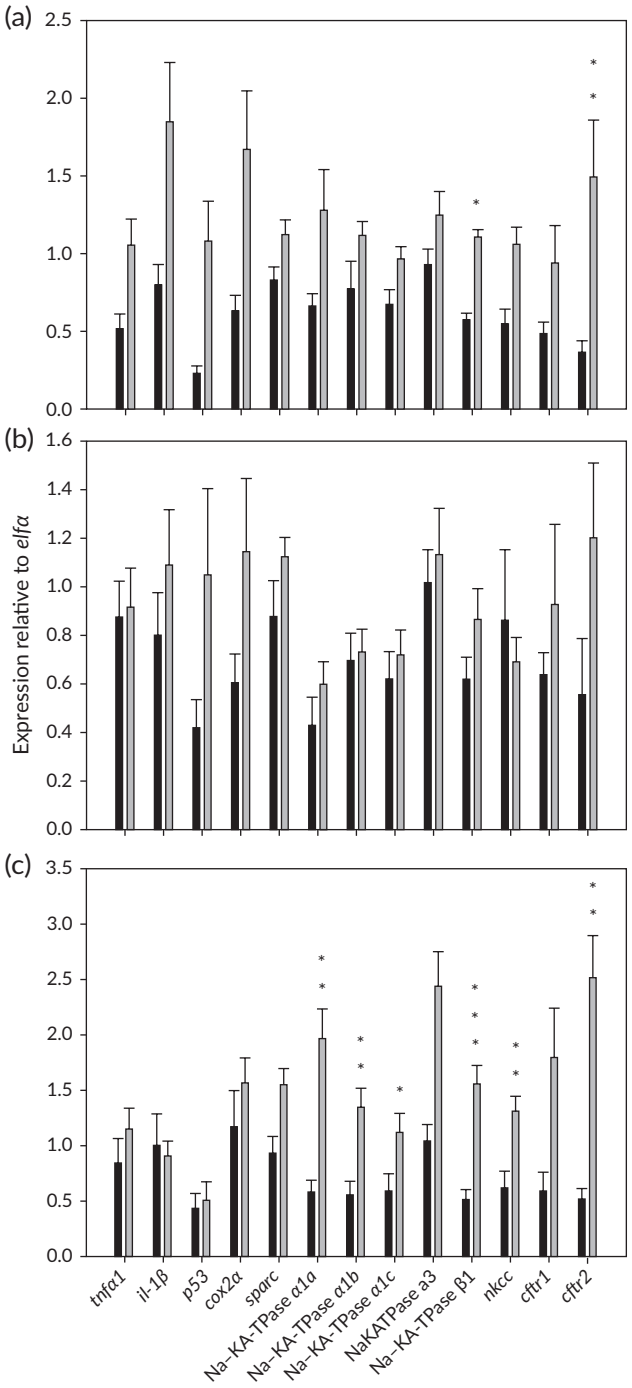

FIGURE 5 Mean (+SE) relative expression of selected genes in *Salmo salar* gills. Gene expression level was quantified by real-time PCR, normalized using the housekeeping gene elongation factor α (*elfα*), and expressed as arbitrary unit where the expression in single exposure is 1. Gills were analysed after a single ($n = 10$) or repeated exposure ($n = 30$) to (a) MS-222 (80 mg l^{-1}) (■) Single, and (▨) Repeat, (b) metomidate (12.5 mg l^{-1}) (■) Single, and (▨) Repeat and (c) AQUI-S (17 mg l^{-1}) (■) Single, and (▨) Repeat. Log_{10}-transformed data were compared by one-way ANOVA. *$P < 0.05$; **, $P < 0.01$; ***$P < 0.001$

3.5 | Gene expression in head kidney tissue

No statistical differences ($P > 0.05$) were seen in the gene expression of any heat shock proteins amplified (*hsp90α1a*, *hsp90α2*, *hsp90β1* & *hsp90β2*) for fish that had received a single dose compared with fish that had been repeatedly anaesthetized prior to lethal overdosing

(Figure 6). Gene expression analysis of the inflammatory cytokines Il-1β, *tnfα1* and *tnfα3* also revealed no significant differences between single dose and repeated dose exposure for metomidate and AQUI-S, however upregulation of *tnfα3* was seen in fish repeatedly anaesthetized with MS-222 when compared with single dose treated fish.

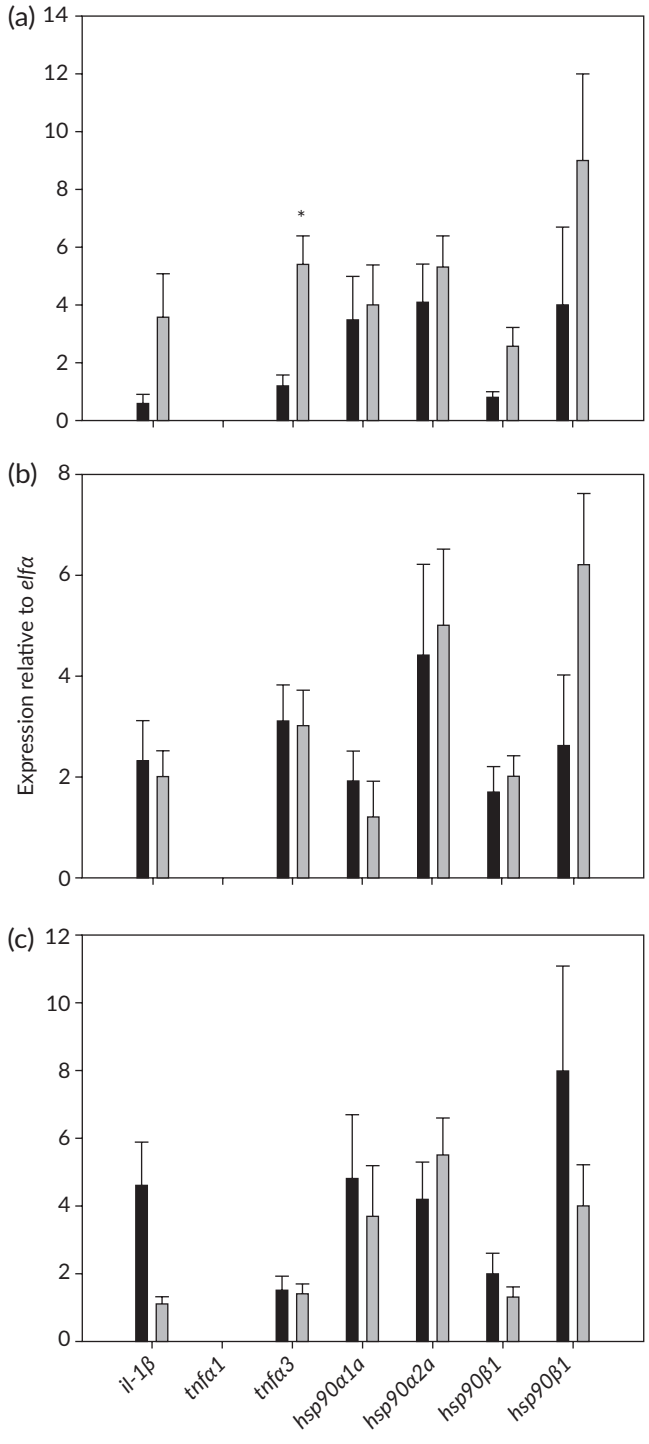

FIGURE 6 Mean (+SE) expression of selected genes from *Salmo salar* head kidney relative to the housekeeping gene elongation factor α (*elfα*). Head kidney was analysed after a single (n = 10) or repeated exposure (n = 30) to (a) MS-222 (80 mg l^{-1}) (■) Single, and (▨) Repeat, (b) metomidate (12.5 mg l^{-1}) (■) Single, and (▨) Repeat and (c) AQUI-S (17 mg l^{-1}) (■) Single, and (▨) Repeat. Log_{10}-transformed data were compared by one-way ANOVA. *P < 0.05

3.6 | Plasma glucose concentration

Plasma glucose concentrations showed no significant differences between fish that had been repeatedly anaesthetized and lethally dosed with their respective anaesthetic compared with undisturbed fish that had only a single lethal dose of respective anaesthetic (Figure 7).

3.7 | Peripheral blood leukocytes

Analysis of counts of 200 leukocytes from each fish utilized in the study showed that there were no significant differences between fish that had been repeatedly anaesthetized with AQUI-S and then euthanized with a single lethal dose *v.* those that had been left undisturbed then euthanized with a single lethal dose (Figure 8).

Metomidate treated fish had no significant differences between the populations of monocytes, thrombocytes or lymphocytes when comparing single and repeated dosing, but repeat exposure of fish had a significantly increased proportion of neutrophils (Figure 8). The opposite was the case for MS-222 treated fish, in which repeatedly exposed fish had a significantly lower proportion of neutrophils (P < 0.05), while the other leukocyte types showed no significant differences (Figure 8).

4 | DISCUSSION

Monitoring gill health is a priority when evaluating the effect on fish of non-lethal monitoring that involves repeated removal from water and exposure to anaesthesia. This is due to the importance of the gills from multiple standpoints: they are the site of uptake for immersion anaesthesia (Hunn & Allen, 1974); their biological importance in regulatory functions (Evans *et al*., 2005); the welfare of the fish (Pettersen *et al*., 2014).

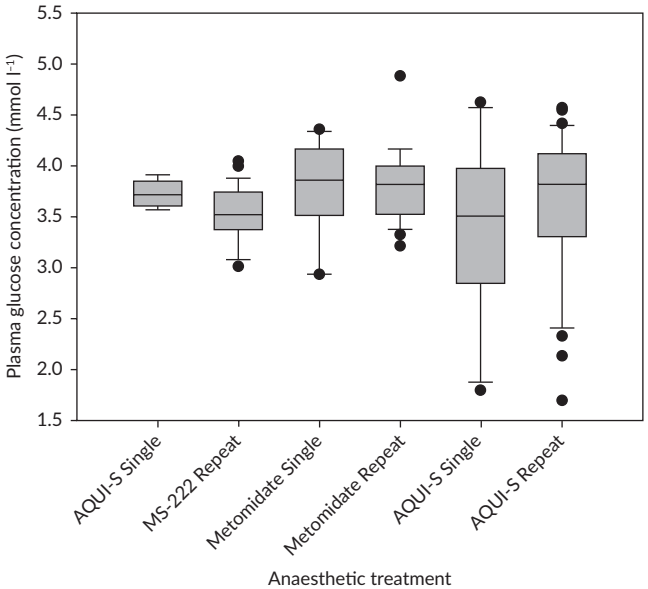

FIGURE 7 Box plot of median (——), 25th and 75th percentiles (□), range (⊤) and outlier (●) glucose concentrations of plasma from *Salmo salar* after single (n = 10) or repeat exposure (n = 30) to the anaesthetics MS-222 (80 mg l^{-1}), metomidate (12.5 mg l^{-1}) and AQUI-S (17 mg l^{-1}). No significant differences were found using a Van der Waerden test

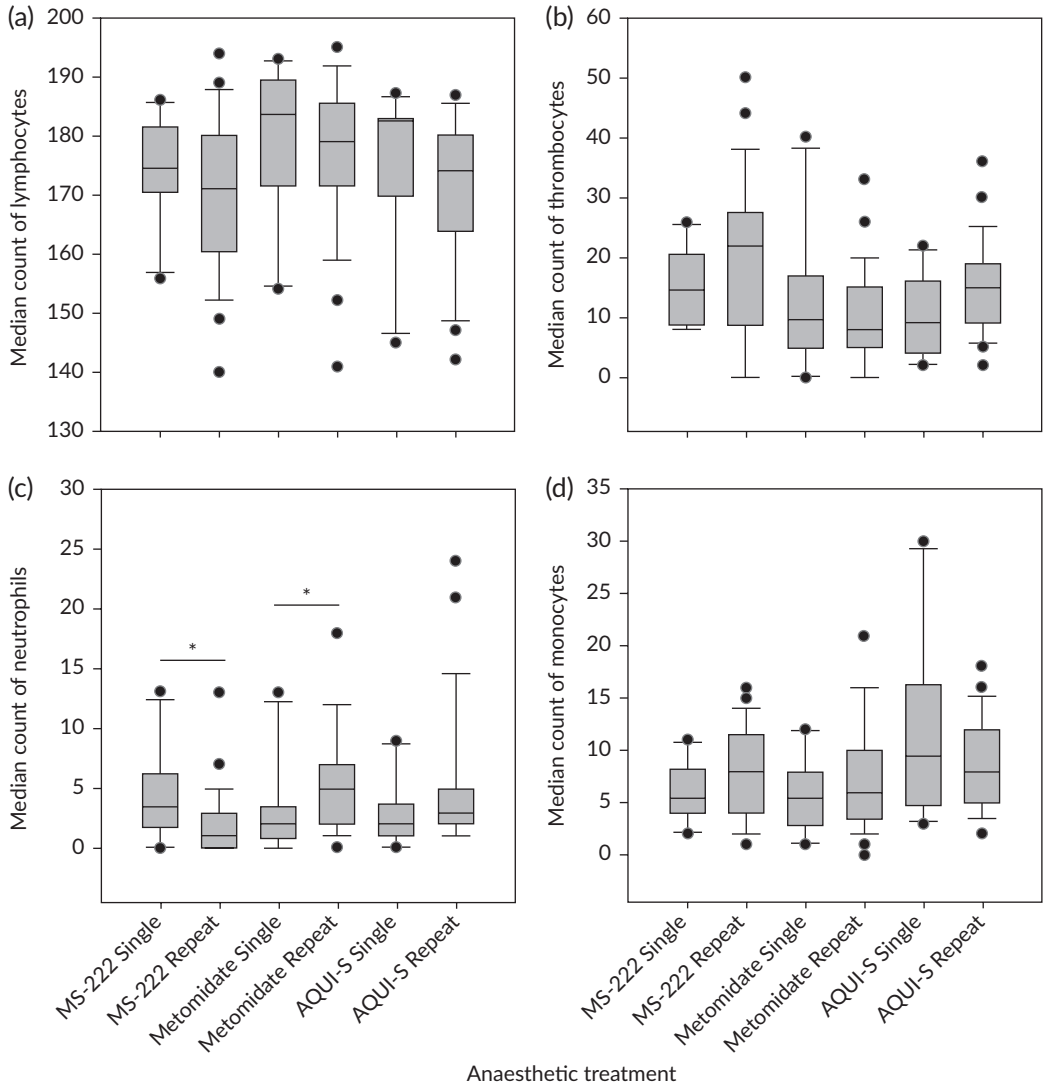

FIGURE 8 Seven Box plot of median (——), 25th and 75th percentiles (□), range (⊤) and outlier (●) leukocytes counts of whole blood smears from *Salmo salar* given single (n = 10) or repeated (n = 30) exposure to the anaesthetics MS-222 (80 mg l^{-1}), metomidate (12.5 mg l^{-1}) and AQUI-S (17 mg l^{-1}). (a) lymphocytes; (b) thrombocytes; (c) neutrophils, (d) monocytes. *, $P < 0.05$

Furthermore, the European aquaculture industry is facing increasing threat from gill-related health issues, such as amoebic (AGD) (Oldham *et al*., 2016), proliferative (PGD) (Durborow *et al*., 2015) gill disease and damage due to phyto and zooplanktonic species (Hallegraeff *et al*., 2017; Treasurer *et al*., 2003). Therefore, any challenge studies investigating individual fish responses to gill insults requires the assurance that host-insult interactions are being analysed, as opposed to reactions to sampling processes (Chance *et al*., 2018). This study focused on assessing the effects of repeated anaesthesia with three different compounds, incorporating the simulation of sample collection wherein fish were removed from water and exposed to air for appropriate periods of time needed for the collection of blood or mucus (repeat sampling was not performed and any effect not assessed here).

No significant increase in induction time was seen for any of the anaesthetics used in this study, showing that repeat anaesthesia of post-smolt *S. salar* does not result in increased tolerance or habituation to the administered doses (Figure 1). This result is in contrast with an increase in concentration needed to repeatedly anaesthetize goldfish *Carassius auratus* (L. 1758) (Posner *et al*., 2013) or angelfish *Pterophyllum scalare* (Schultze 1823) (Mitjana *et al*. 2014) and to the reported decrease for hybrid tilapia [*Oreochromis niloticus* (L. 1758) × *Oreochromis aureus* (Steindachner 1864)] with MS-222 (Smith *et al*., 1999), which suggests that any habituation may be species or dose dependent.

Based on evaluation of the gills histological results (Figure 2) and the levels of gene expression of the inflammatory cytokines *tnfα1* and *il-1β* (Figure 5), there is no evidence that repeat exposure to any of the anaesthetics used induces an inflammatory response in this organ. A lack of histomorphological change in gills of *C. auratus* after repeat exposure to 160 mg l^{-1} MS-222 has been reported previously by Posner *et al*. (2013), but haematological and histological changes were seen in the Siberian sturgeon *Acipenser baerii* Brandt 1869¸ 24 h post a single anaesthetization with 125 mg l^{-1} MS-222. Swelling in the primary and secondary lamellae due to hypertrophy of the epithelial cells was seen, alongside a depletion in leukocyte numbers, namely lymphocytes, neutrophils and eosinophils (Gomulka *et al*., 2008). It should be noted that in-tank anaesthesia was not utilized in these studies, consequently, the stress induced by capture and dosing techniques cannot be ruled out as the underlying cause of the observed changes. Since sturgeons are chondrostean fish they are likely

to have a different haematological profile in comparison with teleosts (Gomulka *et al*., 2008), however fish repeatedly exposed to MS-222 during this study also had a significant decrease in their peripheral neutrophil populations (Figure 8).

Throughout this experiment MS-222 was administered in an unbuffered form; the formation of methanesulphonic acid can reduce the pH of aquarium environments (Smith *et al*., 1999), which leads to adverse reactions in fish such as metabolic imbalances, increased blood pressure, haemoconcentration, epidermal and corneal irritation (Davis *et al*., 2008; Milligan & Wood, 1982; Packer 1979). The salinity of seawater offers some buffering protection from extreme dips in pH (Popovic *et al*., 2012), but the repetitive administration of this unbuffered form may be the cause of the significant upregulation of the pro-inflammatory cytokine *tnfα3* in the head kidney and of Na–K-ATPase *β1* and *cftr2* in the gills (Figures 6 and 7). Any change in seawater pH was not monitored throughout this experiment. Moving forward, it is suggested that a protocol using buffered MS-222 for future individual monitoring challenges should be developed.

A lack of significant differences in the expression of secondary stress response indicators, such as upregulation of isoforms of *hsp90* in the head kidney or significant changes in plasma glucose concentration, after repeat exposure to any of the anaesthetics tested in the present study may suggest that fish do not find the practice of repeated anaesthesia (compared with single overdose of such anaesthetic) cumulatively stressful. Iversen *et al*. (2003) reported that freshwater *S. salar* smolts also had no rise in plasma glucose after a single dose of metomidate (up to 10 mg l^{-1}) or AQUI-S (up to 100 mg l^{-1} active ingredient). However, behavioural (Pirhonen & Schreck, 2003; Readman *et al*., 2013; Wong *et al*., 2014) and blood glucose stress responses (Bourne, 1984; Davis & Griffin, 2004; Larter & Rees, 2017; Molinero & Gonzalez, 1995; Small & Chatakondi, 2005; Soivio *et al*., 1977; Thomas & Robertson, 1991; Wagner *et al*., 2002) have been reported in a variety of fish species exposed to a single dose of the same anaesthetics tested in this study. While the role of in-tank anaesthesia in the reduction of stress has yet to be quantified, it seems likely this methodology plays an important role in minimizing any stress response. A rise in plasma glucose has been strongly linked to increased production of primary stress indicators such as the glucocorticoid steroid cortisol, which was not assessed during this study due to time constraints, lack of required resources and prioritisation of gill-health analyses (Farbridge & Leatherland, 1992; Iversen *et al*., 1998; Pottinger, 2008). Nevertheless, it may be useful to measure the cortisol response of *S. salar* undergoing repeat anaesthesia directly in future studies, especially as it has been shown to be a better indicator of chronic stress in other species, such as the red porgy *Pagrus pagrus* (L. 1758) (Rotllant & Tort, 1997). It should be noted that the level of response of plasma glucose and cortisol to both acute and chronic stressors differ widely between species (Barton, 2002), confounding comparisons of this variable when applying repeat anaesthesia methodology to different species.

Arguably the most concerning result from this study is the significant upregulation of osmotic control marker genes in the gills of fish repeatedly anaesthetized with AQUI-S (Figure 5) and the epithelial lifting in fish following both repeat and single exposure (Table 2 and Figures 2 and 3). Na–K-ATPase activity is required for ion secretion, isoforms of which are upregulated in the fish gill during smoltification (Boeuf & Prunet, 1985; Pelis & McCormick 2001). Cortisol treatment has been shown to induce Na–K-ATPase *α1a* and *α1b* upregulation *in vivo* and *in vitro* in *S. salar* gill (Kiilerich *et al*., 2007; McCormick *et al*., 2008). A decrease of Na–K-ATPase enzyme activity in post-smolt (in seawater) salmonid gills is normally a sign of oxidative stress, often in response to environmental stressors and leads to osmoregulatory failure (Murray *et al*., 2017). Epithelial lifting from the gill capillary, which can be seen as a defence mechanism to reduce superficial surface area contact with the external environment (Mallatt, 1985), has been widely described in fish exposed to environmental stressors (Bruno & Ellis, 1988; Figueiredo-Fernandes *et al*., 2007; Frances *et al*., 2000; Good *et al*., 2017; Jiraungkoorskul *et al*., 2003; Martinez *et al*., 2004; Pretti *et al*., 2006; Skidmore & Tovell, 1972; Smart, 1976; Triebskorn *et al*., 2004) and after bath emersion to levamisole-adjuvanted *Vibrio anguillarum* vaccine (Morrison *et al*., 2001). In this study, fish treated with a single lethal dose of AQUI-S showed double the mean score of epithelial lifting when compared with fish which had been repeatedly anaesthetized with AQUI-S then lethally dosed (mean ± SD = 1.2 ± 0.2 and 0.6 ± 0.2, respectively), but both treatments' scores remain low and reflect light pathological signs (Table 2). A common role of anaesthetic use in fish research is the application of a controlled overdose in humane killing, which leads to wide concentration ranges of AQUI-S reported in the literature, ranging from 12 mg l^{-1}–540 g l^{-1} specifically for post-smolt *S. salar* (Hevrøy *et al*., 2005; Sanden *et al*., 2004). A concentration of 17 mg l^{-1} of AQUI-S was used in this study as recommended for the sedation of mature *S. salar* by the manufacturer (AQUI-S New Zealand LTD), but the duration required to reach desired levels of sedation (mean ± SD = 21 ± 3 min) was, in our hands, double that expected based on guidelines detailed on the manufactures website (AQUI-S, 2018) and > seven and 10 fold that needed for MS-222 and metomidate, respectively (Figure 1). Tanks were fully aerated while fish were exposed to anaesthetic, with approximately 7.3 mg l^{-1} oxygen maintained throughout the study. It is possible that the length of immersion in AQUI-S may be the cause of the epithelial disruption seen, which in turn may have affected hydromineral balance resulting in the significant upregulation of the Na–K-ATPase genes in the repeatedly anaesthetized fish. Interestingly, epithelial lifting has also been reported in gills of control fish euthanized by exposure to a high dose of AQUIS-S (5 ml l^{-1}, equivalent to 5.45 g l^{-1}) for an unspecified period of time during an AGD microarray study, which the authors deemed smoltification or sampling responsible (Morrison & Nowak, 2008). However, in the context of the present results, AQUI-S exposure may be the true cause. It is important to note, however, that epithelial lifting is commonly falsely diagnosed when assessing gill histopathology (Wolf *et al*., 2015), with the separation seen being an artefact arising from conditions of sampling or fixation (Ferguson *et al*., 2006; Wolf *et al*., 2015), but it is unlikely in this case as little to no lifting was seen in MS-222 or metomidate treatments. Speare and Ferguson (1989) found gills fixed with 10% NBF, the fixative used in this study, were more likely to exhibit separation artefacts and therefore future studies seeking to further investigate this effect should make use of alternatives such as Bouin's, Dielrich's or Davidson's solutions (Wolf *et al*., 2015). Further study is needed to elucidate if the length of exposure to AQUI-S can indeed cause osmotic regulatory issues and furthermore, whether a higher concentration would reduce induction times to Stage III Plane

1 anaesthesia and mitigate these observed effects or cause further negative reactions. If individual monitoring is to be widely adopted to improve the investigation of the immune responses in a variety of different species, to a variety of different pathogens, more experiments are required to identify the effects of repeat sampling for each intended host species and challenge condition. Over time, a better picture should emerge to mitigate any extraneous effects due to repeated sampling from the same individual in a challenge model.

In conclusion, this study investigated the effects of repeat anaesthesia for post-smolt *S. salar* as required for individual monitoring, with a focus on gill health. Unbuffered MS-222 gave a significant decrease in peripheral neutrophils, upregulation of *tnfα3* in the head kidney and of Na–K-ATPase *β1* and *cftr2* in the gills. Administration in buffered form for non-lethal sampling challenges may alleviate some of these effects. AQUI-S repeat exposure resulted in upregulation of most osmoregulatory genes examined in the gills and low-level epithelial lifting in both single and repeated dose treatment groups was seen. AQUI-S also required substantially longer times for induction of anaesthesia compared with MS-222 and metomidate. Therefore, further studies on repeat exposure to anaesthetics should be performed with post-smolt salmonids using higher concentrations of AQUI-S to determine if this might mitigate against potentially harmful effects of the longer induction durations required to achieve an appropriate stage of anaesthesia.

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Author contributions

R.C. – ideas, data generation, data analysis, manuscript preparation. G.C. – data generation, manuscript preparation. M.F. – manuscript preparation. P.N. – data analysis, manuscript preparation. T.W. – data generation. C.C. – ideas, data generation, manuscript preparation, funding. C.S. - ideas, manuscript preparation, funding. B.C. - ideas, data generation, manuscript preparation, funding.

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