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Effects of coeliacomesenteric blood flow reduction on intestinal barrier function in rainbow trout *Oncorhynchus mykiss*

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The aim of the current work was to elucidate if there is a connection between stress-induced decrease in coeliacomesenteric artery blood flow (*i.e.* gastrointestinal blood flow; GBF) and disruption of the intestinal primary barrier in rainbow trout *Oncorhynchus mykiss*. Upon initiation of a 15 min acute chasing stress, the GBF decreased instantly by *c.* 92%. The GBF then slowly increased and reached *c.* 28% of resting values at the end of the stress protocol. After the stress was ceased, the GBF slowly increased and returned to resting values within *c.* 45 min. Intestinal permeability assessment in an Ussing-chambers set-up revealed impaired intestinal barrier function 24 h after stress. When the stress-induced GBF reduction was mimicked by an experimental occlusion of the coeliacomesenteric artery for 15 min followed by 24 h recovery, no effect on intestinal barrier function was observed. These results suggest that no direct causal relationship can be found between the GBF reduction and development of intestinal barrier dysfunction following periods of acute stress in this species of fish.

KEYWORDS

acute stress, barrier function, gastrointestinal blood flow, occlusion, salmonids, stress response

1 | INTRODUCTION

The fish intestine is a multifunctional organ, being the main site for up-take of ingested nutrients, ions and fluids but also a major primary barrier against a harsh environment (Bakke *et al*., 2010; Grosell, 2010; Sundell & Rønnestad, 2011; Whittamore, 2012). The luminal content contains a blend of beneficial but also potentially harmful substances, such as an extensive endogenous microbiota, pathogens, proteases and feed antigens. If any of these substances pass across the intestinal epithelium in an uncontrolled way, they can cause disease, tissue damage and inflammatory responses (Knudsen *et al*., 2008; Niklasson *et al*., 2011; Sundh *et al*., 2009, 2010; Sundh & Sundell, 2015). Thus, a key function of the intestinal epithelium is to maintain selective permeability: allowing essentials to pass while blocking potentially detrimental substances from reaching the circulatory system.

The intestinal barrier constitutes three separate layers: the extrinsic barrier, consisting of a highly hydrated mucous gel with immune-active components (Gomez *et al*., 2013; Padra *et al*., 2014; Shephard, 1994), the intrinsic barrier consisting of the enterocytes connected at the apical region by intermembrane complexes known as the tight junctions and the immunological barrier which consists of the gut-associated-lymphoid-tissue (Gomez *et al*., 2013; Rombout *et al*., 2011).

When fish are faced with a stressful situation, a series of responses are elicited that will maintain, restore or adjust internal homeostasis in order for the animal to successfully cope with the situation. Stress is perceived by central nervous system (CNS) and elicits a series of neuro-endocrine responses that are often referred to as the primary stress responses. These responses result in release of catecholamines and corticosteroids (mainly cortisol) into the circulation (Wendelaar Bonga, 1997) as well as an increased adrenergic tone of both the heart (β) and the blood vessels (α) and a decrease in the cholinergic tone of the heart (Sandblom & Axelsson, 2011; Sandblom & Gräns, 2017). Together, these primary responses elicit secondary stress responses in the cardiovascular system, in the form of increased heart rate, increase cardiac output, increased systemic blood pressure

and redistribution of blood flow to oxygen demanding tissues. Furthermore, stress, both acute and chronic, have similar negative effects on the intestinal barrier function in fish as seen in humans and other mammals (Olsen *et al*., 2005; Söderholm & Perdue, 2001; Sundell & Sundh, 2012; Sundh *et al*., 2009, 2010). For example, stress in the form of netting followed by a 4 hr transportation resulted in goblet-cell depletion and epithelial-cell layer detachment from the basement membrane in the common carp *Cyprinus carpio* L. 1758, the Japanese coloured carp, *Cyprinus rubrofuscus* Lacépède 1803, the European eel, *Anguilla anguilla* L. 1758 and the African catfish *Clarias gariepinus* (Burchell 1822) (Szakolczai, 1997). Both Atlantic salmon *Salmo salar* L. 1758 and rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) showed similar changes to the intestinal morphology after acute chasing stress for 15 min (Olsen *et al*., 2002, 2005). Moreover, the acute chasing stress in *O. mykiss* coincided with a functionally impaired intestinal barrier at 4 and 48 h post-stress in the proximal intestine and at 48 h post-stress in distal intestine. In mammals the mechanisms behind stress-induced intestinal barrier dysfunction can involve stress hormones (Spitz *et al*., 1994, 1996; Zheng *et al*., 2017), immune cell activation (Söderholm *et al*., 2002) and inflammation. Also in fish, the stress hormone cortisol is believed to be involved, at least in long-term stress, as slow-release implantation of cortisol for 7 days resulted in barrier dysfunction in *O. mykiss* (Sundell & Sundh, 2012). However, information about the mechanisms behind the rapid, acute (within hours after stress) barrier dysfunction is currently scarce but involvement of enteric nerves have been suggested (Bakker *et al*., 1993).

In fish, as in other vertebrates, the gastrointestinal blood flow (GBF) is of major importance for intestinal functions as it supplies the tissue with oxygen and nutrients and removes metabolic waste products and carbon dioxide. The GBF under resting conditions maintained around 10–40% of cardiac output (Seth *et al*., 2011). However, during acute stress GBF is known to decrease drastically in various species of fish (Dupont-Prinet *et al*., 2009; Gräns *et al*., 2009a, 2009b; Sandblom *et al*., 2012) presumably as blood flow is prioritized to other oxygen demanding organs (*e.g.* brain and muscle tissues) during a fight-or-flight response (Farrell *et al*., 2001; Seth *et al*., 2011).

In mammals, the intestine is very sensitive to decreases in GBF and intestinal ischaemia is apparent if the blood supply to the intestine is inadequate to maintain aerobic metabolism. The aversive effects of ischaemia in mammals include cellular apoptosis and necrosis, dysfunctional tight junctions and loss of cell–cell contact and detachment of epithelium from the basal membrane, leading to impaired intestinal barrier function as observed in increased leakage of luminal antigens across the epithelium (Gonzalez *et al*., 2015; Granger *et al*., 2015). The magnitude of ischaemia induced damages are dependent on the duration and degree of the GBF reduction. Long periods of ischaemia due to almost complete occlusion during medical and surgical procedures, trauma and chock, results in sever epithelial damage (Granger *et al*., 2015). However, intestinal ischaemia and barrier dysfunction have also been reported after experimental reductions of GBF as small as *c.* 20% of normal blood flow (Gonzalez *et al*., 2015). Even strenuous exercise can result in physiological GBF reduction with subsequent ischaemia and barrier dysfunction (Pals *et al*., 1997; van Nieuwenhoven *et al*., 2004; van Wijck *et al*., 2011). In addition to the epithelial damages induced by ischaemia, the restoration of the blood flow, *i.e.* reperfusion, can cause damage to the tissues through formation of endogenous reactive oxygen species concomitant with abundant infiltration of leucocytes (Guan *et al*., 2009). In fish the relationship between a reduction in GBF and intestinal barrier dysfunction is currently unexplored.

As reduced GBF in mammals, as a result of stress, generates similar damages to the epithelium as those observed in fish subjected to stressful situations, the main aim of the current work was to investigate if a reduction of GBF *per se* is involved in the development of intestinal barrier dysfunction in *O. mykiss*.

2 | MATERIALS AND METHODS

2.1 | Experimental animals and holding conditions

Oncorhynchus mykiss of mixed sex were obtained from a local hatchery (EM-lax AB; www.emlax.se) and held at 10°C in 1000 l tanks containing recirculating aerated fresh water. The animals were held under these conditions on a 12L:12D photoperiod for at least 6 weeks prior to experimentation during which they were fed three times a week with dry commercial Protec 4–9 mm trout pellets (Skretting; www.skretting.com). All animals were fasted for at least 1 week prior to experiments. Animal care and experimental procedures were performed in accordance with the guidelines and regulations set by an ethical permit (177-2013) approved by the ethical committee on animal research in Gothenburg, Sweden.

2.2 | Surgical techniques and experimental protocols

2.2.1 | Gastrointestinal blood flow during control, acute stress and recovery periods

For a detailed description of the surgical technique used see Brijs *et al*. (2016). Individual fish (n = 3, mean ± s.e. mass = 627 ± 22 g) were anaesthetized in freshwater (10°C) containing 100 mg l^{-1} MS-222 and buffered with 200 mg l^{-1} $NaHCO_3$. To maintain anaesthesia, the gills were continuously irrigated with aerated water of appropriate salinity containing 75 mg l^{-1} MS-222 buffered with 150 mg l^{-1} $NaHCO_3$ at 10°C. GBF was measured by placing a 2–2.5 mm transit-time blood flow probe (B type, Transonic Systems; www.transonic.com) around the coeliacomesenteric artery. The artery was accessed through a *c.* 25 mm incision. All Transonic blood flow probes were calibrated to 10°C by the manufacturer. Following the instrumentation, fish were individually placed into custom-made Perspex 9.98 l respirometers that were submerged in a 500 l experimental tank with recirculating aerated fresh water at 10.0 ± 0.1°C. The fish were left undisturbed and allowed to recover from surgery for 24 h, during which period any external visual disturbances was minimized by covering the experimental tank with a black lid. After the recovery period, the blood flow probe was connected to a Transonic blood flow-meter (model T206; Transonic Systems). All signals were relayed to a PowerLab 8/30 system (ADInstruments; www.adinstruments.com) and data were collected using ADInstruments Chart 5 Pro 7.2.5 acquisition software, at a sampling rate of 10 Hz. Following the recovery period a

baseline of each fish was recorded for 5 min after which the fish were subjected to 15 min acute stress, *i.e.* adopting the protocol previously used to mediate intestinal barrier dysfunction in *O. mykiss* (Olsen *et al*., 2005), within the respirometer. After the acute stress the fish were given a 45 min recovery period. During whole period of the acute stress and recovery the GBF was measured continuously (total 60 min).

2.2.2 | Acute stress, intestinal barrier function and haematological analyses

To confirm that the adopted acute stress protocol used in part 1 results in intestinal barrier dysfunction, a control experiment was conducted by either netting fish (n = 12, mean ± s.e. mass = 129 ± 5 g) directly from the tank (control) or subjected to the 15 min acute-stress protocol followed by a 24 h recovery period after which they were sampled. The recovery period was chosen based on Olsen *et al*. (2005) and confirmed as efficient in eliciting intestinal barrier dysfunction in a preceding pilot study (data not shown).

Fish were euthanized with a sharp cranial blow and a blood sample was taken by caudal puncture with a heparinized syringe. A ventral incision was made and the intestine was carefully dissected out. The mesenteries and adipose tissue were removed using blunt dissection and the intestine was divided into a proximal and a distal region, separated by the ileorectal valve. The intestinal segments were gently washed and stored in ice-cold Ringer solution for salmonids in freshwater [140 mM NaCl, 2.5 mM KCl, 15 mM $NaHCO_3$, 1.5 mM $CaCl_2$, 1 mM KH_2PO_4, 0.8 mM $MgSO_4$, 5 mM HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid), 10 mM D-glucose, 0.5 mM L-lysine and 20 mM L-glutamine, pH 7.8; www.sigmaaldrich.com] saturated with a gas mixture (99.7% air and 0.3% CO_2) until further processing.

Effects of acute stress on intestinal barrier function and active transport was assessed in parallel using an *in vitro* Ussing-chamber set-up according to (Sundell *et al*., 2003) with modifications as described by (Sundell & Sundh, 2012). In brief, after mounting of the intestinal tissue into modified Ussing-chambers (Grass & Sweetana, 1988), the intestinal segments in were allowed 60 min of recovery. Thereafter, the experiment was started by renewing the Ringer solution on the serosal side and replacing the Ringer solution on the mucosal side with Ringer containing ^{14}C-mannitol (0.04 MBq ml^{-1}) (PerkinElmer; www.perkinelmer.com). From the serosal half-chamber 100 μl of the Ringer was sampled at time points 0, 20, 25 30, 60, 80, 85 and 90 min. Radioactivity was assessed in a liquid scintillation counter (Wallac 1409, PerkinElmer) after adding 5 ml Ultima Gold (PerkinElmer). The intestinal barrier function was measured as the apparent paracellular permeability (P_{app}) of the hydrophilic marker molecule ^{14}C-mannitol and as the transepithelial resistance (TER). Together with TER, continuous monitoring of transepithelial potential (TEP) and short circuit current (SCC) was done every 5 min and used as control of preparation viability. P_{app} was calculated from: $P_{app} = \delta Q \delta T^{-1}(AC_o)^{-1}$, where $\delta Q \delta T$ is the appearance rate of the molecule in the serosal compartment of the Ussing chamber in mol s^{-1}, A is the area (cm^2) of intestinal surface exposed in the chamber and C_o is the initial concentration (mol ml^{-1}) on the mucosal side.

Haematocrit was determined by centrifugation of whole blood using heparinized micro capillary tubes. Whole blood was spun and plasma was collected and stored at −80°C. Subsequently, plasma cortisol was analysed in unextracted plasma using a radioimmunoassay procedure described by Young (1986) using cortisol antibodies (code: S020; Lot: 1014-180,182, Purchased from Guildhay Ltd but no longer in business) validated by Sundh *et al*. (2011).

2.2.3 | Vascular occlusion of the coeliacomesenteric artery

The involvement of the GBF reduction during stress in the development of intestinal barrier dysfunction, was approached by separating the GBF reduction from the neuro-endocrine stress response by surgical occlusion of the coeliacomesenteric artery. Signs of behavioural stress responses were observed during occlusion concurrent with the stress indicators haematocrit, plasma cortisol and heart rate as described in part 2 and whole body oxygen consumption rate ($\dot{M}O_2$) as described below. The fish (n = 12, mean ± s.e. mass = 555 ± 23 g) divided into two groups where both groups underwent surgery before being transferred to the respirometer described above (2.2.1). However, in order to both measure and to fully occlude the gastrointestinal blood flow of the coeliacomesenteric artery transonic flow probes, a 20 MHz Doppler flow crystal mounted in 1.5–2.0 mm silicon cuffs transducer with integral canal for insertion of suture (Iowa Doppler Products; www.iowadoppler.com), were used to measure relative GBF. The reason for using a Doppler flow probes here, as compared with a transonic flow probe was because of its more compact design. This extra space was needed as a 3 mm vascular occluder (PY2 62-0100, Harvard Apparatus; www.harvardapparatus.com) also was attached between the flow probe and the bifurcation from the dorsal aorta. For heart rate measurements, two ECG electrodes (AS 631-2, Cooner wire; www.coonerwire.com) with 5 mm of the insulation removed were used. The un-insulated tip was inserted in a 23 gauge needle (Sterican, B.Braun Medical; www.bbraun.se) and the ECG wire was then bent 180° and temporarily secured to a 1 ml syringe that acted as a handle for the hypodermic needle. The electrodes inserted in a medial position between the pectoral fins where one electrode was directed caudally and the other was directed cranially with the tip located close to the heart. The incision used to access the coeliacomesenteric artery was closed with interrupted 3.0 prolene sutures (Ethicon Inc.; www.ethicon.com). All leads exited the fish through an incision and were secured together with the tube connected to vascular occluder to the back of the fish with 3–0 silk sutures. Following the instrumentation, the fish were individually placed into the respirometers in the experimental tank describe above (2.2.1). The fish were left undisturbed and allowed to recover from surgery for 24 h, during this period any external visual disturbances was minimized by covering the experimental tank with a black lid. All data was collected with a PowerLab 8/30 system (ADInstruments; www.adinstruments.com) using LabChart 7 Pro 7.2.5 software (ADInstruments), at a sampling rate of 40 Hz. The lead from the Doppler flow probe was connected to a directional-pulsed Doppler flow meter (model 545C-4, Iowa Doppler Products). GBF was determined in LabChart as the mean over the last 15 min before occlusion and the first 15 min post occlusion. The ECG electrodes and ground leads placed in the water surrounding the fish were connected to Animal Bio Amps (model FE136, ADInstruments). The sensitivity range (±2 mV), low-pass filter

(50 Hz), high-pass filter (0.3 Hz) and 50 Hz notch filter were adjusted in the Animal Bio Amp to optimize ECG recordings. Heart rate was determined as the mean of the last 15 min before occlusion, during the occlusion and the first 15 min post occlusion from the rate of ventricular depolarization (R peaks) using the ECG module in LabChart. Whole body oxygen consumption rate ($\dot{M}O_2$) was determined using intermittent-flow respirometry as outlined in the literature (Clark *et al*., 2013). Briefly, the partial pressure of oxygen in the water within each respirometer was measured continuously at 1 Hz using a FireSting O_2 system (PyroScience; www.pyro-science.com). Automated flush pumps refreshed the water in the respirometers for 10 min in every 15 min period. The slope of the decline in the partial pressure of oxygen in the water within the respirometers between flush cycles (*i.e.* when the respirometer was closed) was then used to calculate $\dot{M}O_2$: $\dot{M}O_2 = [(V_r - V_f)(\Delta C_{wO2})](\Delta t M_f)^{-1}$, where V_r is the volume of the respirometer, V_f is the volume of the fish (assuming that the overall density of the fish is 1 g per ml of tissue, thus $V_f = M_f$, the mass of the fish), ΔC_{wO2} is the change in the oxygen concentration of the water within the respirometer (C_{wO2} is the product of the partial pressure and capacitance of oxygen in the water and Δt is the time during which ΔC_{wO2} is measured. Following the 24 h recovery period the half of the fish (control group) were removed from the respirometers and euthanized with a sharp cranial blow. In the remaining fish the gastrointestinal blood flow was completely blocked by inflation of the vascular occluder for 15 min. The success of the occlusion protocol was easily verified by visual inspection of the traces in LabChart and by audial inspection, *i.e.* when the Doppler shift disappears (data not shown). Following the occlusion period, the occluder was deflated allowing blood flow to resume and the fish were given a second 24 h recovery period before these fish were also removed from the respirometers and euthanized with a sharp cranial blow. Directly following euthanasia blood from all fish were sampled and processed and the intestine was carefully dissected out to be used in the Ussing-chambers using the same protocols as described above (2.2.2).

2.3 | Statistical analysis

For 2.2.1 descriptive statistics were used to describe the dynamic of GBF in response to an acute chasing stress. For 2.2.2, all variables were compared between the control group (straight from the tank) and the treatment group (24 h post stress) using independent *t*-test. For 2.2.3, between treatment effects were compared for all variables using an independent *t*-test. The within treatment cardiorespiratory effects (*i.e.* $\dot{M}O_2$, heart rate and GBF) in the occluded group where the effects of vascular occlusion on $\dot{M}O_2$, heart rate and GBF were investigated by comparing before, during (not for GBF) and post occlusion using a paired *t*-test. When multiple paired *t*-test were performed (*i.e.* for $\dot{M}O_2$ and heart rate) the *P*-values were adjusted using Bonferroni correction. All statistical analyses were performed in GraphPad Prism 7 (GraphPad; www.graphpad.com). All values are presented as means ± S.E. unless otherwise stated. Statistical significance was accepted at $p < 0.05$.

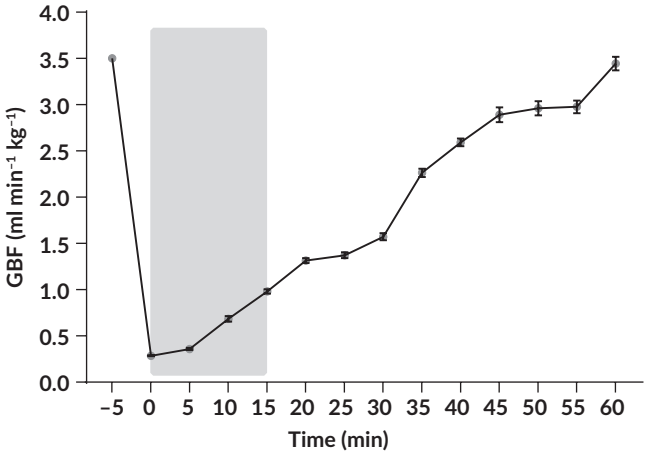

FIGURE 1 The gastrointestinal blood flow (GBF) in *Oncorhynchus mykiss* before (−5), during 15 acute stress (▒) and during 45 min recovery after an acute stress challenge ($n = 3$)

3 | RESULTS

3.1 | Gastrointestinal blood flow during control, acute stress and recovery period

When *O. mykiss* were subjected to 15 min of acute chasing stress the GBF momentarily dropped with *c.* 92% from 3.50 ± 0.03 to 0.29 ± 0.01 ml min^{-1} kg^{-1} (Figure 1). Following this initial drop, GBF slowly started to recover and at the end of the 15 min stress period it had increased from *c.* 8 to 28% of the pre-stress values (0.98 ± 0.02 ml min^{-1} kg^{-1}). Following chasing stress it took approximately 45 min until full (98%) GBF was restored (3.44 ± 0.07 ml min^{-1} kg^{-1}).

3.2 | Acute stress, intestinal barrier function and haematological analyses

The stress protocol used in 2.2.1 resulted in increased P_{app} for mannitol in the distal ($t_{21} = 2.16$, $p < 0.05$), but not the proximal intestine ($t_{19} = 0.37$, $p > 0.05$) compared with unstressed fish (Figure 2). Fish subjected to the 15 min acute stress had significantly higher haematocrit compared with the control group ($t_{22} = 6.235$, $p < 0.001$; 42.5 ± 1.1% *v.* 30.8 ± 1.5%, respectively).

3.3 | Vascular occlusion of the coeliacomesenteric artery

The whole animal measurements showed that during the 15 min occlusion heart rate was significantly decreased from 57.2 ± 1.8 beats min^{-1} before occlusion to 53.3 ± 2.1 beats min^{-1} during the occlusion ($t_{10} = 1.947$, $p < 0.01$; Figure 3(a)). The heart rate was restored 15 min post occlusion to 56.8 ± 1.9 beats min^{-1} and no longer significantly different to the control ($t_{10} = 0.519$, $p > 0.05$). A clear reperfusion was observed 15 min post occlusion as GBF was significantly elevated with 50.8% in comparison with the control ($t_{11} = 3.957$, $p < 0.01$; Figure 3(b)). There were no significant differences in $\dot{M}O_2$ during ($t_{11} = -0.24$, $p > 0.05$) or after

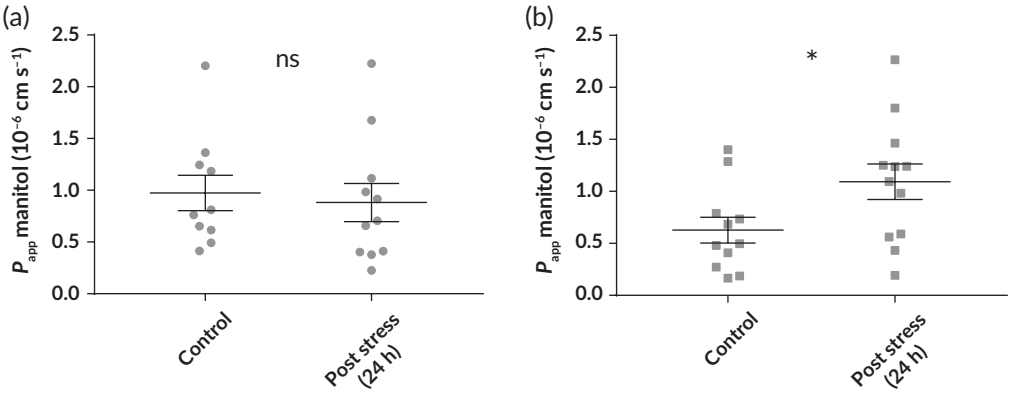

FIGURE 2 The intestinal barrier function in *Oncorhynchus mykiss* assessed as P_{app} for mannitol in (a) the proximal and (b) distal intestine 24 h post stress. Data are presented as individual values, mean (horizontal bar) and ±S.E. Statistical differences: Not significant (ns), $p > 0.05$, *$p < 0.05$, **$p < 0.01$

($t_{11} = -0.20$, p 0.05) occlusion compared with before occlusion ($\dot{M}O_2$ data not shown). No significant treatment effects were found in either the intestinal barrier functions or the haematological analyses, P_{app} (proximal intestine; $t_{21} = 0.927$, $p > 0.05$ or the distal intestine; $t_{22} = 0.560$, P 0.05; Figure 3(c)), TER (proximal intestine; $t_{21} = 0.815$, $p > 0.05$ and distal intestine; $t_{22} = 0.192$, $p > 0.05$; Table 1), plasma cortisol ($t_{22} = 0.108$, $p > 0.05$), haematocrit ($t_{22} = 0.230$, $p > 0.05$).

4 | DISCUSSION

This study investigated the role of GBF on the intestinal barrier functions in fish following acute stress, hypothesizing that stress-induced barrier dysfunction is caused by intestinal ischaemia or reperfusion injury. The results show that when *O. mykiss* are subjected to intense acute stress the GBF is drastically reduced by 92%, is maintained at 70% below normal during stress but returns to resting values 45 min

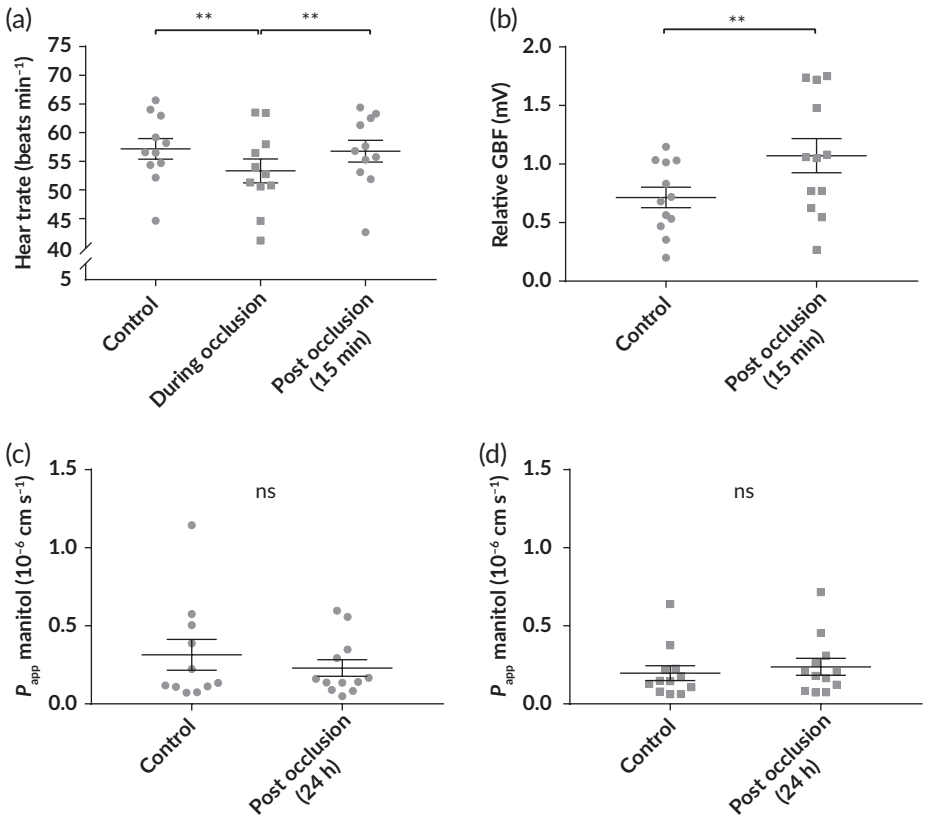

FIGURE 3 The effects of 15 min occlusion of the in *Oncorhynchus mykiss* coeliacomesenteric artery on (a) heart rate in control, during and post occlusion, (b) gastrointestinal blood flow (GBF) in control and post occlusion and the intestinal barrier function of the (c) proximal and (d) distal intestine in control and 24 h post occlusion of the coeliacomesenteric artery. Data are presented as individual values, mean (horizontal bar) and ±S.E. Statistical differences: Not significant (ns), $p > 0.05$, *$p < 0.05$, **$p < 0.01$

TABLE 1 Detailed description of parameters in vascular occlusion of the coeliacomesenteric artery

	Control (mean ± s.e.; n = 12)	24 h post occlusion (mean ± s.e.; n = 12)	t-test P	t	d.f.	F	F-test P
Whole animal stress parameters							
$\dot{M}O_2$ (mL min^{-1})	65.4 ± 12.3	69.4 ± 10.3	0.702	0.393	11		0.083
Cortisol (ng ml^{-1})	43.5 ± 6.4	42.3 ± 9.1	0.916	0.108	22	$2.054_{11,11}$	0.248
Hct (%)	25.6 ± 1.6	25.1 ± 1.0	0.820	0.231	22	$2{,}487_{11,11}$	0.146
Intestinal function parameters							
Proximal intestine							
TER (Ω cm^2)	91 ± 7	84 ± 6	0.424	0.815	22	$1.473_{11,11}$	0.532
TEP (mV)	0.8 ± 0.2	1.1 ± 0.1	0.202	1.314	22	$1.866_{11,11}$	0.316
SCC (μA cm^{-2})	−10.1 ± 2.4	−13.5 ± 1.7	0.252	1.176	22	$1.867_{11,11}$	0.315
Distal intestine							
TER (Ω cm^2)	189 ± 15	194 ± 19	0.950	0.192	22	$1.557_{11,11}$	0.475
TEP (mV)	0.2 ± 0.1	0.3 ± 0.1	0.852	0.190	22	$1.367_{11,11}$	0.613
SCC (μA cm^{-2})	−1.4 ± 0.5	−1.2 ±0.3	0.724	0.357	22	$1.940_{11,11}$	0.287

Note. Hct: haematocrit; $\dot{M}O_2$: Whole body oxygen consumption rate; SCC: short-circuit current; TEP: transepithelial potential difference; TER: transepithelial electrical resistance.

following cessation of stress. Also the stressed fish developed intestinal barrier dysfunction within 24 h. However, when separating the GBF response from the other components of the primary stress response through experimental occlusion of the GBF, no evidence of intestinal barrier dysfunction was found. These results suggest that neither the GBF reduction nor the reperfusion following a period of stress is the direct cause of the development of intestinal barrier dysfunction in *O. mykiss*.

Fish cannot fully perfuse all tissues at the same time but need to prioritise them according to their respective needs (Sandblom & Gräns, 2017; Seth *et al*., 2011). When faced with an acute stressor, the GBF that normally accounts for 20–30% of cardiac output of an unfed fish, drastically drops. This response has been reported in several different species including *O. mykiss* (Axelsson & Fritsche, 1991; Gräns *et al*., 2009a, 2009b; Sandblom *et al*., 2012). We confirmed that this response was initiated when subjecting the fish to the standardized acute-stress protocol that caused intestinal barrier dysfunction in *O. mykiss*.

The development of decreased intestinal barrier function in the distal intestine after 24 h confirmed previous finding on *O. mykiss* (Olsen *et al*., 2005). In contrast, Olsen *et al*. (2005) observed decreased intestinal barrier function in the proximal intestine after 4 and 48 h and in the distal intestine after 48 h, but not after 4 h. This difference could be explained by fish size. In the current study, smaller fish (*c*. 130 g) were used compared with Olsen *et al*. (2005) where *c*. 400 g fish were used. Thus, even though intestinal barrier dysfunction appears to be common consequence of stress (Sundh *et al*., 2009, 2010; Sundh & Sundell, 2015), the specific response pattern may differ between intestinal regions depending on fish size. Further, Olsen *et al*. (2005) showed that fasted *O. mykiss* subjected to acute stress suffered from intestinal barrier dysfunction while continuous feeding mediated barrier protection. As the mechanisms behinds these observations are not elucidated, an involvement of GBF could not be excluded; stress-induced reduction of GBF could be counteracted by the presence of feed in the intestine which is known to trigger postprandial increase in GBF via mechanical and chemical stimuli (Seth *et al*., 2011). This potentially confounding factor was excluded in the current study through the use of fasted fish.

To be able to draw conclusions from the effects of GBF *per se* on the development of intestinal barrier dysfunction, the GBF reduction must be separated from the other components of the primary stress response. In mammals this is somewhat problematic as ischaemia of the gastrointestinal tract induces abdominal pain (Klar *et al*., 2012), which is stressful in itself. In order to circumvent this problem mammalian studies that investigate gastrointestinal ischaemia are often conducted on anesthetized animals (Gonzalez *et al*., 2015; Guan *et al*., 2009). This can be a bias in itself as anaesthesia is known to obstruct both normal gastrointestinal blood flow and motility (A. Gräns, pers. obs.; Gräns *et al*., 2009b). Therefore, when investigating the relationship between intestinal ischaemia on the development of intestinal barrier dysfunction seen in non-anesthetized *O. mykiss* it is essential that the occlusion protocol used does not result in a stress response in itself. To affirm that the protocol did not cause stress, potential changes in haematocrit, heart rate, plasma cortisol and $\dot{M}O_2$ were measured in the present study. The vascular occlusion of GBF did not result in any apparent behavioural signs of stress either during occlusion or reperfusion. The slight increase in heart rate during occlusion is most likely a moderate baroreflex response caused by an increase in systemic blood pressure when the coeliacomesenteric artery is completely occluded and *c*. 20% of the cardiac output is redistributed and thus not a sign of experienced stress. Further, no difference in the physiological indicators of stress (plasma cortisol, haematocrit and $\dot{M}O_2$), could be observed 24 h post reperfusion. Furthermore, if the occlusion were perceived as stressful, the results in 3.1 suggest a substantial decrease in GBF upon reperfusion, while in fact the opposite was observed. Thus, collectively the lack of stress related responses after occlusion and reperfusion clearly suggest that the experimental protocol used successfully separated the effect of decreased GBF from the effects of other stress responses on the intestinal barrier function.

Despite 15 min of complete occlusion of the coeliacomesenteric artery, no negative effects could be observed in the intestinal barrier function. Taken together, the results of the present study indicate that a stress-induced reduction of the GBF through the coeliacomesenteric artery is not responsible for development of the epithelial damages and intestinal barrier dysfunction reported in fish subjected to acute stress (Olsen *et al*., 2002, 2005; Szakolczai, 1997) and verified by the current work. The observed increase in GBF after occlusion nevertheless shows that the intestinal tissue was affected by the blood flow reduction and induces a reactive hyperaemia. A plausible explanation for this observation is that the 15 min occlusion indeed result in ischaemia and a build-up of vasodilatory metabolites from anaerobic glycolysis that dilates arterioles and decrease vascular resistance. Upon reperfusion, the blood flow is elevated until the metabolites are washed out of the tissue which causes the arterioles to regain the normal vascular tone (Granger *et al*., 2015). The hypoxic levels reached are too low or the duration of ischaemia is too short to induce cell apoptosis, necrosis or damages to the tight junction complexes. This is supported by observations in mice *Mus musculus*, where the intracellular pH instantly decreased within minutes after start of occlusion, but were restored 5 min after reperfusion (Guan *et al*., 2009). Equally, in the mouse, no changes in tissue morphology or intestinal barrier function was apparent after the 15 min of occlusion. Furthermore, in the current study, the 15 min occlusion did not affect the active transport functions assessed as TEP and SCC. In conclusion, the results from the current study show that stress-induced reduction of the GBF is not a mechanism behind the stress-induced intestinal barrier dysfunction in *O. mykiss*.

In the absence of GBF reduction as a contributor to intestinal barrier dysfunction in *O. mykiss*, other plausible mechanisms can be put forward involving stress hormones. In mammals dexamethasone, a synthetic glucocorticoid, induces barrier dysfunction by decreasing the levels of protecting IgA levels in the mucus which allows the endogenous microbiota to attach to the epithelium (Spitz *et al*., 1996). Upon attachment, the bacteria induce intracellular signalling pathways that lead to Ca^{2+}-dependent disruption of the tight junctions (Spitz *et al*., 1995). The effect of corticosteroids on barrier function was most severe in the caecum and could be completely abrogated by antibiotic pre-treatment (Spitz *et al*., 1994). These observations are well in line with Olsen *et al*. (2002, 2005), where a stress-induced reduction in the extrinsic mucous layer concomitant with increased epithelial-bacterial interaction was observed. Another suggested mechanism behind stress-induced decreased barrier function was presented in rats, where elevated plasma corticosterone levels induced by chronic stress or subcutaneous injections resulted in intestinal barrier dysfunction mediated by down-regulation of tight junction mRNA and protein expression (Zheng *et al*., 2013, 2017). In these studies, the intestinal barrier dysfunction was only developed in the colon, not in the jejenum, which was suggested to depend on differential expression of glucocorticoid receptors and transcription factors. These findings add an interesting mechanistic aspect to the understanding of stress-induced intestinal barrier dysfunction in *O. mykiss* as well as previous studies in *S. salar* (Sundh *et al*., 2009), where similarly, the intestinal barrier dysfunction only was observed in the distal region. Further studies are needed in order to clarify the role of cortisol in the development of intestinal barrier dysfunction after acute stress in fish.

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Authors' contributions

H.S. contributed with ideas, collected the intestinal barrier-function data, plasma cortisol measurements, data interpretation and manuscript preparation. A.G. and J.B. contributed with ideas, performed the acute chasing protocol, occlusion experiment and collected the gastrointestinal blood-flow data, data interpretation and manuscript preparation. E.S., M.A. L.B and K.S contributed with ideas, data interpretation and manuscript preparation.

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