Fatty acid and lipid class composition in cutaneous mucus of Atlantic salmon, *Salmo salar* (L.)

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The surface mucus of fish is a physical and biochemical barrier that plays roles in osmoregulation, chemical communication, and protection against physical damage (e.g., by abrasion and ultraviolet radiation), chemical insults (e.g., toxins, heavy metals and irritants) and biological threats (e.g., posed by predators, parasites and pathogens) (Shephard, 1994; Ellis, 2001; Alvarez-Pellitero, 2008; Esteban, 2012; Dash et al., 2018; Reverter et al., 2018; Kumari et al., 2019). The major structural constituents of fish cutaneous mucus are mucins (highly glycosylated high molecular weight proteins), in addition to other proteins, carbohydrates, lipids, nucleic acids, and ions like calcium (Shephard, 1994; Brinchmann, 2016). Cutaneous mucus composition has been determined to differing extents for various fish, from gilthead sea bream *Sparus aurat*a (L.) (Pérez-Sánchez et al., 2017) to stingray *Hypanus americanus* (Hildebrand & Schroeder, 1928) (Coelho et al., 2019). However, most studies focused on proteinaceous or immune-relevant constituents, while few studies have characterised the lipid and fatty acid constituents despite influencing mucus properties (Lewis, 1970; Jais et al., 1998; Sato et al., 2008; Rahman et al., 2012; Torrecillas et al., 2019). Earlier studies determined fatty acids for total lipids only, whilst only Torrecillas et al. (2019) analysed the fatty acids in neutral and polar lipid fractions separately, meaning there are no reports detailing the fatty acids within distinct lipid classes of fish skin mucus. Therefore, the aim of this present study was to characterise the fatty acids and lipid classes of the skin mucus of the key farmed species, Atlantic salmon *Salmo salar* (L.).

Cutaneous mucus was collected from nine euthanised Atlantic salmon pre-smolts (each ca. 120 g). Each fish was placed into 10 mL distilled water in a plastic bag and massaged for 2 minutes. Samples were frozen in sampling pots (-20°C, 2 h) and freeze-dried for 72 h. Total lipid was extracted according to Folch et al. (1957). Briefly, each sample was dissolved in 2 mL 0.88% (w/v) KCl, transferred to 50 mL QuickFit™ borosilicate glass test tubes before 16 mL chloroform/methanol (2:1, v/v) was added. Samples were homogenised with an Ultra-Turrax tissue disruptor (Fisher Scientific, Loughborough, UK) and kept on ice for 1 h, before 2 mL 0.88% KCl was added and the sample centrifuged (400 ×*g*, 5 min). The lower layer containing the lipid extract was dried under oxygen-free nitrogen (OFN) and lipid weight determined gravimetrically following overnight

desiccation *in vacuo*. Samples were resuspended in chloroform/methanol (2:1, v/v) containing 0.01% (w/v) butylated hydroxytoluene (BHT) and stored under OFN at -20°C.

Lipid class separation was performed by double-development high-performance thin-layer chromatography (HPTLC) using methyl acetate/isopropanol/chloroform/methanol/0.25% KCl (25:25:25:10:9, v/v) and isohexane/diethyl ether/acetic acid (85:15:1, v/v) as first and second development systems, respectively (Henderson and Tocher, 1992). Total lipids (10–20 µg) were applied to HPTLC plates (Merck KGaA, Darmstadt, Germany) and run to half and full distance using first and second development systems, respectively. Plates were sprayed with 3% (w/v) aqueous cupric acetate containing 8% (v/v) phosphoric acid, before charring (160°C, 15 min). Quantification was by densitometry using a CAMAG-3 TLC scanner (Version Firmware 1.14.16; CAMAG, Muttenz, Switzerland) with winCATS Planar Chromatography Manager, with classes identified by comparison to standards.

Fatty acid methyl esters (FAME) of total lipid extracts were prepared by acid-catalysed transesterification (50°C, 16 h) using 2 mL of 1% (v/v) sulphuric acid (95% Aristar®; VWR Chemicals, Poole, UK) in methanol and 1 mL toluene (Christie, 1993). FAME were extracted and purified according to Tocher and Harvie (1988). To determine the FAME in phosphatidylcholine and free fatty acid classes, total lipid extracts were combined and prepared by TLC (fatty acids in other classes were not determined due to insufficient material). Briefly, total lipid (2–4 mg) was applied to silica gel sixty plates (Merck KGaA, Darmstadt, Germany) and developed to full distance as above. Lipid classes were visualised by spraying with 0.1% (w/v) 2-7-dichlorofluroescin in 97% aqueous methanol (v/v) and viewed under ultraviolet light at 240 nm (UVP® Mineralight® R-52G; UVP Inc. USA, California, USA). Lipid classes were scraped into separate 15 mL QuickFit™ tubes before acid-catalysed transesterification as above. The reaction was stopped with 2% (w/v) potassium bicarbonate in purified water and each sample was washed twice with isohexane/diethyl ether (1:1) + 0.01% BHT. The upper layers from both washes were combined and dried under OFN before re-dissolving in isohexane.

FAME were separated and quantified by gas-liquid chromatography (GLC) using a Fisons GC-8160 (Thermo Scientific, Milan, Italy) equipped with a 30 m × 0.32 mm i.d. × 0.25 µm ZB-wax column (Phenomenex, Cheshire, UK), 'on column' injection, flame ionisation detection and hydrogen as carrier gas. The oven started at 50°C and increased to 150°C at 40°C/min before reaching 230°C at 2°C/min. FAME were identified by comparison to standards and data were processed with Chromcard for Windows (version 1.19; Thermoquest Italia S.p.A, Milan, Italy).

The nine Atlantic salmon skin mucus samples were comprised mostly of neutral lipids (63.5±2.9%, mean ± standard deviation) (Table 1), which is consistent with cutaneous mucus collected from gilthead sea bream where neutral lipids accounted for 53.2–60.0% of total lipids (Torrecillas et al., 2019). Cholesterol/sterols made up the greatest proportion of the lipid classes (24.7±1.7% of total lipids; Table 1), which is not unexpected because these molecules are major components of eukaryotic cell membranes and dead host cells contribute to mucus composition (Brinchmann, 2016). Meanwhile, phosphatidylcholine predominated the polar lipids (15.9±2.6%), with phospholipids collectively composing around 30.1% of total lipids (Table 1). By comparison, Lewis (1970) reported phospholipids in skin mucus from flathead grey mullet *Mugil cephalus* (L.), marine catfish *Plotosus lineatus* (Thunberg, 1787) and dusky flathead *Platycephalus fuscus* (Cuvier, 1829) to contribute 36.1, 48.8 and 62.4% of total lipids, respectively.

The fatty acid profile of total lipid in the salmon mucus consisted saturated (SFA; 38.63±3.84% of total fatty acids), monounsaturated (MUFA; 28.49±1.07%) and polyunsaturated (PUFA; 32.88±4.48%) fatty acids (Table 2). The most abundant fatty acid in the skin mucus was palmitic acid (C16:0; 21.80±1.71%), followed by docosahexaenoic acid (DHA, C22:6n-3; 16.99±3.16%), oleic acid (C18:1n-9; 14.19±1.55%), stearic acid (C18:0; 7.63±0.81%) and eicosapentaenoic acid (EPA, C20:5n-3; 6.41±1.25%). Other fatty acids were at <5% abundance (Table 2) and variations between samples from the nine individuals in the same tank were relatively low. Comparison of the fatty acids in the total lipids of the salmon samples to similar studies is complicated by the influence of intrinsic and extrinsic factors, which affect the composition of the mucus,

such as diet (van der Marel et al., 2010; Jung et al., 2012; Ekman et al., 2015; Torrecillas et al., 2019; Benktander et al., 2020). Nevertheless, the most abundant fatty acid constituents of cutaneous mucus of fish in earlier reports are C18:1n-9, C16:0 and C18:0, which is consistent with this present study (Table 3).

The phosphatidylcholine fraction of the salmon skin mucus was composed largely of PUFA (36.11%), followed by SFA (33.63%) and MUFA (30.26%) (Table 2), with the most abundant fatty acids being C16:0 (25.9%), DHA (19.05%), C18:1n-9 (14.36%), EPA (8.81%) and palmitoleic acid (C16:1n-7; 6.05%); other fatty acids were at <5% abundance (Table 2). Free fatty acids were the third most abundant lipid class in the salmon mucus (14.6±2.5%), followed by the wax/sterol esters (12.6±2.1%), triglycerides (7.0±2.2%), and pigmented material (6.4±1.4%); other lipid classes were detected at <5% of total lipids (Table 1). This relative abundance of free fatty acids in the salmon mucus concurs with Lewis (1970), where these constituted 9.8–23.1% in cutaneous mucus from three fish species. In this present study, the fatty acid profile of the free fatty acids consisted SFA (47.28%), MUFA (28.80%) and PUFA (23.93%), with the most abundant fatty acids being C16:0 (27.87%), C18:1n-9 (14.74%), C18:0 (13.23%), DHA (9.33%) and EPA (5.35%) (Table 2). Free fatty acids are antimicrobial and therefore may explain some of the antimicrobial and anti-parasitic properties of fish cutaneous mucus (Lewis, 1970; Hellio et al., 2002; Alvarez-Pellitero, 2008; Desbois and Smith, 2010; Fuochi et al., 2017; Kumari et al., 2019). However, interestingly, the most abundant fatty acids in the free fatty acids of the salmon mucus have only modest antimicrobial activity, whilst typically more potent PUFA, like DHA and EPA, were present at lower abundance. Still, PUFA may be present at concentrations sufficient to exert meaningful biological activities, especially if synergy with other antimicrobial compounds like antimicrobial peptides or histone fragments is considered (Lee et al., 2009; Martinez et al., 2009; Desbois and Lawlor, 2013; Desbois, 2013). Nevertheless, some fish pathogens are attracted to the lipid components of mucus (O'Toole et al., 1999; Klesius et al., 2008), may adhere to this fluid and its constituents (Magarinos et al., 1995; Padra et al., 2019), and use mucus as a source of nutrients (Guardiola et al., 2014; Shoemaker and LaFrentz, 2015; Shoemaker et al., 2018; Minniti et al., 2019)

125 Lipids influence mucus viscosity and the absolute and relative abundances of lipids (and their fatty acid
126 constituents) affect this and other physical traits, including elasticity, wettability and adhesiveness (Murty,
127 1984). Increased cholesterol is associated with greater viscosity (Galabert et al., 1987), whilst sphingomyelin,
128 phosphatidylserine and phosphatidylinositol are linked to greater rigidity (Girod et al., 1992). Consequently,
129 the lipids in the cutaneous mucus will influence its rigidity, accumulation or dispersal from the surface, and
130 thus the protective potential against pathogens and parasites (Lewis, 1970). Few studies have examined the
131 viscosity of fish-derived mucus (Roberts and Powell 2005; Nordgård et al., 2015) and determination of this
132 and other physical characteristics was beyond the scope of this present study. As such, much still remains to
133 be discovered regarding the influence of the lipids on mucus properties. Recent studies reporting
134 comprehensive characterisation of fish mucus by sophisticated mass spectrometric techniques (Ivanova et
135 al., 2018; Patel et al., 2020) offer an attractive approach to enhance our understanding for the role of lipids
136 in this fluid especially when performed alongside assays of physical and biological properties.
137
138 To conclude, the lipid components of the cutaneous mucus of fish have received little attention and this is
139 the first study to determine the lipid classes in samples from Atlantic salmon. In addition, the fatty acid
140 profiles of total lipid, and free fatty acid and phosphatidylcholine fractions, were determined.
141 Characterisation of the lipid contents of mucus is a first step to understanding the influence that these
142 components exert on the physical and biological properties of this fluid and thus its importance to the fish.
143 In turn, this new knowledge may allow for the mucus contents to be manipulated to augment functions, in
144 particular to protect against biological threats posed by pathogens and parasites.

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Declarations

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Data availability: The data is available from the corresponding author by reasonable request.

Ethics approval: All procedures described herein adhered to the Animals (Scientific Procedures) Act 1986. Ethical approval for this study was granted by the University of Stirling's Animal Welfare and Ethical Review Body (AWERB/1718/035/New Non ASPA).

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311 **Table 1** – Lipid class composition (% of total lipid) of total lipid extracted from skin mucus samples collected

312 from Atlantic salmon pre-smolts (n=9). SD, standard deviation.

313

Lipid class	Mean (%) ±SD	Range (%)
Wax/Sterol esters	12.6 ±2.1	(9.6–14.5)
Triacylglycerols	7.0 ±2.2	(4.4–10.7)
Free fatty acids	14.6 ±2.5	(9.6–18.6)
Cholesterol/sterols	24.7 ±1.7	(22.3–26.9)
Diacylglycerol	4.7 ±1.0	(3.5–6.1)
Total neutral lipids	**63.5 ±2.9**	**(59.5–67.8)**
Phosphatidylethanolamine	3.6 ±0.9	(2.4–5.3)
Phosphatidic acid/Phosphatidylglycerol/cardiolipin	0.0 ±0.0	N/A
Phosphatidylinositol	4.1 ±0.9	(2.7–5.4)
Phosphatidylserine	3.2 ±0.6	(2.3–4.0)
Phosphatidylcholine	15.9 ±2.6	(11.3–17.8)
Sphingomyelin	2.5 ±0.2	(2.2–2.6)
Lysophosphatidylcholine	0.8 ±0.3	(0.4–1.2)
Pigmented material	6.4 ±1.4	(4.6–8.2)
Total polar lipids	**36.5 ±2.9**	**(32.2–40.5)**

314

315

316 **Table 2** – Fatty acid profiles (% total fatty acids) from total lipid, and the free fatty acids and
317 phosphatidylcholine fractions extracted from skin mucus samples collected from Atlantic salmon pre-smolts
318 (n=9 for total lipids; n=1 for free fatty acids and phosphatidylcholine fractions). PUFA, polyunsaturated fatty
319 acids; SD, standard deviation.
320

Fatty acid	Total lipids		Free fatty acids	Phosphatidylcholine
	Mean (%) ±SD	Range (%)	Mean (%)	Mean (%)
14:0	2.89 ±0.33	3.51–2.39	2.16	3.19
15:0	0.98 ±0.25	1.41–0.73	1.27	0.71
16:0	21.80 ±1.71	19.95–24.98	27.79	25.99
17:0	4.23 ±0.85	2.68–5.58	1.32	1.22
18:0	7.63 ±0.81	6.52–8.98	13.23	2.34
20:0	0.30 ±0.10	0.18–0.51	0.55	0.11
22:0	0.47 ±0.16	0.30–0.74	0.69	0.00
24:0	0.34 ±0.12	0.22–0.56	0.26	0.07
Total saturated	**38.63 ±3.84**	**33.34–44.31**	**47.28**	**33.63**
16:1n-9	3.63 ±0.48	3.09–4.44	2.74	4.32
16:1n-7	3.38 ±0.61	2.14–4.34	3.41	6.05
17:1	0.51 ±0.21	0.27–0.81	0.79	0.55
18:1n-9	14.19 ±1.55	13.05–18.04	14.74	14.36
18:1n-7	2.09 ±0.28	1.69–2.47	2.93	1.88
20:1n-11	0.16 ±0.03	0.10–0.20	0.16	0.13
20:1n-9	1.54 ±0.24	1.17–1.90	2.11	1.08
20:1n-7	0.61 ±0.16	0.32–0.78	0.90	0.68
22:1n-11	0.43 ±0.12	0.30–0.62	0.29	0.13
22:1n-9	0.64 ±0.11	0.45–0.78	0.37	0.08
24:1n-9	1.32 ±0.47	0.55–1.83	0.35	1.00
Total monounsaturated	**28.49 ±1.07**	**27.04–30.89**	**28.80**	**30.26**
18:2n-6	2.49 ±0.39	2.02–3.33	2.39	2.32
18:3n-6	0.00 ±0.00	N/A	0.17	0.05
20:2n-6	0.44 ±0.11	0.32–0.64	0.40	0.41
20:3n-6	0.22 ±0.09	0.00–0.31	0.23	0.27
20:4n-6	3.24 ±0.65	2.23–4.01	3.71	2.49
22:4n-6	0.15 ±0.04	0.09–0.22	0.14	0.16
22:5n-6	0.26 ±0.05	0.18–0.34	0.13	0.50
Total n-6 PUFA	**6.80 ±0.73**	**5.52–7.53**	**7.16**	**6.20**
18:3n-3	0.27 ±0.11	0.17–0.49	0.29	0.16

18:4n-3	0.74 ±0.24	0.45–1.17	0.34	0.31
20:4n-3	0.22 ±0.08	0.00–0.26	0.11	0.26
20:5n-3	6.41 ±1.25	3.72–7.97	5.35	8.81
22:5n-3	1.10 ±0.24	0.65–1.51	0.78	1.31
22:6n-3	16.99 ±3.16	10.90–19.97	9.33	19.05
***Total n-3 PUFA*[a]**	**25.73 ±4.25**	**17.19–30.41**	**16.20**	**29.91**
***Other PUFA*[b]**	**0.35 ±0.47**	**0.00–1.30**	**0.56**	**0.00**
Total PUFA	**32.88 ±4.48**	**24.81–37.93**	**23.93**	**36.11**

[a] Includes C20:3n-3 and C21:5n-3; [b] includes C16:2, C16:3 and C16:4

Table 3 – Comparison of most abundant fatty acids in the total lipid extracted from cutaneous mucus of different fish.

Species	Most abundant fatty acids in total lipids	Reference
Haruan (*Channa striata* Bloch, 1793)	C18:2n-6 > C18:1 > C18:0 > C16:0 > C20:4n-6	Jais et al. (1998)
Amberjack (*Seriola dumerili* Risso, 1810)	C16:0 > C18:1n-9 > C22:1n-9 > C18:0 > C24:1n-9	Sato et al. (2008)
Spotted halibut (*Verasper variegatus* Temminck & Schlegel, 1846)	C18:1n-9 > C22:1n-9 > C16:0 > C24:1n-9 > C18:0	Sato et al. 2008
Bluestreak cleaner wrasse (*Labroides dimidiatus* Valenciennes, 1839)	C18:2n-6 > C16:0 > C20:1n-11 > C20:0 > C18:1n-9 > C18:2n-6t > C18:3n-6	Rahman et al. (2012)
Flathead grey mullet (*Mugil cephalus* L.)	C18:1n-9 > C18:4n-3 > C16:0 > C18:3n-6 > C18:3n-3 > C18:0	Balasubramanian and Gunasekaran (2015)
Gilthead sea bream (*Sparus aurata* L.)	C18:1n-9 > C16:0 > C22:6n-3 > C18:2n-6	Torrecillas et al. (2019)
Atlantic salmon (*Salmo salar* L.)	C16:0 > C22:6n-3 > C18:1n-9 > C18:0 > C20:5n-3	This study