Skin Deep: Microscopic Anatomy of Finfish Integument

Diane Elliott
Scientist Emeritus
U.S. Geological Survey, Western Fisheries Research Center, Seattle, WA USA

Further Responses to Participant Questions

The purpose of this document is to provide more complete answers to questions asked at the end of the webinar, with the main focus being the listing of references for additional information on each topic. Some of the questions are edited for brevity or to correct typographical errors or are combined with similar questions.

1) **Are there specific immune cells or specific immune response in skin? Does a shark's skin have special defense mechanisms against water pollution or environmental stressors?**

   Skin immune response: Both innate and adaptive elements of the immune system are present in the integument of fishes. Most studies of integumental immune functions in fish have been conducted with teleosts. Similar to other mucosal tissues (e.g. gill, gut) in teleosts, IgT (also called IgZ) plays a principal role in skin adaptive mucosal immunity, and IgT+ B-lymphocytes represent the predominant B-cell subset in the epidermis (Xu et al. 2013, Proceedings of the National Academy of Sciences USA 110:13097-13102).

   Less is known about adaptive mucosal immunity and other intergumental defense systems in non-teleost fish groups such as elasmobranchs and jawless fishes. Several reviews have been written on innate and adaptive immunity in various fish taxa, including mucosal immunity. Some of these are:

   Esteban, M.A. 2012. 2012. An overview of the immunological defenses of fish skin. ISRN Immunology 2012: 853470 doi:10.5402/2012/853470.

   Flajnik, M.F. 2018. A cold-blooded view of adaptive immunity. Nature Reviews Immunology 18:438-453 doi: 10.1038/s41577-018-0003-9.
   (Note: This review includes comparisons of adaptive immunity in jawless, cartilaginous and bony fishes.)

   Gomez, D., Sunyer J.O., and Salinas, I. 2013. The mucosal immune system of fish: the evolution of tolerating commensals while fighting pathogens. Fish and Shellfish Immunology 35:1729-1739.

   Lazado, C.C. and Caipang, C.M.A. 2014. Mucosal immunity and probiotics in fish. Fish and Shellfish Immunology 39:78-89.

Luer, C.A., Walsh, C.J., and Bodine, A.B.2004. The immune system of sharks, skates and rays. Pages 369-395 In J.C. Carrier, J.A. Musick, and H.R. Heithaus (eds.) Biology of Sharks and their Relatives. CRC Press, Boca Raton, FL.

Mashoof, S., and Criscitiello, M.F. 2016. Fish immunoglobulins. Biology 5:45 doi:10.3390/biology5040045.

Meyer, W., and Seegers, U. 2012. Basics of skin structure and function in elasmobranchs. Journal of Fish Biology 80:1940-1967.
(Note: This review includes a description of leukocytes in elasmobranch skin.)

Smith, N.C., Rise, M.L., and Christian, S.L. 2019. A comparison of the innate and adaptive immune systems in cartilaginous fish, ray-finned fish, and lobe-finned fish. Frontiers in Immunology 10:2292 doi: 10.3389/fimmu.2019.02292.

Shark skin defenses: Information on elasmobranch skin defenses is included in some of the publications listed above. Some research has reported rapid healing of skin wounds in sharks, especially in warmer water (Chin et al. 2015, Conservation Physiology 3: doi:10.1093/conphys/cov062). Pogoreutz et al. (2019, Animal Microbiome 1:9 https://doi.org/10.1186/s42523-019-0011-5) found a similar bacterial assemblages on healthy and injured skin of wild-caught black-tip reef sharks (*Carcharhinus melanopterus*), suggesting the absence of severe bacterial infections or pathogen propagation in areas of skin injury. For information on shark skin defense against intense UV irradiation and sunburn, please see question 8.

2) What is the relation between mast cells (eosinophilic granule cells) and hyperplasia of epithelial and goblet cells of fish after exposure to environmental stressors?

Mast cells (MC, also commonly known as eosinophilic granule cells) actually show a marked diversity in staining properties, with both eosinophilic (acidophilic) and basophilic components in the granules. Staining characteristics can vary by fish species and with different fixation and staining methods (Reite and Evensen 2006, Fish and Shellfish Immunology 20:192-208).

Recruitment of MCs to sites of persistent inflammation seems to be a general response in teleosts; they readily migrate to sites of infection, especially parasitosis (Reite and Evenson 2006). Cases of MC aggregation associated with hyperplasia of epithelial and mucous cells of surface tissues have been observed. For example, Dezfuli and Giari (2008, Journal of Fish Diseases 31:845-852) reported abundant mast cells and rodlet cells, epithelial hyperplasia and mucous goblet cell proliferation near the attachment site of the copepod *Ergasilus sieboldi* on gills of bream (*Abramis brama*). MCs are often seen in close association with granulocytic leukocytes and rodlet cells in sites of infection, and MC degranulation can be induced by exposure to killed bacteria, bacterial products, toxicants or other agents (Sfacteria et al. 2015, Molecular Immunology 63:3-

8). (Note that exposure to environmental stressors may result in increases or decreases in epidermal thickness and numbers of mucous goblet cells, depending on the stressor and fish species (see e.g. Jensen et al. 2015, Journal of Fish Diseases 38:977-992)).

Despite differences in staining properties and tissue distribution of MCs among fish species, MC functions appear to be similar to those of mammals (Sfacteria et al. 2015). MCs are considered to be important initiators and effectors of the innate immune response and regulators of the adaptive immune response (Mulero et al. 2007, Proceedings of the national Academy of Sciences 104: 19434-19439 doi: 10.1073_pnas.0704535104). The various bioactive compounds identified in MC granules of fishes and their functions have been reviewed by Sfacteria et al. (2015). Of the two biogenic amines (serotonin and histamine) present in the granules of mammalian MCs, serotonin has been reported from MCs of several teleost species (Mulero et al. 2007; Sfacteria et al. 2015), but histamine has only been identified in MC granules in higher teleosts of the order Perciformes (Mulero et al. 2007). In Perciformes, the histamine in MCs is able to regulate the inflammatory response by acting on professional phagocytes (Mulero et al. 2007).

3) Which cells are involved in rapid wound healing (in salmonids)? Do high ammonia and nitrate levels in water affect wound healing in fish?

Cells involved in wound healing: Initial wound closure in salmonids and other fishes is accomplished by rapid migration of epidermal epithelial cells from the edges of the wound, as described in the webinar. (Note that these cells are also called keratocytes, keratinocytes, Malpighian cells, filament-containing cells, polygonal cells, principal cells and mucous cells [jawless fishes], among other names). For an extensive literature review of wound healing in fish (including wound prevention/management), please see Sveen et al. 2020 (Reviews in Aquaculture May 2020 p. 1-20 doi: 10.1111/raq.12443).

Water ammonia and nitrate levels and wound healing: I have not found specific references to effects of high ammonia or nitrate levels in water on wound healing. However, high ammonia levels have been shown to affect the skin histology of some fishes. Experimental exposure of sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) to elevated ammonia levels was associated with a general distortion of the epidermis with loss of orientation of basal epithelial cells, vacuolization of epithelial cells and dilation of intercellular spaces (Kalogianni et al. 2011, Journal of Fish Biology 78:1152-1169). An increase in epidermal neutral mucous goblet cells was observed in both species and a reduction in epidermal acid mucous goblet cells was seen in sea bass. In addition, these authors observed dispersion of melanosomes in dermal melanophores associated with exposure to elevated ammonia levels; the effect was most pronounced in sea bream. Elevated nitrate levels may also be associated with increases in the number of epidermal mucous cells in sea bass (Vatsos et al. 2010 European Journal of Histochemistry 54:e22 doi:10.4081/ejh.2010.e22).

Rearing Atlantic salmon (*Salmo salar*) at high density (100 kg/m^3) was shown to delay wound healing in post-smolt Atlantic salmon *(Salmo salar*) (Sveen et al. 2018, Scientific Reports 8:16907 DOI:10.1038/s41598-018-35002-5). Although the delay in wound healing was not associated with elevated ammonia or nitrate levels (which were not reported) or other water quality parameters, effects of high-density rearing on wound healing included an enhanced inflammatory response but poor epidermal organization, reduced mucus production, delayed mineralization of scales and delayed restoration of fibrous tissue and pigmentation.

4) Can a fish's age be determined from the scales? Do fish replace scales one at a time, or in groups, or all at once?

Age determination: Scales are one of the calcified structures frequently used for age determination of teleost fish (see references at end of this section). An advantage of using scales for age determination is that they can be removed non-lethally and are quickly replaced. Usually, scales are collected from particular areas of the body. Scales of teleosts continue to grow as the fish grows, and each scale shows a series of "growth rings" (circuli). During winter, with colder water temperatures, scale growth usually slows and a thicker ring (annulus) is formed. The annuli are used to estimate fish age. Annulus formation can be affected by factors such as water temperature and food supply. Scales may also be lost and regenerated. Age determination via scales may underestimate the age of older or long-lived fishes. Other calcified structures used for age determination of teleosts include otoliths (inner ear bones), vertebrae, spines and opercular bones.

For Actinopterygii, ganoid scales of alligator gars (*Atractosteus spatula*), sectioned and examined for annuli, have also been used for aging, but age determinations may provide overestimates at younger ages and underestimates at older ages (DiBenedetto 2009, MS Thesis, Louisiana State University https://digitalcommons.lsu.edu/gradschool_theses/1304). Examination of otoliths from gars may provide more accurate age estimates than examination of scales (DiBenedetto 2009; Buckmeier et al. 2018, Transactions of the American Fisheries Society 147:639-648) but may underestimate the age of fish older than 10 years (Buckmeier et al. 2018). For age determination of sturgeon, examination of sections of pectoral fin spine sections have been the most practical samples with the highest precision of interpretation (Brennan and Cailliet 1989, Transactions of the American Fisheries Society 118:296-310; Stevenson and Secor 1999, Fishery Bulletin 95:153-166)

Age determination of elasmobranchs is more complicated because they do not have otoliths, placoid scales do not form discernible growth bands, and there is a low level of calcification in cartilaginous body parts (Campana 2014; Carbonara and Follesa 2019; see below). Therefore, vertebrae and fin spines are usually used for aging elasmobranchs (Campana 2014).

Scale loss and regeneration: Elasmoid scales of teleost fishes are regenerated if they are lost due to trauma or other causes, provided that the scale-forming cells lining the empty scale pocket are intact (see Elliott 2000b at end of this document). The number of scales lost (and replaced) at one time depends on the severity of the causative event. Some teleosts such as herring and anchovies (Clupeidae) and halfbeaks (Hemirhamphidae) have deciduous scales that are easily shed, which may aid in escape from predators. Placoid scales of elasmobranchs do not show continuous growth throughout life like elasmoid scales; the placoid scales are replaced as they wear out or are lost.

Following are references on use of scales and other calcified structures for age determination of fish:

Campana, S.E. 2014. Age Determination of Elasmobranchs, with Special Reference to Mediterranean Species: A Technical Manual. Studies and Reviews. General Fisheries Commission for the Mediterranean. No. 94. FAO, Rome. 34 p.

Carbonara, P., and Follesa, M.C. (eds.) 2019. Handbook on Fish Age Determination: A Mediterranean Experience. Studies and Reviews. No. 98. FAO, Rome. License CC BY-NC-SA 3.0 IGO. 192 p.

Chilton, D.E., and Beamish, R.J. 1982. Age Determination Methods for Fishes Studied by the Groundfish Program at the Pacific Biological Station. Canada, Special Publication, Fisheries and Aquatic Sciences 60. 102 p.

Schneider, J.C., Laarman, P.W., and Gowing, H. 2000. Age and growth methods and state averages. Chapter 9 in Schneider, James C. (ed.). Manual of Fisheries Survey Methods II: with periodic updates. Michigan Department of Natural Resources, Fisheries Special Report 25, Ann Arbor.

5) *Tenacibaculum maritimum* is incredibly successful in infecting a wide range of species with different skin anatomies. What characteristics of this pathogens make it so successful at crossing the diversity of skin barriers presented by the various fish species? Why is it so hard to develop a successful long-lasting vaccine to protect the skin mucosa against this pathogen?

Antigenic heterogeneity among isolates of *Tenacibaculum maritimum* may present difficulties for development of vaccines with wide applicability (see e.g. Avendaño-Herrera et al. 2004, Diseases of Aquatic Organisms 58:1-8; van Gelderen et al. 2010, Journal of Applied Microbiology 109:1668-1676). Additionally, phenotypic and genomic analyses have identified a number of factors that are likely involved in the virulence process including adhesion to host mucus, immune escape, invasion, colonization, destruction of host tissue, and nutrient scavenging (Avendaño-Herrera et al. 2006, Diseases of Aquatic Organisms 71:255-266; Perez-Pascual et al. 2017, Frontiers in

Microbiology 8:1542 doi: 10.3389/fmicb.2017.01542). The primary sites of infection are body surfaces such as the head, fins, mouth or flanks, with strong attachment of the bacterium to external skin and mucus prior to invasion and destruction of surface tissues (Avendaño-Herrera et al. 2006). Magriños et al. (1995, Diseases of Aquatic Organisms 21:103-108) reported that *T. maritimum* (formerly called *Flexibacter maritimum*), regardless of origin or degree of virulence, adhered strongly to mucus and resisted the innate bactericidal properties of mucus from three marine fish species. Development of vaccines that are specifically designed to stimulate mucosal immunity (see question 1) and prevent the adhesion to, or colonization and invasion of, host surface mucosal tissues, might be worth investigation (see e.g. Gomez et al. 2013, cited in question 1; Salinas et al. 2015, Developmental and Comparative Immunology 53:105-111; Wilson et al. 2020, p. 811-829 In H. Kiyono and D.W. Pascual (eds.). Mucosal Vaccines, 2nd edn. Academic Press, Cambridge. MA. https://doi.org/10.1016/B978-0-12-811924-2.00048-1). An extensive review of vaccine development strategies is beyond the scope of this webinar. Broad discussions of vaccine development are available in publications such as Gudding, Lillehaug and Evensen (eds.) 2014, Fish Vaccination. Wiley Blackwell, Chichester, UK. 383p.

6) Are there fixatives other than glutaraldehyde-alcian blue for fixation of the mucous cuticle on the skin surface? What is your protocol for best histological sample results?

Mucous cuticle fixation: Glutaraldehyde-alcian blue has become the standard fixative used for preservation of the mucous cuticle for light and electron microscopy (Powell et al. 1992, Journal of Fish Biology 41:813-824). Although there may be other mucus fixatives that I am not aware of, glutaraldehyde-alcian blue has produced good results in our laboratory and in others.

Histological protocols for skin samples: Our laboratory has used a variety of protocols for fixation, processing and staining sections of fish skin tissue. Although we frequently use Carson's modified Millonig phosphate-buffered formalin (Carson et al. 1973, American Journal of Clinical Pathology 59:365-373) as a standard fixative for routine paraffin processing, many common fixatives give good results, depending on the purpose. For most of the scanning electron microscopy (SEM) photomicrographs shown in the webinar (other than specimens fixed in glutaraldehyde-alcian blue for preservation of the mucous cuticle), tissues were preserved with a glutaraldehyde-paraformaldehyde fixative (Millonig 1976, Laboratory Manual of Biological Electron Microscopy, Mario Saviolo, Vercelli, p. 27). Further details of procedures used by our lab for fixation, post-fixation, critical point drying and sputter coating of specimens for SEM are provided in e.g. Elliott et al. (2009) listed at the end of this document.

To make sectioning of scales easier and thereby reduce distortion, skin tissues can be decalcified with EDTA solutions (e.g. Witten et al. 2016, Journal of Fish Biology 88:690-708). Alternatively, for preserving morphological features of all skin layers while facilitating sectioning through large scales, tissues can be embedded in glycol

methacrylate resin instead of paraffin (see e.g. Elliott et al. 2009 at the end of this document). Glycol methacrylate embedding was used for a number of the skin tissue specimens (e.g. Chinook salmon *Oncorhynchus tshawytscha* and goldfish *Carassius auratus*) shown in photos for the webinar. Epon embedding of small sections can also be effective for preserving skin morphology.

The staining protocols we use depend on the integumentary features we are trying to identify. This topic is too broad to be discussed in this document; appropriate references should be consulted for staining of specific integumentary features.

7) Do chromatophores in the dermis of fish skin increase in number as the fish grows?

Specific types of chromatophores can either increase or decrease in number as a fish grows, depending on the types of slow (morphological) color changes that occur as the fish grows and matures. Color changes (both rapid and slow) can also occur during adaptation of a fish to environmental background changes. Morphological color changes are accomplished via alterations in the density (number) and morphology of chromatophores. Increases in pigment cell density may be achieved by proliferation of chromatophores and/or precursor cells and migration/aggregation of those cells in specific areas of the skin; mechanisms may vary by chromatophore type. Decreases in chromatophore density may occur via apoptosis. Slow color changes can also be accomplished by changes in the shape or size of individual chromatophores. Much of the recent research has been conducted with zebrafish (*Danio* rerio). Following are some reviews for further information on mechanisms of morphological color changes in fish integument:

Nüsslein-Volhard, C., and Singh, A.P. 2017. How fish color their skin: a paradigm for development and evolution of adult patterns. Bioessays 39 (3): 1600231. DOI 10.1002/bies.201600231.

Parichy, D.M. 2003. Pigment patterns: fish in stripes and spots. Current Biology 13 (24):PR947-R950. DOI: https://doi.org/10.1016/j.cub.2003.11.038

Patterson. L.D., and Parichy, D.M. 2019. Zebrafish pigment pattern formation: insights into the development and evolution of adult form. Annual Review of Genetics 53:505-530. DOI: https://doi.org/10.1146/annurev-genet-112618-043741

Singh, A.P. and Nüsslein-Volhard, C. 2015. Zebrafish stripes as a model for vertebrate colour pattern formation. Current Biology 25 (2):R81-R92. DOI: https://doi.org/10.1016/j.cub.2014.11.013

Sugimoto, M. 2002. Morphological color changes in fish: regulation of pigment cell density and morphology. Microscopy Research and Technique 58:496-503.

8) Can fish get sunburn? Do the scales help to prevent fish from getting sunburn if they are near the surface of the water?

The dorsal surfaces of fish can indeed be sunburned after excessive exposure to UVB irradiation, especially in shallow water. Sunburn first affects the epidermal tissue external to the scales; thus, scales do not help prevent sunburn damage to the epidermis. Fish may be rendered more susceptible to sunburn by dietary photosensitization (e.g., niacin deficiency) or by certain therapeutants (e.g. florfenicol) (Smith et al. 2019, Skin and fin diseases. p. 97-133 In S.A. Smith (ed.). Fish Diseases and Medicine. CRC Press, New York.)

Fish exposed to excessive UVB irradiation may show loss of mucous goblet cells, and edema and necrosis of the epidermis and dermis (Blazer et al. 1997, Journal of Aquatic Animal Health 9:132-143). "Sunburn cells" with pyknotic or fragmented nuclei may be present in areas of UVB-exposed epidermis (Noceda et al. 1997, Diseases of Aquatic Organisms 31:103-108; Nowak 1999, Bulletin of the European Association of Fish Pathologists 19:290-292). Secondary fungal or bacterial infections may be common in sunburned fish (Blazer et al. 1997; Nowak 1999).

Susceptibility to sunburn varies among fish species. Whereas salmonids, for example, are sensitive to UVB irradiation, other species such as razorback suckers (*Xyrauchen texanus*) and scalloped hammerhead sharks (*Sphyma lewini*) are more resistant to sunburn. Although decreases in epidermal mucous goblet cells have been observed following UVB exposure even in fish species that are relatively resistant to sunburn (Blazer et al. 1997; Kaweewat and Hofer 1997, Journal of Photochemistry and Photobiology B: Biology 41:222-226), resistant fishes have other mechanisms for protection against sunburn. In razorback suckers, increased epidermal thickness is associated with hyperplasia and hypertrophy of ostariophysan-type club cells; Blazer et al. (1997) hypothesized that the club cells contained a substance that protected the fish from UVB irradiation. Other authors have hypothesized a photoprotective effect of melanin in the dermis of the skin of some sunburn-resistant fishes. Scalloped hammerhead sharks exposed to intense UV irradiation in shallow ponds develop darkened skin associated with increased melanin in the dermis (Lowe and Goodman-Lowe 1996, Nature 383:677). Similarly, Rheault et al. (2015, Journal of Fish Biology 87:1248-1253) demonstrated a positive relationship between dermal melanin concentrations in yellow perch (*Perca flavescens*) and water transparency in 11 lakes in Canada. Mueller and Neuhauss (2014, PLoS ONE 9 (1): e87372 doi:10.1371/journal.pone.0087372) reported that exposure of 2-day-old zebrafish embryos to UV light triggered dispersion of melanosomes in melanophores and darkening of the body, presumably a protective response against UV irradiation. Blazer et al. (1997) noted increased accumulations of melanophores in the dermis of razorback suckers exposed to UVB irradiation.

9) How can you differentiate among different cell types viewed by electron microscopy? What is the normal size of different cells in seabream (*Sparus aurata*)?

Skin cell ultrastructure: The cell types present in the skin of fishes differ depending on the species, as outlined in the webinar. A number of publications describe the ultrastructure of cells in the skin (especially the epidermis) of various fish species. Following is a small sample of available articles. For example, ultrastructure of the epidermis of several teleosts was described in three articles published in 1968 by Henrickson and Matoltsy (who also cited earlier publications describing the fine structure of several other fish species, including hagfish [*Myxine*], salmonids [*Salmo*], catfish [*Amiurus*], and flounder [*Hippoglossoides*]). Henrickson and Matoltsy examined the skin of the guppy (*Poecilia* [*Lebistes*] *reticulata*), goldfish (*Carassius auratus*), eel (*Anguilla* sp.) and armored catfish (*Corydoras aeneus*). The Henrickson and Matoltsy articles are:

Henrickson, R.C. and Matoltsy, A.G. 1968a. The fine structure of teleost epidermis. I. Introduction and filament-containing cells. Journal of Ultrastructure Research 21:194-212.

Henrickson, R.C. and Matoltsy, A.G. 1968b. The fine structure of teleost epidermis. II. Mucous cells. Journal of Ultrastructure Research 213-221.

Henrickson, R.C. and Matoltsy, A.G. 1968c. The fine structure of teleost epidermis. III. Club cells and other cell types. Journal of Ultrastructure Research 21:222-232.

Some articles describing the fine structure of salmonid skin are as follows:

Harris, J.E., and Hunt, S. 1975. The fine structure of the epidermis of two species of salmonid fish, the Atlantic salmon (*Salmo salar* L.) and the brown trout (*salmo trutta* L.). Cell and Tissue Research 163:535-543.

Hawkes, J.W. 1974. The structure of fish skin. I. General organization. Cell and Tissue Research 149:147-158.

Hawkes, J.W. 1974. The structure of fish skin. II. The chromatophore unit. Cell and Tissue Research 149:159-172.

Djurdjevič, I., Kreft, M.E. and Sušnik Bajec, S. 2015. Comparison of pigment cell ultrastructure and organisation in the dermis of marble trout and brown trout, and first description of erythrophore ultrastructure in salmonids. Journal of Anatomy 227:583–595.

Some ultrastructural features of elasmobranch skin were described by Meyer and Seegers (2012):

Meyer, W., and Seegers, U. 2012. Basics of skin structure and function in elasmobranchs. Journal of Fish Biology 80:1940-1967.

A few additional articles on ultrastructure of the skin of fishes from various taxa are listed below:

Damasceno, E.M., Monteiro, J.C., Duboc, L.F., Dolder, H., and Mancini, K. 2012. Morphology of the epidermis of the neotropical catfish *Pimelodella lateristriga* (Lichtenstein, 1823) with emphasis in club cells. PLoS ONE 7(11): e50255. doi:10.1371/journal.pone.0050255.

Faílde, L.D., Bermúdez, R., Vigliano, F. Coscelli, G.A., and Quiroga, M.I. 2014. Morphological, immunohistochemical and ultrastructural characterization of the skin of turbot (*Psetta maxima* L.). Tissue and Cell 46:334-342.

Lanzing, W.J.R., and Wright, R.G. 1974. The ultrastructure of the skin of *Tilapia mossambica* (Peters). Cell and Tissue Research 154:251-264.

Ottesen, O.H., and Olafsen, J.A. 1997. Ontogenic development and composition of the mucous cells and the occurrence of saccular cells in the epidermis of Atlantic halibut. Journal of Fish Biology 50:620-633.

Elliott (2000b and 2011b; listed at the end of this document) also cited a number of references that describe the ultrastructure of cells and structures in the skin of fishes from various taxa.

***Sparus aurata* skin cell sizes:** I have not found extensive literature describing the sizes of normal cells in seabream skin. However, Kalogianni et al. (2011, Journal of Fish Biology 78:1152-1169) discussed general skin histology of *Sparus aurata* and the sizes of mucous goblet cells in the epidermis of ammonia-exposed and unexposed fish. Cordero et al. (2017, PLoS ONE 12(6): e0180438. https://doi.org/10.1371/journal.pone.0180438) described differences in the thickness of the epidermis sampled from dorsal and ventral regions of the skin of seabream, and the larger size (surface area, as determined by scanning electron microscopy) of surface epithelial cells from dorsal skin as compared to ventral skin. Ferrer et al. (1999, Histology and Histopathology 14:383-390) described the ultrastructure of three types of chromatophores in the skin of *Sparus aurata* but did not discuss the sizes of the cells.

10) Does the epidermal cell diversity change in salmon for adaptation to anadromy?

Several changes in salmonid skin have been reported to occur during the parr-smolt transformation, but most publications have focused on changes in the dermis, not the epidermis. In the epidermis, mucous goblet cell numbers (densities) decrease in Atlantic salmon during smoltification, with the largest changes observed at the beginning of the parr-smolt transformation period (O'Byrne-Ring et al. 2003, Journal of Fish Biology 63:1625-1630). A study of Atlantic salmon post-smolts sampled at 1 and 4 months after transfer to seawater indicated that epidermal mucous goblet cell numbers increased, and both the epidermis and the stratum compactum of the dermis increased in thickness over the course of the study, indicating gradual enhancement of skin barrier functions after seawater entry (Karlsen et al. 2018, Scientific Reports 8:9510 DOI:10.1038/s41598-018-27818-y).

Changes in the dermis during smoltification of salmonids have been more widely reported. Prominent changes including silvering of the body and development of dark fin margins. The fin margin darkening may be due to changes in photo-responsiveness of melanophores in the fins (Chen et al. 2014, Biology Open 3:1032–1036 doi:10.1242/bio.201410058), whereas the silvering is caused by the deposition of purines (guanine and hypoxanthine) directly beneath the scales and in a deeper layer (likely in the hypodermis) immediately above the skeletal muscle (Johnston and Eales 1967, Journal of the Fisheries Research Board of Canada 24:955-964). The storage of purines in the body may serve a dual purpose: to reduce energy expenditure and water loss required to excrete these compounds in the hyperosmotic environment of seawater, and to provide counter-shading camouflage characteristic of pelagic schooling fishes (Stefansson et al. 2008, Smoltification. Pages 639-681 In R.N. Finn and B.G. Kapoor (eds.) Fish Larval Physiology. Science Publishers, Enfield NH, USA).

The scales of smolts are also looser (more deciduous) in smolts than in parr. This may have no adaptive significance, but rather may be a consequence of the rapid growth rate of smolts and related to the rapid turnover of connective tissue associated with the growth process (Stefansson et al. 2008).

11) Are treatments with commercial products (e.g. EDTA) effective for protecting fish skin? Are salt treatments administered during handling or transport of fish helpful or harmful?

Commercial treatments for protecting fish skin: A recent publication by Vanderzwalmen et al. (2019, Reviews in Aquaculture 11:263-278 doi: 10.1111/raq.12239) reviewed the use of feed and water additives for live fish transport. The paper included a review of literature on dietary supplements intended to enhance the immune system and improve stress tolerance prior to transport, as well as additives used during transport or fish handling procedures to reduce stress or protect the skin by maintaining mucus integrity.

Several additives in commercial water conditioners are intended to prevent deleterious effects of external mucus disturbance, skin abrasion and scale loss, which can result in disturbed osmoregulation and infections with opportunistic pathogens (Wedemeyer 1996, Physiology of Fish in Intensive Culture Systems, Chapman & Hall, International Thompson Publishing, New York, NY, 232 p.). Polymers such as polyvinylpyrrolidone (PVP) or proprietary polymers are purported to bond temporarily to proteins on exposed integumental tissue, forming a protective coating that is displaced as epidermal healing proceeds and surface mucus is regenerated (Wedemeyer 1996). Although several reports have indicated reduction in fish mortality following the use of polymer-based water conditioners for handling or transport (see e.g. Wedemeyer 1996; Harnish et al. 2011, Reviews in Fish Biology and Fisheries 21:43-49; Vanderzwalmen et al. 2019), there is a paucity of peer-reviewed literature on the actual mechanisms and effectiveness of the polymers for maintaining mucus integrity and promoting skin healing (Harnish et al. 2011; Vanderzwalmen et al. 2019). Some water conditioners also contain aloe extracts of the *Aloe vera* plant that are intended to improve immune function and promote wound healing (Harnish et al. 2011; Vanderzwalmen et al. 2019). One study with matrinxã (*Brycon amazonicus*) showed that *Aloe vera* powder dissolved in transport water at doses up to 2 mg/L resulted in dose-dependent enhancement of leukocyte respiratory burst activity at 2 h of transport, but the effects were no longer apparent at the end of 4 h of transport (Zanuzzo et al. 2012, Revista Brasileira de Zootecnia 41:2299-2302). Though further studies of *Aloe vera* use for fish transportation are warranted (Vanderzwalmen et al. 2019), Harnish et al. (2011) cautioned that this additive could reduce water quality or oxygen levels via the addition of organic matter, and that reports of potential toxicity also need to be addressed.

Prolonged aqueous exposure to sublethal concentrations of heavy metal ions such as copper and cadmium can cause a variety of pathological changes in the epidermis and dermis of fish (see e.g. Iger et al. 1994a, Aquatic Toxicology 29:49-64; Iger et al. 1994b, Archives of Environmental Contamination and Toxicology 26:342-350). Ethylenediaminetetraacetic acid (EDTA) is included as a chelating (metal binding) agent in some commercial water conditioners to sequester metal ions in water and thereby reduce their toxicity Khangarot 1981, Current Science 50 (5):246-248; (Harnish et al. 2011). For example, addition of EDTA to water was shown to result in a marked reduction in copper toxicity to guppies (Khangarot 1981). However, prolonged aqueous exposure to EDTA may also result in reduction of tissue levels of certain trace minerals required for body functions in fish (Nicula et al. 2011, Animal Science and Biotechnologies 44(2):40-44).

A serious issue in evaluating the effectiveness of commercial products for fish handling and transportation is that very few empirical studies have been conducted and published in peer-reviewed literature (Harnish et al. 2011; Vanderzwalmen et al. 2019). Harnish et al. (2011) stressed a need for comparative studies of available water conditioners to explore their efficacy for reducing mucus loss, promoting skin healing,

and reducing infection in fish. Studies should also examine potential sublethal and lethal effects that these conditioners may have on fish.

Salt treatments administered during handling or transport: Salt (NaCl) is commonly added to water to mitigate transport stress in freshwater fishes. The stress of transport itself (in addition to surface mucus and tissue disruption) can cause changes in osmoregulation (Barton and Iwama 1991, Annual Review of Fish Diseases 1:3-26). Salt added to fresh water reduces the difference between the internal osmolality of the fish and that of the transport water and thereby reduces the physiological workload required for the fish to maintain homeostasis (Nikinmaa et al.1983, Aquaculture 34:93-99). In addition, Tacchi et al. (2015, Aquaculture 435:120-127) reported differences in skin mucus and surface bacteria between rainbow trout (*Oncorhynchus mykiss*) transported for 5 h in water to which salt was added (5 g NaCl/L) compared to fish transported for 5 h in fresh water. Scanning electron microscopic analysis suggested that transportation in salt-added water reduced the discharge of mucus from surface goblet cells. Further analyses indicated a ~10-fold increase in skin-associated bacteria in the group transported in salt-added water compared to a ~50-fold increase in bacteria in the group transported in fresh water, and the skin mucus of the saltwater transport group also showed significantly higher ability to inhibit growth of *Vibrio anguillarum in vitro.*

Although the addition of NaCl to transport water appears to be clearly beneficial for some fish species, different species inhabit a range of salinities and exhibit differences in osmoregulatory capacity from stenohaline to euryhaline. Therefore, the value of NaCl as a water additive for transportation will always be very dependent on the fish species and life stage (Vanderzwalmen et al. 2019). A table of examples of studies that have investigated the effects of NaCl addition to water during transport is included in Vanderzwalmen et al. (2019).

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