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Author(s)	Kudo, Hideaki; Shinto, Masakazu; Sakurai, Yasunori et al.
Citation	Chemical Senses, 34(7), 617-624 https://doi.org/10.1093/chemse/bjp042
Issue Date	2009-07-08
Doc URL	https://hdl.handle.net/2115/43306
Rights	This is a pre-copy-editing, author-produced PDF of an article accepted for publication in Chemical Senses following peer review. The definitive publisher-authenticated version in Chemical Senses 2009 34(7):617-624 is available online at: http://chemse.oxfordjournals.org/cgi/content/short/34/7/617 .
Type	journal article
File Information	Kudo_CS_HUSCAP.pdf



Morphometry of olfactory lamellae and olfactory receptor neurons during the life history of chum salmon (*Oncorhynchus keta*)

Hideaki Kudo¹, Masakazu Shinto¹, Yasunori Sakurai², and Masahide Kaeriyama¹

¹Laboratory of Strategic Studies on Marine Bioresource Conservation and Management and

²Laboratory of Marine Ecology, Faculty of Fisheries Sciences, Hokkaido University,

Hakodate, Japan

Correspondence to be sent to: Hideaki Kudo, Faculty of Fisheries Sciences, Hokkaido

University, 3-1-1, Minato-cho, Hakodate 041-8611, Japan

E-mail: hidea-k@fish.hokudai.ac.jp

Telephone and Fax: +81-138-40-5602

Abstract

It is generally accepted that anadromous Pacific salmon (genus *Oncorhynchus*) imprint to odorants in their natal streams during their seaward migration, and use olfaction to identify these during their homeward migration. Despite the importance of the olfactory organ during olfactory imprinting, the development of this structure is not well understood in Pacific salmon. Olfactory cues from the environment are relayed to the brain by the olfactory receptor neurons (ORNs) in the olfactory organ. Thus, we analyzed morphometric changes in olfactory lamellae of the peripheral olfactory organ and in the quantity of olfactory receptor neurons (ORNs) during life history from alevin to mature in chum salmon (*O. keta*). The number of lamellae increased markedly during early development, reached 18 lamellae per unilateral peripheral olfactory organ in young salmon with a 200 mm in body size, and maintained this lamellar complement after young period. The number of ORNs per olfactory organ was about 180,000 and 14.2 million cells in fry and mature salmon, respectively. The relationship between the body size (fork length) and number of ORNs therefore revealed an allometric association. Our results represent the first quantitative analysis of the number of ORNs in Pacific salmon, and suggest that the number of ORNs is synchronized with the fork length throughout its life history.

Key words: salmon, olfactory receptor neuron, olfactory lamella, development, migration.

Introduction

Fish olfaction plays a prominent role in feeding (Valentinčič 2005), migration (Hasler et al. 1978), social interaction such as schooling (Hemmings 1966) and kin discrimination (Rajakaruna et al. 2006), and reproduction (Olsén and Liley 1993). In particular, the homing migration of anadromous Pacific salmon (Genus *Oncorhynchus*) to the natal stream is one of the most interesting phenomena in fish biology. It is generally accepted that salmon imprint to odorants of their natal streams on downstream migration (Stabell 1992), and use olfaction to identify these streams during spawning migration (Wisby and Hasler 1954; Ueda et al. 1998; Shoji et al. 2000). Despite the importance of the olfactory organ for olfactory imprinting, the neurological and molecular biological mechanisms of these phenomena are not well understood. In some teleost fish, the peripheral olfactory organ, known as the olfactory rosette, is composed of olfactory lamellae covered with olfactory epithelia (Zeiske et al. 1992; Hansen and Zielinski 2005). As in other vertebrates, teleost olfactory receptor neurons (ORNs) relay olfactory information to the brain (Satou 1992). Previous reports of Pacific salmon have described the number of olfactory lamellae (several species: Pfeiffer 1963, rainbow trout, *O. mykiss*: Halama 1982), as well as the ultrastructure of the olfactory organ in chum and masu salmon (*O. keta* and *O. masou*: Yamamoto and Ueda 1977). However, these reports were from a comparative morphological viewpoint and included mature phase or few developmental life stages and body sizes. Few attempts have been made to investigate the developmental processes of the olfactory organ throughout the life history of salmon (Kalinina et al. 2005). Densities of ORNs per unit surface area (1 mm²) of the olfactory epithelium were 158, 376, and 24 thousand neurons in chum salmon, masu salmon, and Arctic charr

(*Salvelinus alpinus*: not Pacific salmon), respectively (Kalinina et al. 2005; Thommensen 1983). Furthermore, the number of ORNs in the olfactory organ is unknown even in mature fish of any salmon species.

In the present study, we quantified olfactory lamellae and estimated the numbers of ORNs in chum salmon from the alevin to mature in order to clarify the developmental processes of olfactory organs throughout the life cycle of Pacific salmon.

Materials and methods

Animals

The morphology of the chum salmon olfactory organs was examined during eight different developmental periods of its life history: i) alevin, ii) fry, iii) pre-fingerling, iv) post-fingerling, v) young, vi) immature, vii) maturing, and viii) mature. The classification of alevin to young phases was based on Kaeriyama (1986). Gonadal development was an index of maturity after the immature period. Alevins were obtained by rearing in an indoor freshwater glass tank (60 L) at 12°C from fertilized eggs (eyed eggs). Fry and pre-fingerlings were collected in freshwater using an electronic shocker (Electrofisher LR-24; Smith-Root, Vancouver, WA, USA) from the Yurappu River, southern Hokkaido, Japan and were provided by the Yurappu Salmon Hatchery, Hokkaido Salmon Propagation Association, from April to May 2006. Post-fingerlings were collected using landing nets in the coastal waters off Shiretoko Peninsula from June to July 2006 aboard the training ship (T/S) *Ushio-Maru*, Hokkaido University. Salmon of life stages not obtained by sampling were derived from the above-mentioned rearing. Young were obtained by rearing in indoor seawater fiber-reinforced plastic tanks (500 L) at 12°C from the above fry period from April 2007 to July 2008. Immature high-sea salmon were collected by angling in the Bering Sea (33°N, 175°W, 33° and 34°N, 180°W) from August to September 2008 aboard the research vessel *Hokko-Maru*, Fisheries Research Agency. Maturing salmon were collected by angling in the coastal waters off Shiretoko Peninsula in September 2007 aboard the T/S *Ushio-Maru*. Mature spawning salmon were collected by hand from the above river in December 2007. Body size (fork length) data of the chum salmon are summarized in Table

1.

Fish were anesthetized with 2-phenoxyethanol (200 mg/L), and the olfactory rosette, including the olfactory lamella, and olfactory bulb, including the olfactory nerve bundle, were surgically isolated.

Morphometric procedures

To count the number of olfactory lamellae per olfactory rosette, rosettes were fixed by immersion with 40 g/L paraformaldehyde (PFA) in 0.1 mol/L phosphate buffer (pH 7.2; PB) for 72 h at 4°C, and then dehydrated with 70% ethanol in double distilled water (DDW). These samples were observed using a binocular dissecting microscope (SMZ1500; Nikon, Tokyo Japan).

To estimate the number of ORNs per olfactory rosette, olfactory nerve bundles including the olfactory bulb were fixed by immersion in a mixture of 20 g/L PFA and 25 g/L glutaraldehyde in 0.1 M PB for 16 h at 4°C. They were then postfixed in a solution of 10 g/L osmium tetroxide in the same buffer for 2 h at 4°C before being dehydrated in a graded series of acetone and embedded in epoxy resin. Frontal semi-thin sections of posterior olfactory nerve bundles proximal to the olfactory bulb were prepared on an ultramicrotome (Ultracut N, Reichert-Nissei, Tokyo, Japan) and stained with a mixture of 5 g/L methylene blue, 5 g/L azure-II, and 5 g/L borax in DDW. We measured the area of the frontal section in each olfactory nerve bundle (area of olfactory nerve bundle: AB, μm^2) using a microscope (Eclipse 80i, Nikon, Tokyo, Japan) equipped with a digital camera (EOS 5D, Canon, Tokyo, Japan) and ImageJ 1.36b software (NIH, USA). In this study, non-axonal areas (consisting of blood vessels, fibrocytes, endoneurium, and olfactory ensheathing cells) were removed

from the above areas. After the measurement of AB, ultra-thin sections were prepared on the above ultramicrotome using the same samples and stained with saturated uranyl acetate and lead citrate. Numerical assessments of axon densities were performed using the modified method of Kreutzberg and Gross (1977). They were then examined over ten frames from each sample using a H-7000 electron microscope (Hitachi, Tokyo, Japan), and we quantified the number of olfactory nerve axons (number of axons in electron micrograph: NE) in three randomly chosen frames from each sample (area of electron micrograph: AE, 70-110 μm^2) using electron micrographs (mag. x 5000). Total numbers of olfactory nerve axons (NA) per olfactory rosette were calculated by:

$$\text{NA} = \text{AB} \times (\text{NE} / \text{AE})$$

In the present study, the number of ORNs per olfactory rosette was determined based on NA because each ORN projected a single axon to the olfactory bulb.

Statistical analysis

All data are expressed as means \pm SEM. Differences between data were assessed using the Mann Whitney *U*-test. Differences among data were analyzed using the Kruskal-Wallis test, and, subsequently, Dunn's multiple range tests for combinations of two data sets. Statistical analysis was performed using Prism 4.0a (GraphPad Software, San Diego, CA, USA). A *P*-value < 0.01 denoted a significant difference.

Results

Development of the olfactory rosette

Varying numbers of lamellae radiated from a rostro-caudally oriented median depression of the olfactory rosette. After the fingerling period, the caudal lamellae was the most developed, and the lamellae decreased in size from caudal to rostral (Figure 1). After the young period, each lamella showed a concave and convex margin. Both flat sides of lamellae were thrown into secondary folds, which ran parallel to one another from the concave to convex margin (Figure 1D).

The mean number of olfactory lamellae per rosette increased markedly from 3.6 ± 0.2 lamellae during the alevin stage to 11.8 ± 0.15 in the post-fingerling stage. After the young period, the number of lamellae remained at approximately 18, and did not differ significantly ($p > 0.1$; Figure 2). The relationship between body size (fork length) and the number of olfactory lamellae per olfactory rosette is shown in Figure 3. The number of lamellae increased until about 200 mm in body size was reached during the early developmental stage. After the salmon reached a body length of 200 mm, the meant number of olfactory lamellae reached a plateau at 18 (16-21 lamellae). No significant differences between males (17.8 ± 0.53 , $n=10$) and females (17.5 ± 0.39 , $n=13$) were detected in lamellae numbers after the immature period ($p > 0.1$).

Histology of the olfactory nerve bundles

The area of the olfactory nerve bundles was viewed in frontal sections by light microscopy (summarized in Table 2). Axons of the ORNs predominated in these frontal sections of

olfactory nerve bundles (Figure 4). Connective tissues and olfactory ensheathing cells were localized in the nerve bundles. Blood vessels over 50 μm in diameter and a developing network of connective tissues appeared after the immature period. By electron microscopy, mitochondria and neurotubules were often observed in the axons. The turnover of ORNs may have contributed to the degenerated appearance of a small number of axons. The axonal diameter (excluding mitochondria) ranged from 80 to 200 nm (Figure 5). There was no significant density in axonal density (number of axons per one μm^2 ; summarized in Table 2) throughout the eight life history stages.

Total ORN counts during the life history

The numbers of ORNs present during each developmental period was evaluated by counting axonal profiles in olfactory nerve bundles (shown in Figure 6). Changes in the numbers of ORNs revealed significant differences throughout the life history ($p < 0.001$), although there were no significant differences among the immature, maturing, and mature periods ($p > 0.05$). No significant differences between males ($15.8 \times 10^6 \pm 1.34 \times 10^6$ neurons, $n=9$) and females ($15.4 \times 10^6 \pm 1.39 \times 10^6$ neurons, $n=10$) were detected in ORN numbers after the immature period ($p > 0.5$). A comparison of the numbers of ORNs per unilateral olfactory organ in chum salmon, other teleosts, and several mammals is summarized in Table 3. Analysis of the relationship between logarithmic values for body size (fork length) and the number of ORNs per olfactory rosette (shown in Figure 7), revealed that the number of ORNs increased with advancing fork length. Relationships between the number of ORNs and fork length under 250 mm ($n=111$, $r^2=0.95$, $F=989$, $P < 0.001$), and between the number of ORNs and fork length over 300 mm ($n=28$, $r^2=0.20$, $F=6.33$, $P < 0.05$) followed the allometric equations of: In

(number of ORNs) = $2.02 \times \ln(\text{fork length}) + 4.34$, and $\ln(\text{number of ORNs}) = 0.57 \times \ln(\text{fork length}) + 12.81$, respectively. An inflection point was indicated at 342 mm in fork length and 10.1×10^6 neurons using both allometric formulae (Figure 7).

Discussion

Previous studies of chum salmon indicated that the numbers of olfactory lamellae in alevin, juvenile (fry+fingerling), and mature salmon were 3-4, 11, and 18, respectively (Kalinina et al. 2005; Yamamoto and Ueda 1977), and that of young salmon was also 18 (Pfeiffer 1963).

The present results were consistent with these values, and provide a detailed profile of the increasing numbers of lamellae during the salmon's life history. The present developmental profile of olfactory lamellae was similar to that in Arctic charr (another salmonid; Olsén 1993), although the number of olfactory lamellae differed between these two species when the lamellar count reached a plateau during the young phase. The present observations suggest that newly generated lamellae sprouted from the rostral-basal part of the olfactory rosette during early life period. Most fry chum salmon start seaward migration (Kaeriyama 1986; Salo 1991). This period is important for imprinting to its natal stream, although the mechanisms responsible for imprinting remain unknown. There are five lamellae present in the olfactory organ during the fry stage, when the chum salmon are imprinting to the odour of the natal stream. These five lamellae, which contact the natal stream, are also present during seaward migration during the fry stage. In other words, of the approximately 18 lamellae in the olfactory organ in homing mature salmon that are able to discriminate their natal stream; only 5 caudal lamellae contact the natal stream water during imprinting.

While the number of olfactory lamellae didn't change at about 18 lamellae, the number of ORNs increased after the young period in the present study. Previous reports indicated that the density of ORNs per unit surface area didn't change markedly in the olfactory epithelium of chum salmon (Kalinina et al. 2005). The formation of secondary folds of the olfactory lamellae contributed to the increase in the surface area. However, the convex area of

secondary folds did not contain sensory epithelia including the ORNs (Yamamoto and Ueda 1977; Olsén 1993). These results suggested that the increase in the number of ORNs reflected the increase in the surface area of sensory epithelia (i.e., the olfactory epithelia) with the growth of olfactory rosettes. Secondary lamellae may be related to the maintenance of the space and water current in the contiguous gap between each lamella by the preclusion of lamellar adhesion.

The number of ORNs in mature chum salmon was larger than that in other teleosts (Gemme and Døving 1969; Kreutzberg and Gross 1977; Yamamoto 1982) and humans (Moran et al. 1982), although fewer than in mammals with a highly sensitive olfactory system such as the dog (Menco 1980), rabbit (Allison and Warwick 1949), and rat (Andres 1965). The olfactory organ in chum salmon may be more developed than that other teleosts, although their body sizes differ in above reports. Large numbers of axons projecting to the brain indicated that the number of synapses increased in the brain of mature chum salmon. Hence, the central olfactory nervous system of chum salmon may be more developed in comparison with other teleosts. On the other hand, it became clear that salmon could imprint to their natal stream using a small number of ORNs in the fry period before seaward migration. This simple neural network may carry out imprinting of the natal stream in chum salmon fry. During spawning migration, a markedly developed olfactory organ may be necessary for discriminating the natal stream, as well for responding to reproductive pheromones (e.g., Yambe et al. 2006). The olfactory nerve bundles contained very low numbers of efferent axons such as the terminal nerves (salmon-type gonadotropin-releasing hormone; Kudo et al. 1994; 1996). It seems reasonable to suggest that the number of axons in the olfactory nerve bundle reflected the number of ORNs because the peripherally projecting axons were very

scarce. The peak value for ORN abundance occurred during the maturing stage in chum salmon that were captured in coastal waters, although this value was not significantly different from the number of ORNs seen in mature salmon during the homing migration. In fact, the body size range in maturing was larger than that in mature salmon in the present study.

Based on the *in vivo* uptake of 5-bromo-2'-deoxyuridine, as in mammals, sockeye salmon (*O. nerka*) ORNs undergo repeated cell turnover, and differentiate from olfactory epithelial basal cells (Yanagi et al. 2004), and cell proliferation in the olfactory epithelia was activated in the parr-smolt transformation period (i.e., just before seaward migration) in coho salmon (*O. kisutch*; Lema and Nevitt 2004). However, the present study failed to reveal a significant ORN increase during seaward migration between the fry and pre-fingerling periods. The increase in cell proliferation, observed by Lema and Nevitt (2004) may have reflected changes in cell death and proliferation during ORN turnover taking place shortly before the chum salmon's seaward migration. Our results showed that the number of ORNs was synchronized with the body size in chum salmon throughout the life history. The relationship between the number of ORNs and body size shifted from positive to negative allometric growth at an inflection point (342 mm in fork length, 10.1×10^6 neurons). This body size may be related to the phase shift from growth to sexual maturation in chum salmon.

In Pacific salmon, two morphologically distinct ORN types, ciliated and microvillous ORNs, exist according to the ultrastructure of the olfactory knob in the dendrites of ORNs (Yamamoto and Ueda 1977). Recently, crypt ORNs were observed in brown trout (*Salmo trutta*; Castro et al. 2008). However, the present conventional ultrastructural observation failed to distinguish between these ORN types. Although functional information on these

ORN types in salmon is scarce (e.g., Sato and Suzuki 2001), further cytophysiological analysis is required to assess the cell proportion of each ORN type in the olfactory epithelium during the salmon's life history,

Several researchers reported biochemical and molecular biological investigations regarding the expression of some olfactory-related molecules in the olfactory organs of Pacific salmon (e.g., Dittman et al. 1997; Hino et al. 2008; Kudo et al. 1999; Shimizu et al. 1993). However, these quantitative or semi-quantitative analyses of expression were carried out without taking the number of ORNs in the homogenized samples of olfactory organs into consideration. Knowing the number of ORNs that are present during the specific life stages is useful for calculating expression levels of specific molecular probes on a per ORN basis.

In summary, we provided details of olfactory lamella formation and the first quantitative analysis of the number of ORNs in Pacific salmon, and suggested that the number of ORNs is synchronized with body size throughout its life history. This basic information will be a useful tool for investigating mechanism responsible for homing migration in salmon.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science (JSPS; No. 2058018808), Grant-in-Aid for Scientific Research (A) from the JSPS (No. 18201006), and by the Akiyama Life Science Foundation Research Grant 2007. We thank the Hokkaido Salmon Propagation Association and Dr. Mitsuhiro Nagata, Hokkaido Fish Hatchery, for providing juveniles and fertilized eggs of chum salmon.

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Table 1. Body size, approximate age, and sampling site of each developmental period of chum salmon in the present study.

Developmental period	Fork length (mm)	Body weight (g)	Age	Number of samples	Sampling site or source
Alevin	31.3±0.63	2.58±0.07	1-2 months	28	Freshwater rearing in laboratory
Fry	43.4±0.40	5.58±0.16	3-5 months	118	Yurappu River
Pre-fingerling	67.6±3.79	2.23±1.09	4-5 months	127	Yurappu River
Post-fingerling	97.3±1.40	8.33±0.38	5-6 months	80	Coastal waters off Shiretoko Peninsula
Young	180±2.99	52.7±2.60	1 year	54	Seawater rearing in laboratory
Immature	476±12.0	1306±105.1	3-4 years	18	Bering Sea
Maturing	616±28.9	2921±367.3	3-5 years	14	Coastal waters off Shiretoko Peninsula
Mature	695±14.3	3494±292.5	3-5 years	13	Yurappu River

Table 2. Morphometric parameters for the estimation of the number of ORNs in each developmental period of chum salmon in the present study.

Developmental period	Number of individuals	Area of the olfactory nerve bundle (μm^2)	Density of axons per μm^2 in the olfactory nerve bundle
Alevin	9	$9.40 \times 10^3 \pm 0.81 \times 10^3$	11.8
Fry	32	$14.0 \times 10^3 \pm 0.91 \times 10^3$	12.6
Pre-fingerling	28	$24 \times 10^3 \pm 1.6 \times 10^3$	13.2
Post-fingerling	16	$40 \times 10^3 \pm 3.7 \times 10^3$	12.0
Young	26	$25 \times 10^4 \pm 1.4 \times 10^4$	13.7
Immature	10	$92 \times 10^4 \pm 5.8 \times 10^4$	11.3
Maturing	7	$1.28 \times 10^6 \pm 0.09 \times 10^6$	14.5
Mature	13	$1.03 \times 10^6 \pm 0.06 \times 10^6$	12.7

Table 3. Comparison of known numbers of ORNs per olfactory organ on one side among chum salmon, other teleosts, and several mammals.

Species	Number of ORNs (million neurons)	Reference	Remarks
Dog (beagle)	200	Menco 1980	
Pig	34	Gasser 1956	
Rat	26	Andres 1965	
Rabbit	25	Allison & Warwick 1949	
Human	3	Moran et al. 1982	
Chum salmon (mature)	14.2	Present study	BL ^a : 690 mm
Chum salmon (fry)	0.18	Present study	BL: 44 mm
Pike (<i>Esox lucius</i>)	5.1	Kreutzberg & Gross 1977	BL: 800 mm
Burbot (<i>Lota lota</i>)	6-9	Gemme & Døving 1969	BL: 300 mm
Medaka (<i>Oryzias latipes</i>)	0.04	Yamamoto 1982	BL: 30 mm

^aBody length

Figure legends

Figure 1. External morphological development of olfactory rosettes and lamellae in chum salmon using a dissecting microscope. After the fingerling period, the caudal lamellae developed the most (seen at the top in Figures 1A-C), and the lamellae decreased in size from caudal to rostral. During the maturing stage, there were secondary folds on the olfactory lamellae. A. fry (fork length [FL]= 44.7 mm), B. pre-fingerling (FL=57.1 mm), C. maturing (FL=837 mm), D. single lamella shown in the rectangle in Figure 1C. Arrowheads indicate the rostral part of the raphae in the olfactory rosettes. Arrows indicate the secondary folds in the olfactory rosettes in Figure 1D. Bars: 500 μ m.

Figure 2. Changes in the number of olfactory lamellae on olfactory rosettes during the life history of chum salmon. The mean number of olfactory lamellae per olfactory rosette increased markedly until the post-fingerling period. There was no significant difference in the number of lamellae during the young, immature, maturing and mature stages, when the lamellar number remained at approximately 18 ($p>0.1$). Data represent the mean \pm SEM values. n: number of samples.

Figure 3. Relationship between the body size (fork length) and number of olfactory lamellae per olfactory rosette in chum salmon. There was a steep increase in the number of lamellae during the early life stages, when the chum salmon were shorter than 200 mm (fork length). The number almost reached a plateau at 18 lamellae after 200 mm in body size.

Figure 4. Photomicrographs of the frontal semi-ultrathin sections through the posterior olfactory nerve bundles proximal to olfactory bulb in chum salmon stained with methylene blue-azure II. These frontal sections of the olfactory nerve were

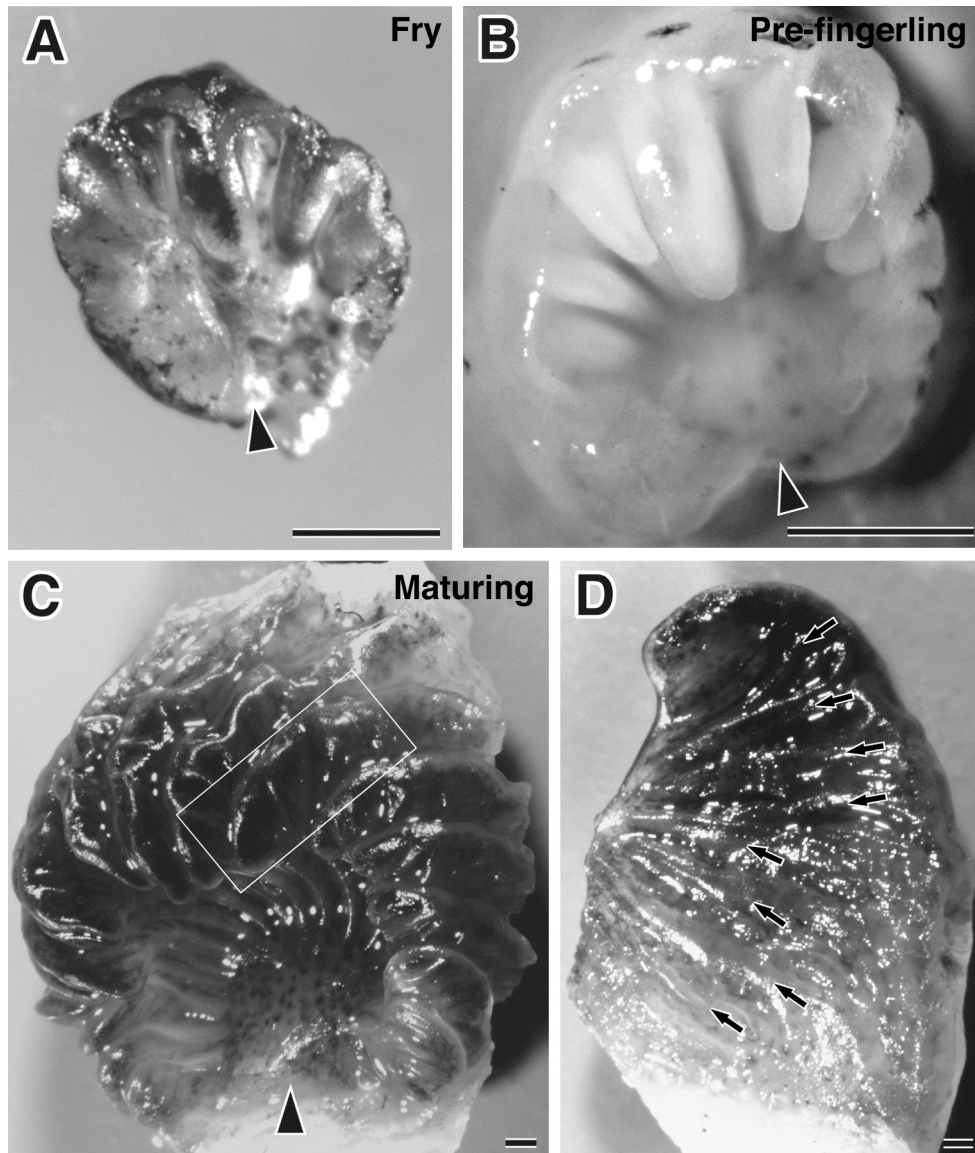
predominantly filled with ORN axons. Blood vessels over 50 μm in diameter (arrowheads) and the development of a network of connective tissues appeared from the immature period. A. fry, B. post-fingerling, C. young, D. immature, E. maturing. Bars: 100 μm .

Figure 5. Electron micrographs of the frontal ultra-thin sections through the olfactory nerve bundles in immature chum. *Inset* shows the micrograph at high magnification.

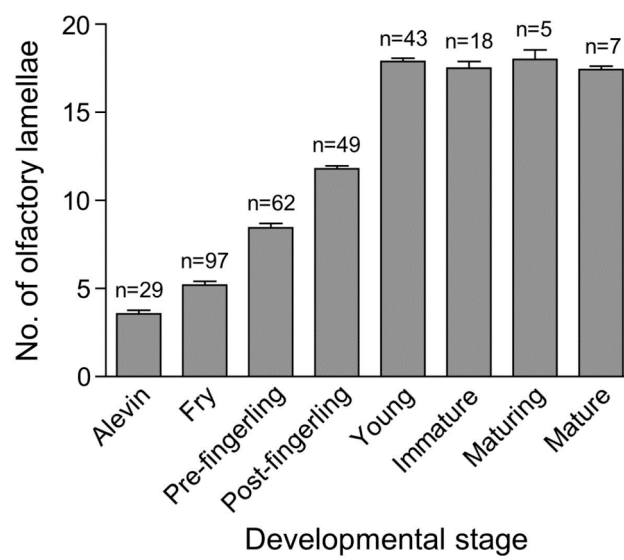
Mitochondria (M) and neurotubules (arrowheads) were often observed in the axons. A small number of axons degenerated due to the turnover of ORNs. Cross sections of the axons, which do not include mitochondria, were 80-200 nm in diameter. Bars: 200 nm.

Figure 6. Change in the number of ORNs per olfactory rosette in chum salmon during their life history. Numbers of ORNs in each developmental period were evaluated based on the number of axons in olfactory nerve bundles. Changes in the numbers of ORNs indicated significant differences throughout the life history ($p < 0.001$), although they showed no significant differences among the immature, maturing, and mature periods ($p > 0.05$). Data represent the mean \pm SEM values. n: number of samples.

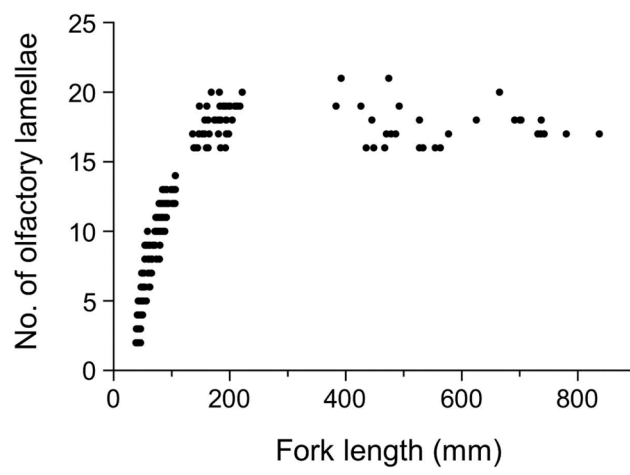
Figure 7. Relationships using logarithmic values between the body size (fork length) and number of ORNs per olfactory rosette in chum salmon. The number of ORNs increased with advancing fork length. Lines represent the allometric formula (solid line, under 250 mm in fork length: $n=111$, $r^2=0.95$, $F=989$, $P < 0.001$; dashed line, over 300 mm in fork length: $n=28$, $r^2=0.20$, $F=6.33$, $P < 0.05$). An inflection point was indicated at 342 mm in fork length and 10.1×10^6 neurons using both allometric formulae.



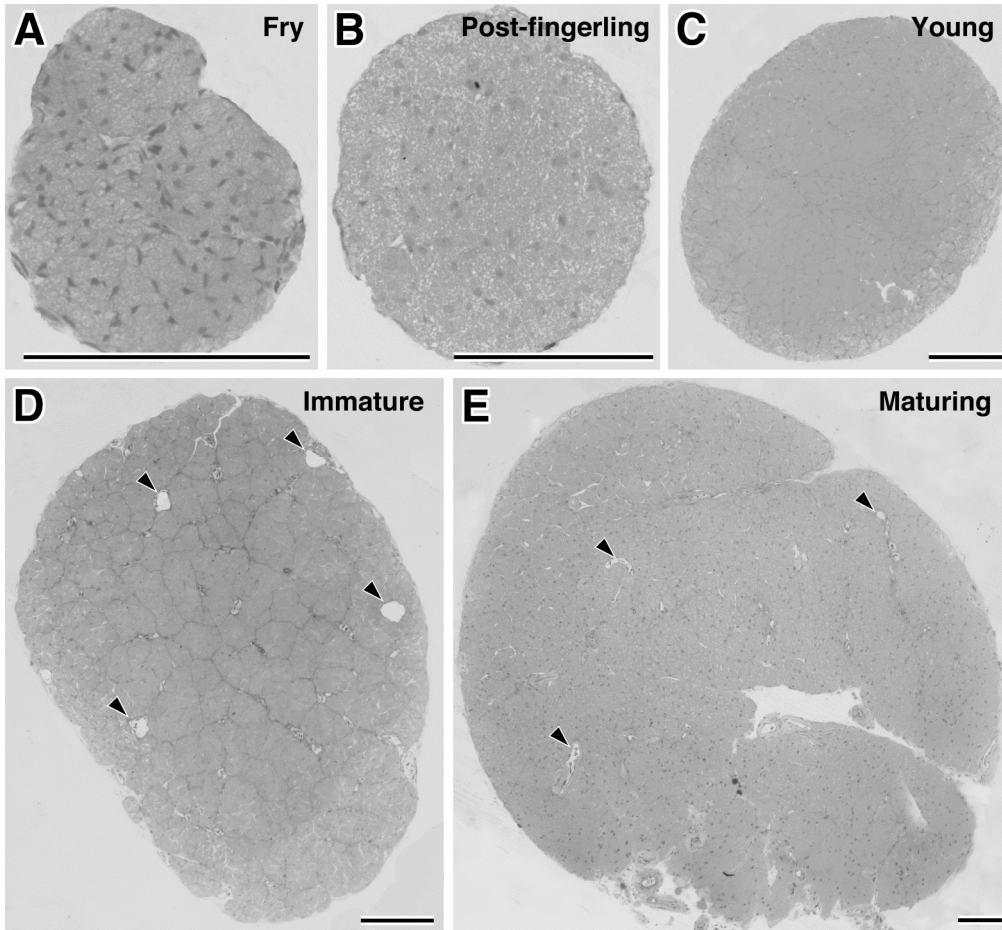
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Figure 1**



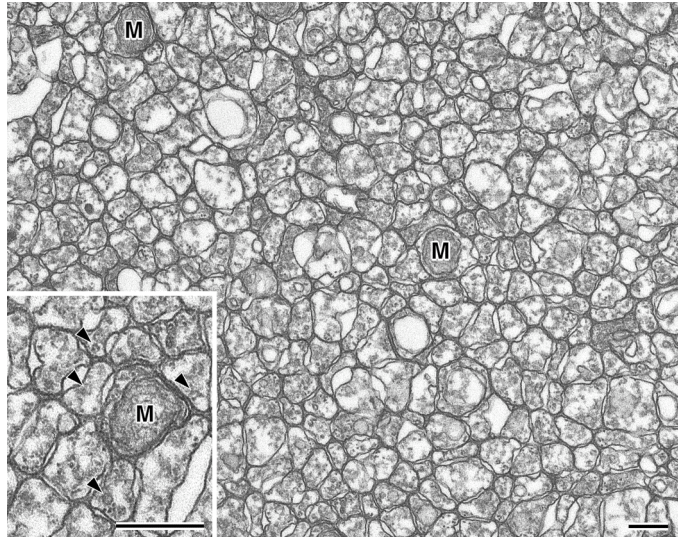
Kudo et al.
Figure 2



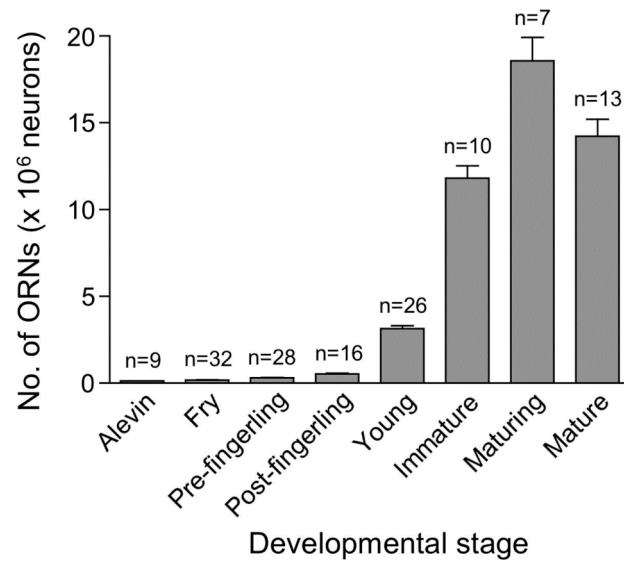
Kudo et al.
Figure 3



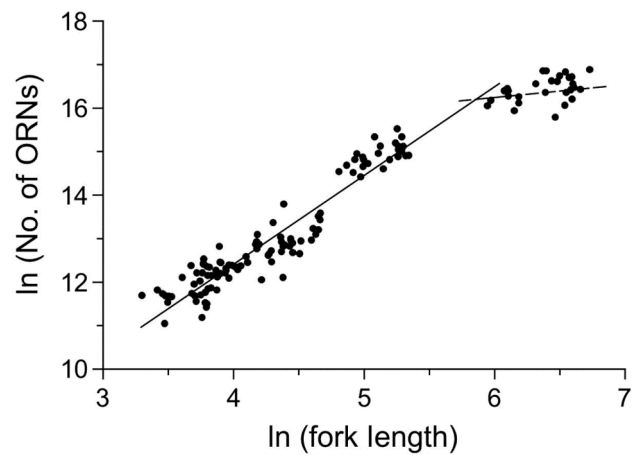
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Figure 4



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Figure 5



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Figure 6



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Figure 7