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Étude de la squelettogenèse chez la lamproie marine (*Petromyzon marinus*)

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a	arcualia
ac	annular cartilage
ad	anterior dorsal cartilage
an	annular cartilage
al	anterior lateral cartilage
ap	apical cartilage
apl	apical lateral cartilage
B	branchial region
ba	branchial arch
Bi2	second order bifurcation
bp	basitrabecular process
br	branchial ring
C	caudal fin
c	chondrocyte
cc	central chondrocyte
cn	**chondrone**
co	copula

D1	first dorsal fin
D1Bi	bifurcation of the fin rays in the first dorsal fin
D1R	presence of fin rays in the first dorsal fin
D2Bi	bifurcation of the fin rays in the second dorsal fin
D2R	presence of fin rays in the second dorsal fin
D2	second dorsal fin
dmr	dorsomedian rod
ECM	extracellular matrix
ee	elastica externa
eh	extra-hyal
ELBi	bifurcation of the fin rays in the epichordal lobe of the caudal fin
ELR	presence of fin rays in the epichordal lobe of the caudal fin
ep	epitrematic bar
eppr	epitrematic protuberance
fr	fin ray
HLBi	bifurcation of the fin rays in the hypochordal lobe of the caudal fin
BLR	presence of fin rays in the hypochordal lobe of the caudal fin
hy	hypotrematic bar
hyb	hypobranchial bar
hypr	hypotrematic protuberance

inpr	inferior process
l	lacunae
lr	dorsolateral rod
lw	lateral wall of the neurocranium
mde	mediodorsal vertebral elements
MDS	multidimensionnal scaling
n	notochord
nc	nasal capsule
ns	notochordal sheath
nt	neural tube
nu	nucleus
oc	otic capsule
P	predorsal region
p	piston
pa	parachordal
pc	peripheral chondrocyte
PC1	first principal component
PC2	second principal component
pd	posterior dorsal cartilage
per	pericardial cartilage

PF	posterior fusion of fin rays
pl	posterior lateral plate
soa	subocular arch
soap	pillar of the subocular arch
st	stylet
sub	subchordal bar
supr	superior process
TL	Total length (longueur totale) en mm
TL_{50}	Longueur totale à laquelle 50 % des individus ont développé la structure d'intérêt
tr	trabecula
va	velar arch
vmr	ventromedian rod
vp	velar plate

INTRODUCTION GENERALE

Les vertébrés représentent un groupe monophylétique caractérisé par la présence d'éléments vertébraux, de cellules des crêtes neurales ainsi que de placodes neurogéniques [1-3]. Deux grands groupes caractérisent les vertébrés : (1) les agnathes, soit le groupe de chordés ne possédant pas de mâchoires, et (2) les gnathostomes, soit les animaux à mâchoires [2,4-6]. Les Pétromyzontiformes (i.e., lamproies) et les Myxiniformes (i.e., myxines) représentent les deux taxa d'agnathes encore vivants ; ceux-ci sont considérés comme des vertébrés basaux car ils ne possèdent pas de squelette minéralisé, ni de canaux et de sillons sensoriels [2]. Les agnathes ont de nombreuses caractéristiques plésiomorphes : un neurocrâne simple, une notochorde comme seul support axial, un squelette entièrement cartilagineux sans aucune trace de minéralisation, une narine médiane, ainsi que l'absence de mâchoires, de nageoires et ceintures pectorales et pelviennes [7]. Les Myxiniformes ont longtemps été classés comme craniates, soit des organismes plus basaux que les vertébrés possédant un neurocrâne pour protéger le cerveau [8]. Cependant, des études récentes ont démontré la présence d'éléments vertébraux ventraux dans la région caudale d'une espèce de myxine (*Eptatretus burgeri*) [9,10].

Les hypothèses moléculaires et morphologiques concernant les relations phylogénétiques entre les Pétromyzontiformes, les Myxiniformes et les gnathostomes sont conflictuelles depuis des décennies. Malgré plusieurs publications à ce sujet, il est encore difficile de déterminer si les cyclostomes forment un groupe monophylétique basal comme le veut l'hypothèse moléculaire (Fig. 1A) [4,11-14], ou bien si les cyclostomes sont paraphylétiques par rapport aux gnathostomes comme le stipule plusieurs hypothèses morphologiques (Fig. 1B) [8,15,16]. Une hypothèse alternative est que les Myxiniformes seraient le groupe le plus proche des gnathostomes (Fig. 1C). Cependant, des études plus récentes combinant données morphologiques et moléculaires supportent la monophylie des

Cyclostomata [4,9,10,17-19]. Les relations phylogénétiques entre les lamproies, myxines et gnathostomes représentent plus que jamais un sujet chaud dans le domaine de la biologie évolutive et de la paléontologie ; l'étude du développement squelettique pourrait nous apporter de nouvelles informations qui pourraient aider à éclaircir cette question phylogénétique.

Le groupe des Pétromyzontiformes actuel incorpore 10 genres, 40 espèces, dont 18 seulement sont parasitaires [20,21]. Les lamproies possèdent une distribution anti-tropicale [20]; 34 espèces sont localisées exclusivement dans l'hémisphère Nord (Petromyzontidae) [22], alors que les autres espèces sont présentes dans l'hémisphère Sud (Geotriidae, Mordaciidae) [23]. Il existe quelques espèces de lamproies indigènes au Québec, dont deux non-parasitaires (Lamproie du nord, *Ichthyomyzon fossor* ; Lamproie de l'est, *Lethenteron appendix*) et trois parasitaires (Lamproie brune, *Ichthyomyzon castaneus* ; Lamproie argentée, *Ichthyomyzon unicuspis*; Lamproie marine, *Petromyzon marinus*) [21]. La lamproie marine, la plus grande espèce parasitaire de lamproies, s'attaque principalement aux téléostéens [24,26], mais également aux cétacés (e.g., rorquals communs, épaulards) [27] ; des cas d'attaques sur des requins ont aussi été répertoriés [28].

En Ontario, la lamproie marine a envahi les Grands Lacs vers la fin des années 1930 [26]. Quelques années après cette invasion, les populations de touladis (*Salvelinus namaycush*) et de lottes (*Lota lota*) furent les premières à décliner [30,31], suivies des populations de truites arc-en-ciel (*Oncorhynchus mykiss*), de grands corégones (*Coregonus clupeaformis*) [32] et de catostomidés (*Catostomus* spp. et *Moxostoma* spp.) [26]. Les lamproies marines se reproduisent en grand nombre lorsqu'elles se trouvent dans un milieu favorable [26]. Cette espèce représente un bel exemple d'un prédateur parfaitement adapté à son nouvel environnement en raison de l'absence totale de prédateur ou de pression *top-down*. Plusieurs plans de gestion ont été établis afin de contrôler les populations croissantes de lamproie marine. Le TFM (3-trifluoromethyl-4-nitrophenol), un lampricide sélectif visant les ammocètes dans leur développement juste avant la métamorphose, a largement été utilisé entre 1958 et 1978 [26,33]. Cependant, les dommages liés à l'utilisation d'un tel

produit sont nombreux; en effet, le TFM affecte également les macroinvertébrés, les amphibiens, les plantes aquatiques ainsi que les ammocètes d'espèces de lamproies indigènes [34,35]. Grâce à notre étude, l'obtention d'une séquence développementale particulière à la lamproie marine pourrait nous aider à mieux caractériser et comprendre sa biologie générale et son comportement, ce qui pourrait nous permettre de cibler cette espèce de façon plus sélective lors d'ultérieures tentatives de contrôle.

La lamproie marine possède un cycle de vie avec des stades bien particuliers. L'éclosion des œufs s'effectue en eau douce pour tous les Pétromyzontiformes [25,36], soit environ deux semaines après la ponte [25,36-38]. Après les 1-3 semaines pendant lesquelles s'opère le développement des différents systèmes [25,37,38], la prolarve effectue une migration en aval loin du nid afin de trouver un substrat approprié pour s'y enfouir [25,36] ; celui-ci est normalement composé de vase ou de sable très fin [25,38]. Une fois la prolarve enterrée dans les sédiments, celle-ci entame un nouveau mode de vie sédentaire et microphage (i.e., stade ammocète). La larve ammocète possède un réseau de cirrhi oraux au niveau du capuchon oral lui permettant de filtrer les diatomées et détritus propres à sa consommation [36,39,40]. L'ammocète peut rester de 3 à 8 ans enfouie dans le substrat [25,36]. L'ammocète peut effectuer d'autres migrations occasionnelles afin de coloniser de nouveaux habitats plus favorables [25,41]. Effectivement, il a été observé que les ammocètes plus grosses semblent être capables de se déplacer sur une plus longue distance [41]. Même si cette étape de vie est caractérisée par un faible taux métabolique, les ammocètes grandissent de façon régulière au fil des années [41]. Cependant, le taux de croissance dépend énormément des ressources disponibles et des conditions environnementales [36] ; il est aussi important de spécifier que la grosseur d'une ammocète n'est pas directement liée à son âge [42,43].

Toutefois, peu de choses sont connues sur le rôle des nageoires pendant la migration en aval des ammocètes, ainsi que sur le développement des nageoires médianes elles-mêmes. Les Pétromyzontiformes possèdent une ou deux nageoires dorsales en plus d'une nageoire caudale. Il n'existe aucune nageoires paires, ni de nageoires anales *contra* les

observations effectuées par Piavis [37,38] ; cependant, deux cas de femelles adultes possédant une nageoire anale supportée par des rayons cartilagineux ont déjà été répertoriés chez *Petromyzon marinus* [44,142]. Pour les espèces possédant deux nageoires dorsales, telle que la lamproie marine, la différentiation de celles-ci se déroule uniquement lors de la métamorphose [45,46]. Chez plusieurs espèces de Pétromyzontiformes, la première dorsale est séparée de la seconde dorsale ; cependant, toutes deux restent liées par une fine membrane [21,46]. Les lamproies possèdent des éléments squelettiques dans les nageoires médianes. Ces rayons cartilagineux sont alignés parallèlement les uns aux autres ; il a été observé que les rayons de la nageoire caudale sont fusionnés proximalement (Fig. 2) [1] ; toutefois, il n'est pas connu à quel moment se produit leur développement ni comment celui-ci se déroule. Vladykov [47] a observé des ammocètes avec des rayons cartilagineux supportant les nageoires, indiquant que le développement de ces éléments se déroulerait dans un stade plus précoce.

Peu de choses sont connues au sujet de ces rayons qui ne présentent aucunes caractéristiques propres aux autres types de rayons présents chez des taxa plus dérivés (i.e., actinotriches, cératotriches, lépidotriches, camptotriches). Les actinotriches et cératotriches sont entièrement cartilagineux, tandis que les lépidotriches et les camptotriches sont ossifiés et minéralisés, respectivement [48,49]. Suite à des analyses histochimiques, il a été déterminé que les camptotriches, occupant la position classique d'un rayon dans la membrane des dipneustes et possédant une composition cellulaire différente des autres rayons connus, pouvaient être considérés comme un type de rayon à part entière [49,50]. Dans le cas des lamproies, les études histologiques et histochimiques des rayons sont inexistantes. Certains auteurs, considérant l'aspect primitif de ces rayons, ont préféré leur donner le terme de « radiaux » [51-53]. Considérant l'absence totale de documentation à ce niveau, il est difficile de déterminer si les structures cartilagineuses des nageoires des lamproies possèdent davantage les caractéristiques d'un rayon ou celles d'un radial. Notre étude permettra de déterminer quels types de structures sont présents dans les nageoires médianes de *Petromyzon marinus* ainsi que donner des informations quant à la mise en place de leur patron développemental.

La dernière année de l'ammocète est une phase préparatoire où la croissance est presque complètement arrêtée [25,29], permettant à la larve d'emmagasiner des réserves de lipides en prévision des importantes demandes énergétiques qu'engendre la métamorphose [54-56]. L'initiation de la métamorphose est provoquée par un signal transmis à l'ammocète via un ensemble de facteurs liés à l'environnement, telle que la température de l'eau [54,57]. La métamorphose peut durer plusieurs mois et implique des changements profonds au niveau de la morphologie externe (e.g., développement de yeux proéminents, différentiation des nageoires dorsales, changement dans la forme des pores branchiaux, pigmentation, taille du museau) [29,46,58] et interne (e.g., modification des systèmes digestifs, rénaux, musculo-squelettiques) [59,60]. Dans le cas des espèces non parasitaires, le système digestif deviendra entièrement non-fonctionnel; les jeunes adultes ne disposeront que de quelques mois afin de s'accoupler avant de mourir [29]. Youson et Potter [58,61] ont établi un staging de la métamorphose pour la lamproie marine, basé entièrement sur des traits morphologiques externes. Ce staging est universellement utilisé de nos jours afin de discriminer les ammocètes pendant leur métamorphose en se basant uniquement sur leur apparence externe. Cependant, un staging basé sur un autre système, tel que le système squelettique, n'a jamais été abordé dans la littérature sur la métamorphose. Notre étude sera la première à se pencher sur la présence et l'absence d'éléments squelettiques afin de discriminer des stages de métamorphose chez la lamproie.

Une fois métamorphosés, les jeunes adultes émergent des sédiments et migrent en aval dans le but de se nourrir [54]. La période entre la fin de la métamorphose et le début de l'alimentation peut durer entre 4 et 10 mois [25,41]. Les nouvelles pièces squelettiques développées pendant la métamorphose servent principalement à l'alimentation de la lamproie (i.e., squelette buccal) [62,63]. L'adulte peut s'accrocher aux téléostéens grâce au disque buccal muni de rangées de dents labiales cornées pouvant être unicuspides, bicuspides ou même multicuspides [21,39,63]. La « langue » de la lamproie est composée de pièces cartilagineuses aux rebords tranchants (i.e., laminae linguaux longitudinaux, lamina linguale transversale) supportées par le piston [53,64,65]. L'emplacement et le nombre de ces dents, ainsi que la morphologie des lamina linguaux sont communément

utilisés comme critère taxonomique chez les Pétromyzontiformes [20,21,62]. De nombreux muscles s'insèrent aux niveaux des pièces cartilagineuses buccales, permettant un mouvement du piston vers l'avant et l'arrière [64]; ainsi, la partie apicale de la langue s'accroche à la chair de l'hôte et le mouvement du piston permet de la déchirer et d'avoir accès au sang [21,29,39,64]. Le mécanisme d'alimentation de l'adulte dépend principalement de l'action combinée de plusieurs éléments squelettiques et musculaires au niveau du squelette buccal [7,64]; de ce fait, chaque élément est important pour l'alimentation de la lamproie adulte.

La notochorde est particulièrement bien développée chez les Pétromyzontiformes adultes [66,67]. Cette structure représente le seul support axial et possède un rôle de stabilisation; ces fonctions sont particulièrement importantes pour les chordés possédant un corps allongé et une nage ondulatoire [66,68]. Comparativement aux ammocètes, la notochorde des lamproies adultes est plus large et plus rétrécie antérieurement [66]. Les adultes possèdent également des paires d'éléments vertébraux cartilagineux, communément appelés arcualia. Ces éléments se développent durant la métamorphose et sont présents tout le long de la notochorde [64,66,69,70]. Les arcualia sont présents au nombre de deux paires par myomères [21,45]. Le nombre total d'arcualia varie d'une espèce à l'autre ; dans le cas de la lamproie marine (*Petromyzon marinus*), jusqu'à 130 arcualia peuvent être dénombrés [70]. À part la morphologie générale de certains arcualia, très peu d'informations sont disponibles sur ces éléments vertébraux. Alors que Strenger [71] affirme que les arcualia ne possèdent aucune utilité particulière, il a été néanmoins démontré que ces éléments servent de point d'ancrage pour les fibres de collagènes présentes au niveau du squelette axial [69] ; aucune autre fonction ne leur est connue pour l'instant. Certains émettent l'hypothèse que les arcualia seraient homologues aux arcs neuraux des gnathostomes [7,53]. Le développement des arcs neuraux et hémaux des poissons primitifs s'effectue à partir de quatre paires d'éléments vertébraux de base [2,68]. Une paire d'éléments dorsaux, appelée basidorsaux, se développe dorsalement à la notochorde et chaque élément se rejoint dorsalement au tube neural dorsal afin de produire l'arc neural [68]. Les éléments situés ventralement à la notochorde, appelés basiventraux, se joignent aussi distalement au niveau

post-abdominal afin de créer les arcs hémaux. Chaque basidorsaux sont séparés par des interdorsaux, tandis que les basiventraux sont séparés par les interventraux. Ces éléments vertébraux de base sont majoritairement présents chez les vertébrés [68]; cependant, les Pétromyzontiformes ne possèdent aucun élément vertébral ventral et intervertébral [66,70], tandis que les Myxiniformes ne possèdent pas d'éléments dorsaux, ni intervertébraux [9,10]. Il est ainsi difficile de pouvoir établir des homologies entre les éléments de ces groupes plus basaux et les véritables vertèbres présentes chez les gnathostomes. Notre étude sera la première à se pencher sur le patron développemental et la morphologie cellulaire des arcualia chez une espèce de Pétromyzontiformes ; grâce à ces informations, nous seront en mesure de produire de meilleures comparaisons avec les éléments vertébraux des gnathostomes.

Le système épidermique [72-76], uro-génital [77,78], endocrinien [79-82] et squelettique [83-89] ont été décrits pour plusieurs espèces de lamproies ; cependant, la lamproie marine (*Petromyzon marinus*) représente l'un des modèles le plus utilisé dans les études liées au développement. Kaensche [90], Parker [91], Nestler [92] et Bujor [93] sont les premiers auteurs ayant décrit l'anatomie et le développement du crâne durant l'embryogenèse chez *Petromyzon.* Le développement de la tête et du pharynx chez la lamproie a également été étudié [85,87,94,95], notamment afin de comprendre la segmentation et l'apparition de certains caractères dérivés des gnathostomes (e.g., mâchoire). De nombreuses études ont aussi décrit le développement du squelette neurocrânien [83,87,89,94,96-98] et branchial [88,97,99] des prolarves dès les premiers jours post-éclosion. Quelques études se sont aussi penchées sur le squelette en général durant la métamorphose [93,94,100,101].

La composition cellulaire et la morphologie générale du cartilage chez la lamproie a également été étudié au niveau de certains éléments neurocrâniaux et branchiaux [69,97,102-104], mais jamais au niveau post-branchial. Le cartilage est un tissu présent chez tous les vertébrés; bien que possédant une composition cellulaire très différente, des tissus cartilagineux ont été identifiés chez plusieurs groupes d'invertébrés (e.g., Cnidaria,

Polychaeta, Mollusca, Arthropoda) et de chordés (e.g., Cephalochordata) [105-107]. Le cartilage des vertébrés apparait dans les stades précoces du développement (i.e., chondrogenèse) ; celui-ci sera éventuellement ossifié suite au processus d'ossification endochordale, se minéralisera ou bien persistera à l'âge adulte en possédant une fonction de support squelettique [108]. Dans le cas des Pétromyzontiformes et des Myxiniformes, le squelette restera cartilagineux toute leur vie [7,64]. Le cartilage est formé de chondrocytes encastrés dans une matrice extracellulaire. La matrice extracellulaire est composée de fibres de collagène fibrillaire (e.g., type I, II, V) ou non-fibrillaire, ainsi que de protéoglycans (i.e., mucopolysaccharides) [107]. Il a été démontré que le collagène de type II, encodé par le gène Col1A2, est la protéine la plus présente dans le cartilage des vertébrés supérieurs [109]. Le cartilage hyalin est le type ayant été le plus étudié ; en effet, les études sur les caractéristiques histologiques et histochimiques sont moins abondantes au niveau des autres types de cartilage (e.g., fibreux, élastique). Notre étude se penchera sur la caractérisation de la morphologie cellulaire du cartilage des éléments squelettiques postcrâniens (i.e., rayons, arcualia) ; cette étude sera la première à notre connaissance à traiter en détails de ces éléments. De telles informations sont importantes à acquérir sur des groupes plus basaux, où le squelette cartilagineux persiste tout le long de la vie, afin de comparer avec les autres types de cartilages retrouvés chez les gnathostomes.

Les études sur la squelettogenèse sont nombreuses chez les ostéichthyens. Ces études relèvent majoritairement du domaine de la biologie évolutive du développement (évo-dévo) et nous donnent des informations cruciales permettant de comprendre le caractère évolutif de certaines conditions développementales [110-119]. Dans un contexte morphologique, l'étude de la squelettogenèse aide à comprendre les causes des malformations ostéologiques chez les poissons d'intérêt économique (e.g., espèce menacée, d'élevage) [120-130].

Aucune étude publiée à ce jour n'a documenté la squelettogenèse durant plusieurs stades de vie consécutifs chez *Petromyzon marinus*. De plus, aucune information sur la séquence développementale des arcualia et des éléments squelettiques des nageoires médianes n'est disponible. Le type de cartilage présent dans ces structures n'a également

jamais été documenté, contrairement aux autres éléments squelettiques localisés au niveau du squelette neurocrânien, branchial et buccal [60,102,131,132]. En analysant la morphologie générale et la structure cellulaire de ces structures pendant leur développement, il serait possible d'effectuer de meilleures comparaisons et établir des homologies avec des structures présentes chez les gnathostomes (e.g., arc neuraux, lépidotriches).

L'objectif principal de la présente étude est de décrire la squelettogenèse au niveau du squelette neurocrânien, buccal, branchial, axial et appendiculaire pour trois différents stades de vie de *Petromyzon marinus* (i.e., ammocète, ammocète en métamorphose, adulte). Cette étude a également pour but d'investiguer le développement, la morphologie générale et la composition cellulaire des éléments postcrâniens, soit les arcualia (i.e., éléments vertébraux dorsaux) et les rayons des nageoires médianes.

La lamproie marine (*P. marinus*) représente un bon sujet de recherche vu l'abondance de littérature disponible sur le développement et l'embryologie de celle-ci. Nous possédons un bon échantillonnage de spécimens appartenant à trois stades de vies (ammocète, ammocète en métamorphose, adulte) qui nous a été prêté d'une collection muséale (CMNFI, Gatineau). Cet échantillonnage contient des populations provenant de l'Ontario (Bassin du Lac Huron) et du Québec (Rivière Sainte-Anne). Des observations *in toto* (i.e., « cleared-and-stained ») et histologiques seront produites sur les spécimens afin d'obtenir des informations sur la mise en place du patron de développement, ainsi que sur la morphologie cellulaire des arcualia et des rayons des nageoires médianes.

Figures

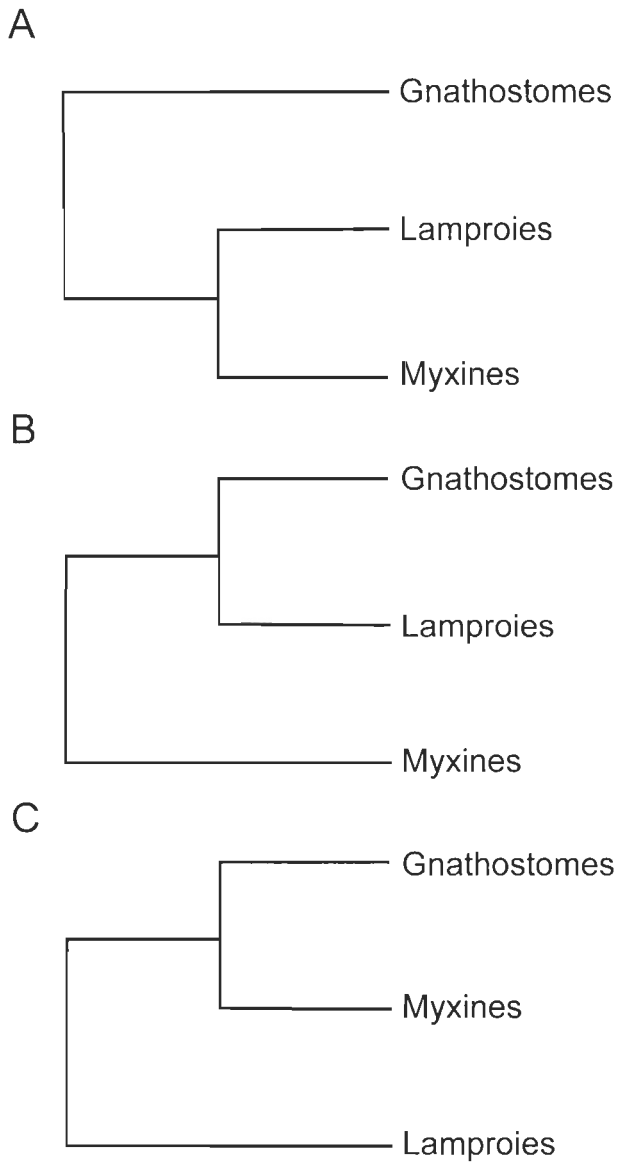

Figure introductive 1. Hypothèses des relations phylogénétiques entre gnathostomes, lamproies et myxines. (A) Lamproies et myxines formant un groupe monophylétique, les cyclostomes, (B) les lamproies sont plus proches des gnathostomes que les myxines et (C) les myxines sont plus proches des gnathostomes que les lamproies.

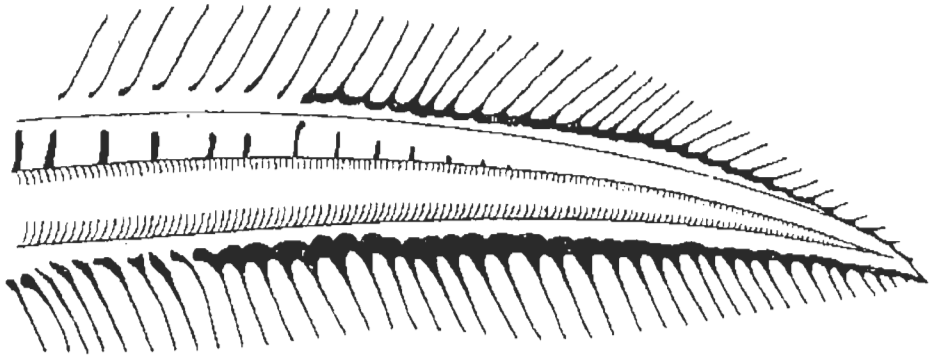

Figure introductive 2. Éléments squelettiques et arcualia terminaux dans la nageoire caudale d'un Pétromyzontiformes. D'après Tretjakoff [70].

CHAPITRE 1.
ETUDE DE LA SQUELETTOGENESE ET DE LA METAMORPHOSE CHEZ LA LAMPROIE MARINE (*PETROMYZON MARINUS*)

Par Catherine Morel, Richard Cloutier et Claude B. Renaud

1.1 RÉSUMÉ EN FRANÇAIS DU PREMIER ARTICLE

Dans un contexte évolutif, l'étude du développement squelettique nous permet de déterminer les conditions développementales les plus communes et les plus dérivées afin de comprendre leur homologie et leur évolution. Cependant, ce type d'étude est peu disponible pour les vertébrés non-ostéichthyens plus basaux, telles que les lamproies (Pétromyzontiformes). Nous avons décrit la squelettogenèse chez la lamproie marine (*Petromyzon marinus*) pour trois stades de vie consécutifs (ammocète, ammocète en métamorphose et adulte) ainsi que la morphologie générale et cellulaire des éléments postcrâniens (i.e., arcualia, rayons). Les éléments squelettiques ont été observés sur des spécimens colorés au bleu alcian (i.e., coloration *in toto*) d'ammocètes (n = 79), d'ammocètes en métamorphose (n = 14) et d'adultes (n = 19) provenant de l'Ontario (Bassin du Lac Huron) et du Québec (Rivière Sainte-Anne). Afin de visualiser la morphologie cellulaire des éléments postcrâniens, des coupes histologiques d'une ammocète et d'un adulte ont été produites, puis colorées à l'hématoxyline et éosine. Le développement des rayons cartilagineux est dépendant de la taille de l'ammocète. Les rayons se développent en premier antéropostérieurement dans la seconde nageoire dorsale, puis bidirectionnellement dans la nageoire caudale et antéropostérieurement dans la

première nageoire dorsale. Un développement asynchrone a été détecté au niveau des éléments buccaux, ainsi qu'entre les différents systèmes squelettiques. Les arcualia possèdent deux centres de développement : (1) antéropostérieurment au niveau du squelette branchial et (2) bidirectionnellement au niveau de la seconde nageoire dorsale. Le cartilage des arcualia est composé de chondrocytes groupés aléatoirement et encastrés dans une matrice extracellulaire territoriale mince d'épaisseur variable. Cette morphologie cellulaire ressemble à la lamprine mais diffère de celle des rayons, où les chondrocytes sont cuboïdes, étroitement empilés et retenus par une matrice extracellulaire périphérique. Nous avons observé deux morphologies cellulaires au niveau des éléments squelettiques postcraniens : (1) discoïdale (type I) et (2) pentagonale (type II). Nous avons observé la présence d'un cartilage notochordal avec une morphologie de type II dans la région caudale pour tous les stades étudiés. Ce cartilage, présent bilatéralemment au tube neural, ne représente pas une fusion d'arcualia au niveau caudal ; ce cartilage pourrait être le résultat d'une chondrogenèse notochordale. La morphologie générale et cellulaire des rayons de *P. marinus* est fort différente de celle de tous les autres types de rayons connus ; considérant leur position dans la nageoire ainsi que leur complexité (e.g., bifurcation de second ordre), ces éléments devraient être considérés comme de véritables rayons plutôt que des radiaux. Nous ne pouvons certifier que les arcualia des lamproies représentent une forme homologue de l'arc neural ; cependant, le développement antéropostérieur des éléments vertébraux situés au début du squelette axial représente une condition plésiomorphe. Cette étude est la première à décrire la squelettogenèse parmi trois stades, tout particulièrement sur la structuration et la morphologie cellulaire des arcualia et des rayons d'une lamproie.

L'article sera soumis durant l'hiver 2014 au journal *PLoS One*. En tant que premier auteur, j'ai réalisé l'ensemble des observations et des analyses. J'ai élaboré et choisit les méthodes d'analyses en collaboration avec mon directeur. Mon directeur, le deuxième auteur, a eu l'idée originale de ce projet, est responsable de la logistique du laboratoire et de la révision de l'article ; mon codirecteur, le troisième auteur, a contribué à l'interprétation des résultats et a révisé cet article.

1.2 A STUDY OF THE SKELETOGENESIS AND METAMORPHOSIS IN THE SEA LAMPREY (*PETROMYZON MARINUS*)

Abstract

The skeletogenesis of numerous osteichthyans has been investigated in an evolutionary biology context, but rarely in non-osteichthyan basal vertebrates. We describe the skeletogenesis of the Sea Lamprey (*Petromyzon marinus*) for three life stages and the patterning, general and cellular morphology of the postcranial elements (i.e., arcualia, fin rays). Skeletal elements were studied through Alcian blue-stained specimens (79 ammocoetes, 14 metamorphosing ammocoetes and 19 adults) and cross-sections stained with hematoxylin and eosin. The development of fin rays is size dependent. Fin rays appear first in the second dorsal fin, anteroposteriorly, then bidirectionally in the caudal fin and anteroposteriorly in the first dorsal fin. Asynchronous development among skeletal elements and skeletal systems was detected. Arcualia possess two centers of development: (1) anteroposteriorly in the branchial region and (2) bidirectionnally in the region of the second dorsal fin. Arcualia are composed of chondrocytes randomly grouped and embedded into a territorial extracellular matrix. This cellular morphology is similar to lamprin but differs from that of fin rays, which consists of closely-stacked cuboid chondrocytes with a thin peripheral extracellular matrix. We describe two cellular morphologies of cartilage: (1) discoidal (type I) and (2) pentagonal (type II). A type II notochordal cartilage was detected in the caudal region for all three stages. This cartilage, present bilaterally to the neural tube, has never described before and may likely be the result of a notochordal chondrogenesis. The general and cellular morphology of *P. marinus* fin rays is unique; considering their position in the fin and their complexity (e.g., second order bifurcation), we infer that these elements should be considered as true rays rather than radials. It is not clear if arcualia are homologous to the neural arches or a precursor; however, the anteroposterior development of dorsal vertebral elements seems to represent a plesiomorphic condition. Our study is the first to assess the description of the skeletal development in three stages and the patterning and cellular morphology of the arcualia and fin rays in a lamprey.

Keywords : skeletogenesis, fin rays, Sea Lamprey, arcualia, patterning, cellular morphology, notochordal cartilage, asynchronous development

Introduction

The lampreys (Petromyzontiformes) represent one of the last two living clades of the jawless fishes. This group possesses skeletal peculiarities that are of great interest to evolutionary biology. The presence of an extremely simple neurocranium, seven branchial arches, dorsal vertebral elements and an entirely cartilaginous skeleton represent examples of interesting plesiomorphic traits [1-6]. The development of these structures can be followed through the stages of the lamprey life history.

The life history of the Sea Lamprey (*Petromyzon marinus*) includes: (1) the prolarval stage lasting 1-3 weeks after hatching, during which first skeletogenesis leads to the formation of the larval skeleton [1,7-13]; (2) the ammocoete larval stage, during which the microphagous larva is buried in soft deposits of streams and rivers for many years [14-16]; (3) the metamorphosing stage, during which important anatomical and physiological changes occur in the ammocoete in order for it to acquire the adult phenotype [7,17-23] and (4) the adult stage, where the ectoparasitic lifestyle begins prior to sexual maturation and upstream migration [16,23,24].

Development of the neurocranial and branchial skeleton occurs during the prolarval skeletogenesis and has largely been described for *P. marinus* (Fig. 1). The branchial skeleton is the first system to form [8,9,12,13,25]. The branchial basket is composed of seven branchial arches, interconnected dorsally by the subchordal bar and ventrally by the hypobranchial bar. Above each gill sac, an epitrematic bar points forward and a hypotrematic bar unites the mid-region of every branchial arch [1]. The first branchial arch forms at 13 days post-fertilization (dpf), followed by the arches 2-7 that develop posteriorly until 20 dpf [7,9,12,25]. At 18 dpf, the mucocartilage starts to form in the oral hood [13]. The mucocartilage, unique to ammocoetes, is composed of transparent elastic fibres that form a loose connective tissue [13,26,27]. The mucocartilage is generally regarded as an unique adaptation to the burrowing habit and the microphagous lifestyle of the ammocoete

[1,26]. At 20 dpf, the trabecula represents the first neurocranial element to appear, followed by the formation of the basitrabecular process and parachordals at 24 dpf [9,12]. The nasal and otic capsules finally form near 25 dpf [9]. Between 25 dpf and 33 dpf, only condensation of chondrocytes is observed [9]. At 33 dpf, all the neurocranial and branchial elements that need to be present are fully developed as the ammocoete is either buried or able to burrow [9]; this corresponds to the developmental stage 18 described by Piavis (Fig. 1) [10,28].

Development of the median finfold also occurs during the prolarval stage starting at 12 dpf [11]. Differentiation of the lobes of the caudal fin is achieved around 70 dpf [11]; the caudal fin forms an epichordal lobe and a hypochordal lobe that meet at the posterior extremity of the notochord. The epichordal lobe is continuous with the second dorsal fin (D2) whereas the hypochordal lobe reaches the cloaca. On the other hand, the dorsal fin fold divides into two discrete dorsal fins only during metamorphosis [5,21]. The anal fin is normally absent *contra* observations by Piavis [10,28]. Exceptionally, an anal fin supported by fin rays has been reported in two female specimens of *Petromyzon marinus* [29,30]; the presence of an anal fin may represent an atavistic expression of a plesiomorphic condition [31]. The first dorsal fin (D1) is located anteriorly to D2 and is at least two times shorter and lower than D2. The median fins of the Petromyzontiformes are known to possess cartilaginous fin rays that serve as an internal support [32]. Some authors have used "fin radials" to term these cartilaginous elements [8,32]. Vladykov [33] observed the presence of numerous fin rays in the dorsal and caudal fins of larval eastern American lampreys (Petromyzontidae), suggesting that the chondrogenesis of these cartilaginous elements may have occurred in the ammocoete stage or earlier; however, the exact moment of the chondrogenesis of these elements is still unknown. In fact, while the microphagous larva is buried, chondrogenesis is ongoing because condensation and differentiation of chondrocytes are still observed in the branchial skeleton [9]. However, chondrogenic events occurring after 63 dpf are unknown. In fact, no study has found new skeletal elements in ammocoetes prior to metamorphosis; it is generally assumed that only somatic growth occurs during the intervening years. Previous authors [34,35] reported migration of neural

crest cells into the embryonic dorsal fin fold in early developmental stages of *Lampetra japonicum* (= *Lethenteron camtschaticum*) and *Lampetra reissneri* (= *Lethenteron reissneri*). Since neural crest cells have an important role in cartilage formation of the neurocranial and branchial skeletons [36], it is plausible that these cells are the precursors of cartilage formation in the median fins in latter stages of development (i.e., ammocoete). Extensive descriptions of the development and morphology of the fin rays were never documented for any Petromyzontiformes; thus, the patterning and the type of cartilage forming the fin rays remain unknown.

After the prolarval skeletogenesis, metamorphosis is the second most important developmental event during the life history of the Sea Lamprey. Metamorphosis occurs around mid-July or August and is highly influenced by both environmental and hormonal cues [37,38]. A total length of 120 mm must normally be reached prior to metamorphosis [39,40]. Ammocoetes between 3 and 8 years-old undergo metamorphosis; however, metamorphosis in 2 year-old ammocoetes has also been reported [39]. The time required for the completion of metamorphosis in *P. marinus* ranges between 3 to 4 months [20,23]. In order to become prespawning adults, ammocoetes undergo important transformations in both their external and internal morphologies. A staging of metamorphosing ammocoetes was adopted for *P. marinus* and is based on external morphology such as the eye diameter, mouth shape, dorsal fin height and body pigmentation [21,41]. However, such staging has never been tested on other systems (e.g., skeletal system); it is not known whether the skeleton remodels at the same rate as the external morphology.

During metamorphosis, mucocartilage is the first tissue to transform [7]. Most of the mucocartilaginous elements are destroyed [7,17,42,43]. The mucocartilage can also revert to the condition of an embryonic mesenchyme (i.e., blastema) [1,17,42,43]. New cartilaginous elements will arise from the blastema located in the oral aperture in order to form the buccal skeleton. The formation of the buccal skeleton represents the most distinctive transformation among all skeletal systems since the neurocranial and branchial skeletons undergo only minor transformations [7,43]. The buccal skeleton of the adults

possesses peculiar cartilaginous elements associated with the feeding function [1,44,45]. The annular cartilage surrounds and supports the buccal cavity [1,8,14]. A pair of spinous cartilages, the stylets, are articulated posterolaterally on the annular cartilage; these elements provide a point of insertion for the muscles responsible for the positioning of the buccal funnel [8]. Medioventrally to the annular cartilage is the copula, a T-shaped plate that serves as an important point of insertion for muscles that control the orientation and movements of the piston [1]. The anterior tip of the piston carries apical cartilages on which are located the transverse lingual lamina and two longitudinal lingual laminae, the combination of which is otherwise referred to as the rasping "tongue-like" organ [1,31,44,45]; these laminae possess sharp cusps that enable the penetration and removal of the flesh of the preys [1,31,45]. The dorsal cartilages consist of the anterior dorsal and the posterior dorsal. The posterior dorsal slightly overlaps the posteriormost region of the anterior dorsal cartilage; together, these plates form the roof of the buccal cavity [1]. The relative movement of these two plates changes the volume of the hydrosinus and plays an important role in the suction mechanism [1,8].

Adult lampreys possess paired dorsal perichordal cartilages located along the notochord [31,46]. These elements, termed arcualia, develop from the condensation of sclerotomes in discrete locations during metamorphosis [5,21,47]. Arcualia are arranged as two pairs per myomere and can reach up to 130 arcualia in the case of *Petromyzon marinus* [5,31,48]. While Strenger [49] claimed that the cartilaginous arcualia have no important function, Potter and Welsch [47] confirmed that arcualia act as an attachment for collagen fibres in adult *Lampetra fluviatilis*. Tretjakoff [48] and Goodrich [50] also confirmed that arcualia each possess a foramen through which passes the posterior branches of the vagus - an important nerve associated with the branchial region [50]. Owing to their shape and position, arcualia are often assumed to be homologous to higher-vertebrate neural arches [1,5,14]. However, no study has ever focused on the developmental pattern and the cellular morphology of these elements. It is not known whether arcualia are formed of hyaline cartilage, as seen in the embryonic neural arches of some gnathostomes [51] or of another specialized type of cartilage unique to Petromyzontiformes. Moreover, the development of

these vertebral elements has never been observed during metamorphosis.

Adult lampreys are known to possess at least two types of unique cartilaginous tissues. One type of cartilage is known as lamprin and is mainly present in the piston, annular cartilage, as well as in the neurocranial elements [52-54]. Lamprin is composed of highly hydrophobic proteins and contains no recognizable collagen fibres [52,53,55-57]. The lamprin tissue shows a strong resemblance to elastin, a structural element found in elastic cartilage of higher vertebrates [52]. Another type of cartilage was found in the trabecular, branchial and pericardial cartilages of adults [56,58]. This cartilage is extremely cellular and does not possess any collagen fibres; it is composed of cyanogen bromide-insoluble proteins that also show similarities to elastin [55]. However, a recent study demonstrated the expression of two Col1A2 orthologues (i.e., Col1A2a, Col1A2b) in the branchial arches, arcualia, notochord and notochordal sheath during the chondrogenesis of *Petromyzon marinus* [59]. This discovery rejects what has been described in previous studies and raises the possibility that the skeleton of lamprey may be entirely collagenous [59].

Lampreys represent one of the two extant groups of jawless fish and are viewed as basal vertebrates. The phylogenetic relationships among lampreys, hagfishes and gnathostomes have been controversial for a long time. Morphological and molecular phylogenetic hypotheses between lampreys, hagfishes and gnathostomes are not congruent [3,60,61]. Morphology-based investigations support lampreys being more closely related to gnathostomes than they are to hagfishes [4,31,62,63]. On the other hand, molecular data suggest that lampreys and hagfishes form a monophyletic group known as the Cyclostomata [64-67]. However, the most recent analyses combining both morphological and molecular data tend to support the monophyly of the cyclostomes, mainly as a result of the overwhelming number of molecular characters [6,68-70]. New developmental characters could potentially help to resolve this debated evolutionary question.

Chondrogenesis and ossification patterns are fairly well documented in a diversity of gnathostomes in the context of evolutionary [71-80] and morphological [81-91] studies.

However, lamprey skeletogenesis through consecutive life stages is poorly described and relies on a few classical morphological studies based on sectioned specimens. None of these studies have focused on the complete overview of the chondrogenesis on whole-mount specimens with a particular attention to the development of arcualia and cartilaginous fin rays. The developmental, histological and morphological aspects of the postcranial skeleton are still poorly understood. Our study is the first to investigate the development of arcualia and to describe the patterning and the general morphology of these elements throughout the axial skeleton. We also are the first to describe the development and the morphology of the skeletal elements forming the appendicular skeleton (i.e., fin rays). The determination of the cellular morphology and the patterning of arcualia and fin rays will enable us to propose comparisons and homologies with gnathostome elements (e.g., neural arches, lepidotrichia).

The main objectives of this study are: (1) to describe the skeletogenesis of the neurocranial, branchial, buccal, axial and appendicular (i.e., median fins) elements in three consecutive stages (i.e., ammocoetes, metamorphosing ammocoetes and adults) and (2) to describe the patterning and the general and cellular morphology of the postcranial elements (e.g., arcualia, fin rays).

Materials and Methods

Specimens

Specimens of Sea Lamprey (*Petromyzon marinus*) used in this study are housed in the Fish Collection of the Canadian Museum of Nature (CMNFI). The lampreys were sampled from Lake Huron Basin (ON, Canada) and the St. Anne River (Qc, Canada). In addition, ammocoetes collected from the Old Woman River (ON) in July 2012 were provided by the Bayfield Institute (Fisheries and Oceans Canada). The Maurice-Lamontagne Institute also provided an adult collected from the St. Lawrence River (Qc) during the summer of 2010. Specimens were fixed in 4% buffered formaldehyde and stored in 70% ethanol. Total length (TL), the distance between the tip of the snout and the posteriormost part of the caudal fin [31], was measured with a digital caliper.

Histology and Skeletal Preparation

Whole-mount specimens of ammocoetes (4 populations, n = 79, TL = 118-154 mm), metamorphosing ammocoetes (2 populations, n = 14, TL = 125-144 mm) and young adults (4 populations, n = 21, TL = 114-153 mm) were cleared-and-stained (C&S) following Potthoff's protocol [92]. Alcian blue (0.3%) in acid solution was used to color the cartilaginous structures. A structure was considered formed when uptaking the blue stain. Specimens were examined under a Leica MZ16A binocular microscope equipped with a Qicam digital camera with a CCD sensor (Meyer Instruments, TX).

To determine the skeletogenesis through the stages, the presence of 46 skeletal elements was observed for ammocoetes and 136 elements for metamorphosing ammocoetes and adults. Each element was coded into a binary database; developmental events occurring in median fins (e.g., formation of rays, bifurcation of rays) were also coded. Two possible

developmental states were observed: (0) absence and (1) presence. Drawings were made using a camera lucida mounted on a Leica MS9.5, then were vectorized with Inkscape (Version 0.48, Free Software Foundation, 1990) and corrected using Adobe Photoshop Element (Version 9.0.3, Adobe Systems Incorporated, 2013).

Samples of branchial, predorsal, D2 and caudal regions (see definitions below) of an ammocoete (Old Woman River, AMPMH-01, TL = 132.9 mm) and an adult (St. Lawrence River, ADPMH-01, TL = 270.5 mm) of *P. marinus* were selected for histological preparation. Samples were processed in a Shandon Citadel 2000 automated tissue processor (www.thermoscientific.com). They were dehydrated in graded ethanol solutions (20–100%), transferred to xylene/ethanol and finally pure xylene. Samples were then transferred to a 50/50 xylene/paraffin solution and impregnated with melted paraffin under a vacuum. Samples were embedded in Paraplast Plus (www.mccormickscientific.com) and sectioned at 7 μm intervals. Sections were processed through regressive staining using standard haematoxylin and eosin (H&E) (http://www.sigmaaldrich.com). Sections were observed with a Leica DMLB microscope and images were taken with an AmScope MU1000 microscope digital camera using Toupview software (Version 3.7, AmScope, 2013).

Statistical Methods

To visualize the order of developmental events in the median fins of ammocoetes, generalized linear models were fitted against the binary dataset of skeletal development. These logistic regressions can describe the relationship between the continuous predictor variable (TL) and the random component (i.e., developmental states for each element) and give an estimate of the TL at which half of the specimens possess the developed element (TL_{50}). This computation was adapted from the SL_{50} of Fischer-Rousseau *et al.* [75] and Grünbaum *et al.* [77]. The significance of the logistic regression was tested using χ^2-statistic. Only elements with a significant model ($p < 0.05$) were considered for analysis. In

order to minimize a potential interpopulational variation, TL_{50} was performed only on the population of St. Anne River (n = 54, TL = 19-129 mm).

To visualize variation within morphometric traits among metamorphosing ammocoetes, a principal component analysis (PCA) was run on a covariance matrix of log_{10}-transformed morphometric traits. Nine morphometric traits adapted from Renaud [31] were measured on metamorphosing specimens (Fig. 2). PCA provides a good way to visualise and detect structure relationships and variance among descriptors. The Kaiser-Gutman criterion was used to determine the number of statistically interpretable axes. Eigenvectors of each trait were collected for the retained axes. Allometric growth is present if the eigenvector for a morphometric trait is noticeably different from the others [76].

Shapiro-Wilk and multinormality tests were performed on the nine log_{10}-transformed morphometric traits. The Shapiro-Wilk normality test was not significant for PNL and MW ($p > 0.05$). However, without those traits, the dataset was not close to the significance threshold ($p = 0.002$); on the other hand, by keeping those traits, the dataset was closer to being significant ($p = 0.02$). Both were therefore kept for the analysis. Colinearity among variables was evaluated based on correlation coefficients. HL and PNL were perfectly correlated ($r_s = 1$), suggestive of high colinearity. Since HL represents a redundant measurement (HL = SL + EL + POL), it was removed from the analysis.

To test the staging based on the presence of skeletal structures in metamorphosing ammocoetes, a nonmetric multidimensional scaling with stable solution from random starts (metaMDS) was used. This analysis was performed on a Dice binary distance matrix that includes the data of both metamorphosing ammocoetes and adults. Points in this ordination space represent the specimens. The number of dimensions with the lowest stress was selected for each matrix.

All statistical analyses were performed with RStudio (version 0.97.551, RStudio Inc., 2009, Synvale, USA).

Nomenclature and Definitions

Nomenclature for the Sea Lamprey skeleton follows that of Jollie [8], Bardack and Zangerl [93], Hardisty [1], Yao *et al.* [12], Richardson and Wright [11], Martin *et al.* [9] and Richardson *et al.* [5].

Arcualia are defined as the primary cartilaginous elements forming dorsally along the notochord in vertebrates [51,94]. During embryonic development, the first mesenchymal cells gather around the notochord, forming discrete blocks of cartilage [51]. These structures, mainly formed of hyaline cartilage, develop into neural arches in derived groups of fishes [51,95,96]. This term is also used to designate the dorsal vertebral elements in the Petromyzontiformes [5,31,43,48]. We defined mediodorsal vertebral elements as the cartilaginous structures present alongside arcualia; these elements are attached dorsally to the notochord and positioned inwardly to the arcualia.

The development of arcualia was categorized in terms of their position along the body axis. The branchial region (B) starts at the first branchial arch and finishes at the level of the seventh branchial arch inclusively. The predorsal region (P) covers the area from the seventh arch to the first fin ray of the first dorsal fin (D1). The D1 region (D1) contains the first to the last ray of the first dorsal fin. The D2 region (D2) is delimited between the first and the last ray of the second dorsal fin; since the second dorsal fin is continuous with the caudal fin, we considered the shortest ray of the posteriormost region of D2 as the limit between D2 and the caudal fin. The caudal region (C) encompasses the last fin ray of the second dorsal fin to the posterior extremity of the notochord. Each region was grossly separated into three zones: (1) anterior, (2) middle and (3) posterior. Development of arcualia was defined as follows: absent if no arcualia is observed (0); weakly developed if arcualia are small cartilaginous nodules that are barely visible along the notochord (+); moderately developed if arcualia are rather small but well visible along the notochord (++) and well developed if arcualia are noticeably long and large (+++).

Fin rays are defined as the structures internally strengthening and supporting the fins

[51,97,98]. Rays can be formed of cartilaginous, mineralized and osseous tissues. Four kinds of rays have been described to date. Lepidotrichia, present in actinopterygians and sarcopterygians with the exception of dipnoans, are flexible rays composed of successive mineralized hemisegments connected together by ligaments [97,98]. The distal ends are dichotomously branched. Actinotrichia are present between the most distal hemisegments of the lepidotrichia. This kind of ray consists of short, tapered cartilaginous rods that are generally distally branched [97,98]. Actinotrichia can also be found in the adipose fin of some Salmonidae and Siluridae [98]. Ceratotrichia are flexible, unsegmented cartilaginous rods present in the fins of chondrichthyans. Those rays are longer and thicker than actinotrichia and are usually branched distally [97,99,100]. Lastly, camptotrichia, present only in dipnoans, consist of straight cylindrical rods arranged in two asymmetrical rows [51,99]. This kind of ray is usually dichotomous at the margin and is composed of acellular fibrous tissues and mineralized bones [51]. Despite the terms that have been used in previous studies (i.e., radials) [32] we will use the term fin ray for lampreys in the present study.

Staging of metamorphosis was based on morphological traits described by Youson and Potter [20].

Results

Our description of the skeletogenesis of *Petromyzon marinus* focuses on three consecutive periods of development: (1) ammocoete, (2) metamorphosing ammocoete and (3) adult. For each period, the description is organised in terms of four anatomical systems: (1) neurocranium, (2) branchial skeleton, (3) axial skeleton and (4) appendicular skeleton.

Ammocoetes

Skeletal Development

The number, position, shape and relative proportion of the skeletal elements of the neurocranial, branchial and axial skeletons remained unchanged for specimens from all populations, ranging from 19 to 150 mm TL. However, the appendicular skeleton showed progressive development in the number and the position of fin rays as the size of ammocoetes increases.

Based on fin ray development, seven developmental steps were designated. Step 1 (Fig. 3A) is defined by the absence of cartilaginous rays in fins, a condition observed in specimens inferior to 30 mm TL. Starting at 31.6 mm TL_{50} (Fig. 4; $p = 1.187e-07$), step 2 corresponds to the formation of the first fin rays in the anteriormost region of D2 (Fig. 3B). Fin rays consist of unsegmented, short and cylindrical cartilaginous rods formed by continuous stacking of flat chondrocytes. Fin rays are not supported by endoskeletal elements; thus, no connection exists between the fin rays and the axial skeleton.

In step 3, fin rays develop anteroposteriorly in D2 (Fig. 3C); rays are present between 75% and 100% of the length of D2. In step 4, fin rays start to form bidirectionally in the

epichordal and hypochordal lobes at the posteriormost region of the caudal fin (TL_{50} = 34.4 mm, p = 1.691e-10); simultaneously, fin rays have appeared in the anteriormost region of D1 (Fig. 3D). Step 5 represents the completion of the development of rays and is marked by the presence of well-developed rays in every fin (Fig. 3E). The presence of short, unifid rays provides an oval shape to the caudal skeleton (Fig. 5A). However, caudal fin rays are not yet reaching the margin of the fin. Condensation of prechondrogenic cells is visible in the distal extremity of the fin rays, whereas the proximal ends of fin rays have slightly enlarged and extended anterodorsally to the neural tube and anteroventrally to the notochord. Rays located near the tip of the notochord are noticeably shorter for both epichordal and hypochordal lobes, leaving the posterior end of the neural tube and the notochord uncovered (Fig. 5B).

In step 6, fin rays of the caudal fin are larger and dichotomously branched (Fig. 3F); however, this latter condition is not observed on every fin ray, even in the later developmental step. Our observations suggest that bifurcation occurs posteroanteriorly in the caudal fin. The origin of bifurcation is located in the distal part of the ray but also in the proximal region on rare occasions. The bifurcation gives the typical well-rounded shape to the caudal skeleton (Fig. 5C). Bifurcation appears in the hypochordal lobe (TL_{50} = 59.2 mm, p = 1.384e-11) before the epichordal lobe (TL_{50} = 64.8 mm, p = 1.334e-09). Proximal ends have further extended anteriorly along the notochord and neural tube. Fusion of proximal parts among adjacent rays resulted in the formation of cartilaginous median rods in both epichordal and hypochordal lobes. In every specimen, the ventromedian rod is noticeably thicker than the dorsomedian rod. The median rods were observed only in the latter half of the caudal fin.

In step 7, posterior fusion between rays can be observed at the tip of the notochord (Fig. 3G, TL_{50} = 78.7 mm, p = 5.766e-08). Distal fusion between the last rays of the epichordal and hypochordal lobes leads to the formation of a thin cartilaginous rod at the tip of the notochord (Fig. 5D). Bifurcation is present in D2 (TL_{50} = 67.6 mm, p = 2.854e-11) and subsequently in D1 (TL_{50} = 115.9 mm, p = 0.001). During this step, second order

bifurcation appears in both lobes of the caudal fin (TL_{50} = 100.3 mm, p = 0.001); a new branching is formed symmetrically or asymmetrically on the existing bifurcation of the ray (Fig. 6A). Second order bifurcation appears first in the epichordal and hypochordal lobes, in addition to be occasionally observed in D2 in longer specimens (Fig. 6B).

The epichordal and hypochordal lobes are composed of approximately 50-60 and 70-80 fin rays, respectively. The sequence of branched-unbranched fin rays in both lobes is variable among specimens. Anterior extension of fin rays in the epichordal lobe is spatially limited by the presence of D2; the hypochordal lobe is longer than the epichordal lobe and therefore contains more fin rays. We also noticed that the posteriormost part of the notochord was generally slightly curved upwards in specimens not having reached step 7 (Fig. 5). Specimens longer than 78.7 mm TL possess a fairly straight notochord.

The development of fin rays is correlated with the size of specimens as suggested by the results from TL_{50}, demonstrating the existence of a relationship between the length of ammocoetes and the progression of fin development (Fig. 4). These results, combined with general observations on C&S specimens, provides a general sequence of development: (1) D2, anteroposteriorly; (2) caudal fin (epichordal and hypochordal lobes), bidirectionally and (3) D1, anteroposteriorly. Successive skeletal events (e.g., bifurcation and fusion of fin rays) appear as ammocoetes are increasing in size.

Closely-stacked rectangular cells forming the fin rays were visible with both Alcian blue (Fig. 7A) and H&E staining (Fig. 8A). Histological sections provided additional information about the cellular morphology of these chondrocytes. Fin rays are formed by a stacking of cuboid-shaped chondrocytes and are approximately five-cells wide (Fig. 8A, Fig. 9B). Very little extracellular matrix (ECM) was observed in this peculiar type of cartilage and lacunae were totally absent. Prechondrogenic cells are normally present prior to the expression of cartilage extracellular matrix; thus, the chondrocytes observed may still be immature.

Fin rays and branchial arches are similar at the macroscopic and microscopic levels (Fig. 7, Fig. 8). Both structures are composed of closely stacked and elongated chondrocytes with very little ECM; however, the stacking and the shape of chondrocytes in the branchial arches are slightly more regular between rows (Fig. 8B). On the other hand, chondrocytes in fin rays vary in their size and shape, more particularly in the peripheral zone (Fig. 8A).

The dorsomedian rod is located dorsally to the neural tube (Fig. 9A,C). Central chondrocytes are globular and visibly larger compared to those located in periphery. Groupings of chondrocytes with a similar cellular morphology are present bilaterally on the notochord (Fig. 9A,D), underlining the presence of cartilaginous elements that were not observed on C&S specimens. These elements were found only in the posteriormost region of the caudal fin, attached to the elastic layer of the notochord.

The ventromedian rod is lying ventrally to the notochord and is formed by a random stacking of globular cells (Fig. 9E-F). This rod is two to three times higher than the dorsomedian rod. Chondrocytes located distally are noticeably different from those in the center. These cells represent the proximal extremity of the fin ray, anchored in the ventromedian rod. Gradation in shape and size is visible among cells, as well as the delimitation between cells of the ventromedian rod and the base of the fin ray (Fig. 9G).

Metamorphosing Ammocoetes

Staging

Staging of specimens S3-1 to S3-9 was consistent with the stage 4 of Youson and Potter [20]; these specimens possessed fused lips, an oval-shaped oral disc and small, underdeveloped fimbriae (See Appendix). Specimens S3-10 to S3-14 were classified as stage 5 based on the presence of well-developed fimbriae, a slit-like mouth and an elongated snout (see Appendix).

PCA provides a descripton of the global interaction among traits (Fig. 10). The first principal component (PC1) explains 71.4% of the total variation, whereas PC2 explains 20.1% (Table 1). PC1 primarily corresponds to the general growth of metamorphosing ammocoetes, with a clear allometric change of proportion of the mouth (MW, ML). Moreover, traits related to the size of the mouth (MW, ML) contribute for most of the variation on PC1 (Fig. 10, Table 1). Length traits tend to be grouped and inversely correlated with width traits (MW, IL). The interocular distance (IL), branchial length (BL) and postocular length (POL) do not contribute to a great deal of variation in PC1 and PC2. PC2 corresponds to the shape variation (Fig.10, Table 1). The mouth width (MW) and the prenostril length (PNL) contribute for most of the variation on PC2, whereas the postocular length (POL) contrasts slightly with the remaining variables on PC2.

MDS analysis showed that the classical staging by Youson and Potter [20] is not consistent with the staging based on the presence of skeletal elements (Fig. 11). No clusters appeared among stage-4 and stage-5 specimens on the ordination diagram; however, adults (S4) and metamorphosing ammocoetes (S3) are greatly dissimilar, as shown on dimension 1. The longest specimen that belonged to stage 4 (S3-9) is the only metamorphosing specimen to be greatly dissimilar from other specimens; it is not clustering either with adults or with the other metamorphosing ammocoetes.

Skeletal Development

The sequence of formation and modification of skeletal elements was variable among specimens. The neurocranium was already well developed by stage 4. The elements of the branchial skeleton were entirely formed, with the exception of the pericardial cartilage (per) and the small cartilaginous protuberances located on the epitrematic and hypotrematic bars (eppr, hypr). Those small triangular elements were only present dorsally on the epitrematic bar (ep) and ventrally on the hypotrematic bar (hy), halfway between the branchial arches (Fig. 13). These protuberances were never observed at the level of the first branchial arch, where the epitrematic and hypotrematic bars fuse into a loop. Their presence is variable along the branchial skeleton; they can be present on every epitrematic and hypotrematic bars or present on the epitrematic bars only. The development of the pericardial cartilage was also variable within stages. Condensation of chondrocytes was observed more importantly in the pillars attaching the pericardial cartilage to the seventh branchial arch. No clear relationship was observed between the stage, the number of developed protuberances and the presence of the pericardial cartilage.

Most of the elements of the buccal skeleton were already formed by stage 4, with the exception of the anterior dorsal cartilage (ad), posterior dorsal cartilage (pd), the anterior lateral plate (al) and the posterior lateral plate (pl); in fact, these elements did not consistently develop in the same order. We observed that the development of some skeletal elements is unsynchronized among metamorphosing specimens. The anterior dorsal cartilage is generally more developed than the posterior dorsal cartilage; however, the posterior dorsal is noticeably longer and more developed in some specimens (S3-5, S3-6, S3-7). In stage 4, the anterior lateral plate appears before the posterior lateral plate for most of the specimens; however, the anterior lateral plate is absent whereas the posterior lateral plate is well developed in specimen S3-9. These results underline the absence of a clear order or sequence of formation for a given skeletal element at the level of the buccal skeleton. Such asynchrony has also been observed among skeletal systems, regardless of the metamorphosis stage. The pericardial cartilage can be well developed in some

specimens, whereas the arcualia are weakly developed in the branchial region; in other specimens, the pericardial cartilage is poorly developed whereas arcualia are well developed.

Trends in the proportions of the skeletal elements can be observed within a particular stage of metamorphosis (Fig. 13). The most obvious proportional changes occur at the level of the buccal and branchial skeletons. The branchial basket of stage-5 ammocoete is more compressed and elongated than in stage 4 (Fig. 13A-B). The dorsal and ventral parts of the branchial arches are curled up, bringing the hypobranchial bar (hyb) closer to the hypotrematic bar (hy); the epitrematic bar (ep) is also closer to the subchordal bar (sub). In both stages, the subchordal bar tends to move under the notochord and is barely visible, except above the branchial arch 7. From stage 5 to the adult (Fig. 13 B-C), dorsal cartilages of the buccal skeleton (ad, pd) have extended straight and are positioned horizontally, resulting into a pronounced elongation of the snout; note that the posterior dorsal has grown noticeably longer than the anterior dorsal. The annular cartilage (an) is larger and higher anteriorly, whereas the lateral sides are pitted. The stylet (st), posterior lateral (pl) and anterior lateral plates (al) have grown thicker and longer. The piston (p) is larger and has curled inwardly between the subocular arches (soa). The anterior part of the notochord has slightly curved ventrally, resulting in the curvature starting immediately behind the otic capsule (oc). The branchial rings (br), the epitrematic (eppr) and hypotrematic (hypr) protuberances are well developed and are present along every branchial arch, except for the first arch in the case of the protuberances. The pericardial cartilage is also entirely formed. The copula and the neurocranial elements have remained globally unchanged between stage-5 ammocoetes and adults.

Morphology and Development of the Arcualia and the Mediodorsal Vertebral Elements

A dark Alcian blue staining of arcualia indicates the presence of glycosaminoglycans. Arcualia developed in the branchial region (B) first, indicating the location of the first center of development (Table 2). The number of arcualia formed in this region varied from 6 to 12 regardless of the metamorphosing stage. We also observed that arcualia decreased in size as one progressed posteriorly along this region. Mediodorsal vertebral elements were present on rare occasions alongside the third, fourth and/or fifth arcualia of the branchial region (Fig. 13B-C); thus, most of the specimens did not possess mediodorsal elements.

The morphology of the first arcualium is distinctive from the others; it is noticeably larger and its distal extremity is always bifid rather than unifid. Region B3 always develops after B1 and B2 since arcualia in this section were mostly weakly developed or absent (Table 2). Two specimens (S3-12, S3-13) out of 14 possessed arcualia in the predorsal region (P), more specifically in P1. These results indicate that the development of arcualia occurred anteroposteriorly in the branchial region, starting immediately above the first branchial arch. Larger stage-4 specimens (S3-8, S3-9) did not possess arcualia in B3, a condition that differs from the smaller specimens that belong to the same stage. These observations suggest that the number of developed vertebral elements is not strictly related to length.

Unlike stage-4 ammocoetes, stage-5 specimens possessed arcualia in region D1 and D2. Moderately-developed arcualia were present in D2-2 for most of the specimens of this stage; weakly-formed elements were also observed on D1-3, D2-1 and D2-3. The presence of a greater number of arcualia in these regions is suggestive of a more developed axial skeleton; thus, stage-5 ammocoetes are likely more developed. The presence of vertebral elements in D1 and D2 also confirms the existence of a second center of development.

Fin Rays, Median Rods and Dorsolateral Cartilage

Second order bifurcations are present mostly in the posterior region of the caudal fin and regular bifurcation is present in D1 and D2. In both stages, the median rods located in the caudal fin have grown conspicuously larger and thicker (Fig.14). The ventromedian rod is still more important than the one present in the epichordal lobe. Two different populations of cells were noticed in the caudal region. The first population, forming the fin rays, is composed of regularly stacked cells with a rectangular shape; the same morphology was observed previously in the fin rays of ammocoetes. The second population contains large, pentagonal cells that are randomly arranged. Chondrocytes with this morphology were observed in the median rods of the caudal fin, but also dorsolaterally to the notochord. The agglomerations of pentagonal chondrocytes along the notochord are forming irregular plates of cartilage in the posteriormost region of the caudal fin (Fig. 14); this dorsolateral cartilage was observed on every C&S specimens.

Adults

Skeletal Development

No major changes in the size, shape, position and relative proportion were observed in the neurocranial, buccal and branchial skeletons among the 19 C&S adults of *P. marinus*; note that two specimens were damaged during the skeletal preparation. The only noticable changes among all skeletal elements correspond to the condition of the epitrematic and hypotrematic protuberances located in the branchial skeleton. These small triangular cartilages were observed at first on the epitrematic bar of the last branchial arch, then were developing anteriorly along the following epitrematic rods. Hypotrematic

protuberances are forming after the epitrematic protuberances and were also observed developing anteriorly.

Morphology and Development of the Arcualia and the Mediodorsal Vertebral Elements

Arcualia were also categorized in terms of body sections for the adults (Fig. 15A). The first 7-8 arcualia located in the branchial region were always the largest and the most developed (Table 3). Generally, arcualia are well developed in region B1, B2 and B3; only a few specimens (S4-5, S4-11, S4-17) have weakly-developed arcualia in B3 (Table 3). Besides the size and the typical "hook"-shape, the general morphology of arcualia in the branchial region is variable (Fig. 15B). The distal extremity is either unifid, bifurcated or trifurcated; it can either point anteriorly or posteriorly. The proximal extremity of the arcualia is generally well rounded and perforated, as observed previously in metamorphosing specimens. Mediodorsal vertebral elements are also present in this region and vary greatly in size and shape. The shape of the mediodorsal vertebral elements is either elongated, short, crumpled, notched or twisted. Their size varies as being the same height as the arcualia, whereas other elements are small, stocky and barely visible along the notochord. These elements were present only in the branchial region, mostly alongside the third to the seventh arcualia. Some specimens possessed two mediodorsal vertebral elements between two consecutive arcualia.

The P, D1 and D2 regions contain two pairs of arcualia per myomere. The development is generally more advanced in D1 and D2 regions compared to region P; arcualia in P are generally weakly to moderately developed (Table 3). Arcualia in D2 region are mostly well developed; however, arcualia in D2-2 are slightly more developed than those present in D2-1 and D2-3. Region D1 also possesses well-developed arcualia in D1-1 and moderately to weakly developed arcualia in D1-2 and D1-3. The general morphology of the arcualia in regions P, D1 and D2 was fairly constant. Thin and elongated

arcualia are regularly spaced along the notochord (Fig.15C). The proximal ends of arcualia are rounded whereas the distal extremities are sharp and unifid.

Weakly to moderately developed arcualia were observed in the caudal region (C) for some specimens (S4-6, S4-7, S4-9, S4-12, S4-18, S4-19). This condition suggests that arcualia in the caudal region are the last to develop. Even in the largest specimens (S4-18 and S4-19), arcualia were generally weakly to moderately developed in C1 and weakly-developed or absent in C2. No arcualia was present in the terminal section of the caudal region (C3). Unlike the trunk region, the caudal region contains arcualia with variable morphologies. Region C1 and C2 are composed of arcualia with a great variation of size and shape, in addition to being irregularly spaced along the notochord (Fig. 15D). The arcualia located in C2 are dispersed from each other and their distal extremities are inclined posteriorly towards the notochord.

The cellular morphology of the arcualia differs from the condition observed in the fin rays; chondrocytes are embedded in a territorial extracellular matrix of variable thickness (Fig. 16A,B,C). This pericellular matrix forms a highly branched network surrounding the chondrocytes. No subperichondrial region was observed in this tissue, indicating an absence of differentiating zone at the periphery of the cartilage. Dark-purple colored nuclei of mature cartilaginous cells (i.e., chondroblasts) are clearly visible within the lacunae. Chondrocytes are forming isogenous groups of cells (i.e., chondrones) that are randomly arranged. The proximal end of the arcualia is attached to the elastica externa of the notochord (Fig. 16C); it is not continuous nor fused with the external sheath of the notochord. The size and shape of chondrocytes are hardly visible within the lacunae.

Fin Rays, Median Rods and Dorsolateral Cartilage

Second order bifurcations are present in most of D2 and in the posterior region of the caudal fin, but never in D1. The proximal region of the rays has grown thicker compared to

the condition in ammocoetes, whereas the distal branched extremities have become noticeably thinner. Fin rays are larger than those found in ammocoetes since they possess a minimum diameter of 12 cells (Fig. 17C,D). The cartilage forming fin rays is highly cellular; territorial extracellular matrix is only present at the periphery and no lacuna is present. The ratio nucleus:cytoplasm is slightly higher in these chondrocytes compared to the ones found in the dorsal and ventral median rods. Peripheral growth is confirmed by the presence of small and elongated chondrocytes along the border of the fin ray. Many cells are gathered in pairs, indicating they are presumably still dividing. Chondrocytes mostly possess a cuboid shape that slightly differs from the globular morphology of the cells found in the median rods.

Chondrocytes are observed on each side of the neural tube in the caudal region (Fig. 17A). These dorsolateral cartilaginous rods (lr) are approximately two cells-wide and are fused distally to the dorsomedian rod (dmr), as previously observed in ammocoetes. A thin pericellular matrix surrounding the chondrocytes is present in both dorsomedian and dorsolateral rods (Fig. 17A,B). These groups of chondrocytes are similar to that of arcualia in terms of their position on the notochord; however, arcualia are supposed to be totally absent in the terminal region of the caudal fin. The cellular morphology of these groups is obviously different from the cartilage observed in arcualia. This group of chondrocytes suggests the presence of a cartilaginous structure that was not observed on C&S specimens.

Discussion

Our study is the first to describe the developmental events in the median fins in specific stages of the life history of *Petromyzon marinus* (i.e., ammocoete, metamorphosing ammocoete, adult). The development of the appendicular skeleton was categorized into seven developmental steps. We demonstrated a clear relationship between the developmental events in fins and the total length of ammocoetes. We also described the

morphology of the skeleton during two different developmental stages (i.e., stage 4, stage 5) in metamorphosing ammocoetes. We detected an asynchronous development among some buccal elements and also among skeletal systems. Moreover, we are the first to provide information about the development and cellular morphology of the arcualia and fin rays of a lamprey.

Patterning and Morphology of the Appendicular Skeleton

Previous studies have extensively described the skeletogenesis of *Petromyzon marinus;* the formation of skeletal elements starts at 13 days post-fertilization (dpf) and is achieved around 33 dpf [5,9-11], which corresponds to stage 18 as described by Piavis [10,28]. Considering that all skeletal elements of the ammocoete are present by that stage [7,9,14,43], we assumed that the skeletal development occurring afterwards was limited to somatic growth. However, our data showed that skeletogenesis was still ongoing during the ammocoete stage; the appendicular skeleton develops in a predetermined sequence of seven steps that is size (i.e., total length) dependent. We are the first to demonstrate a direct relationship between the size of an ammocoete and the skeletal development of the median fins. Size was found to be a better predictor than age for skeletal development in numerous osteichthyans [101,102]. Since developmental progress and growth respond similarly to environmental variation, body size represents a cumulative measure of both processes [102]. The size is therefore a good proxy to discriminate ammocoete since it directly reflects the progression of the development in the median fins. This peculiar relationship was not found in metamorphosing ammocoetes and adults.

Our results showed that fin rays of *P. marinus* developed first in the second dorsal fin (step 2-3), followed by the caudal fin and the first dorsal fin (step 4-5). Fin rays of the first and second dorsal fins always developed anteroposteriorly. These results contrast with the condition in actinopterygians, where the endoskeletal and exoskeletal elements develop

first in the caudal fin [84,86,87,89,101,103]. In these derived groups, bidirectional development of the skeletal elements is the most common pattern found in the dorsal, anal and caudal fins [98,103]. In fact, only a few taxa (i.e., Carangidae, Scombridae) are known to possess anteroposterior development at the level of the dorsal fins [103]. However, fin rays of the caudal fin developed bidirectionally starting at the posteriormost region in *P. marinus*; observations of this highly conserved pattern in *P. marinus* corroborate the plesiomorphic condition of this condition.

Even if the second dorsal fin and the caudal fin are not distinctly separated in *P. marinus,* their developmental independence is obvious. Our results showed that fin rays were developing in both dorsal fins at different times and did not follow the same differentiation pattern. The longitudinal elongation of fins is often the result of the fusion of all median fins, as seen in many different groups of fishes (e.g., lampreys, hagfishes, pleuracanth sharks, Polypteriformes, Anguilliformes, Ophidiiformes) [103,104]. Such fins are highly specialized and are derived from separate, short-based fins [104]; thus, it is normal to observed different differentiation patterns. The fusion between the second dorsal and caudal fin may represent a derived condition and does not necessarily imply a developmental dependency.

Descriptions of the general morphology of the appendicular skeleton in lampreys are scarce in literature. The presence of median cartilaginous rods, bifurcation and posterior fusion of fin rays in the posteriormost region of the caudal fin have previously been observed by Tretjakoff [48] Goodrich [32,48] and Jollie [8]. Posterior fusion of the rays occurred at the caudal fin level in step 7 ammocoetes. Jollie [8] described this condition as a skeletal mass enclosing the end of the notochord and the neural and haemal canal, with "fin radials" extending out from this mass to the margin of the caudal fin. Fusion of skeletal elements in the caudal complex represents an adaptation that has been observed in many osteichthyans: the fusion of modified uroneurals and epurals in the Salmonidae, resulting in the formation of the stegural [77]; fusion of hypurals to form a hypural plate in advanced teleosts (e.g., atherinomorphs, gasterosteids, scombrids) [105,106] and the urostyle of

Perciformes, formed by several urocentra, fused with hypurals elements [107]. In every case, the fusion of posterior elements has a specific purpose for the propulsive locomotion. However, Petromyzontiformes possess an undulatory swimming and are known to possess a poor propulsive ability [24,108]. We observed that the posterior end of the notochord was slightly curved upward in smaller ammocoetes, whereas more advanced ammocoetes (i.e., step 7) with a posterior fusion possessed a fairly straight notochord. We hypothesise that the function of the posterior fusion in *P. marinus* might help to increase the lateral stabilisation of the notochord.

The morphology of the caudal skeleton of *P. marinus* is rather similar to that of hagfishes (e.g., *Myxine glutinosa, Eptatretus burgeri* and *Bdellostoma* sp.). Ota *et al.* [70] have recently showed that cartilaginous median rods are present dorsally to the neural tube and ventrally to the notochord in addition to bifurcation in the fin rays in the caudal fin of *E. burgeri*. In this species, the posterior fusion occurs between both median rods at the posteriormost end of the caudal region, enclosing the tips of the notochord and the neural tube. In the different species of hagfishes for which the information is available (i.e., *M. glutinosa, E. burgeri* and *Bd.* sp.), the median rods are noticeably larger than the ones found in *P. marinus*. Since the skeletogenesis of the median fins has never been studied in hagfishes, we cannot determine if the median rods result from the fusion of the proximal ends of the rays, as was seen in *P. marinus*. Owing to the difficulty of outgroup comparison at this level of the craniate phylogeny, we cannot determine whether the similarity in the caudal skeleton of hagfishes and lampreys constitute shared synapomorphies or symplesiomorphies.

It is not known why such posterior fusion and bifurcations of the fin rays occur in ammocoetes since their locomotion activities are rather limited [15]; ammocoetes occasionally leave their burrows for downstream migration in order to colonize new favorable habitats [38]. The tail length of ammocoetes increases abruptly within the first year of the larval life; an increase in the height of both dorsal fins was also observed in more advanced ammocoetes [108]. This period of anatomical changes leads to an improved

hydrodynamic efficiency and is followed by a steady growth for the rest of the larval stage [108]. These observations perfectly corroborate our results; as the size of the ammocoete increases, more skeletal elements are added and further developmental events occur in the median fins. Ammocoetes with higher and stronger fins will eventually possess a better swimming efficiency and thus be able to migrate greater distances.

Since the appendicular skeleton is the only portion of the skeleton that does not exhibit any changes among the three stages, we believe that has an adaptive value for the ammocoete to invest energy into the development of its fins prior to metamorphosis. In fact, ammocoetes need to stock lipids a year prior to entering metamorphosis in anticipation of this period of intense series of transformations [41]; to develop their appendicular skeleton during that stage would likely divert energy reserves needed for other processes.

Fin Rays

Lamprey fin rays have sometimes been called "fin radials" [61,109]. It has been uncertain whether the cartilaginous rods observed in the median fin lobes of lampreys are true rays or fin radials. Fin rays are endochondral elements internally supporting and strengthening the fins [32,91,97], whereas radials are defined as endochondral elements linking the median fins of gnathostomes to the axial skeleton [51,91]. Fin rays are articulated proximally to the radials [51]. The fin rays of *P. marinus* show gross similarities to lepidotrichia; in both cases, the fin rays are present from the base to the border of the fin, thus internally supporting the fin. Both types of fin rays possess distal bifurcation that results in the emergence of sister rays; moreover, branched or unbranched fin rays can coexist within one fin [110]. We observed the presence of second order bifurcation of fin rays in both second dorsal and caudal fins. This condition was found in the caudal lepidotrichia of the zebrafish [111] and in the pectoral fin rays of some batoids [112]. Fossil actinopterygians (e.g., *Cheirolepis canadensis*) [113] and sarcopterygians [114] are also

known to possess fin rays with second to fifth order of bifurcation. Only Bendix-Algmreen [115] noticed first order bifurcation of radials in paired fins of Cladoselache and other forms of bradyodonte. However, as far as we know, there is no case of second order bifurcation in radials. Considering their position within the fins, the presence of both first and second order of bifurcation and their peculiar histologic morphology, the endochondral elements of *P. marinus* do possess the characteristics of true rays rather than fin radials.

The fin rays of *P. marinus* are not segmented and never mineralize, bringing their condition closer to that of the ceratotrichia. Another interesting feature of the fin ray of *P. marinus* is the absence of endoskeletal elements to connect to the vertebral column. The presence of a gap between the axial and the appendicular skeletons in selachians and Rajiformes is considered to be derived [32]; this condition is explained by the need to possess a fin with an independent action for a more active swimming mode of life. For now, we can only hypothesize that this condition in *P. marinus* may also be derived.

The fin rays of *P. marinus* are the sole skeletal elements of the appendicular skeleton and develop proximodistally in order to reach the margin of the fin; this developmental pattern is consistent with the development of lepidotrichia of actinopterygians and dipnoans [51,98,103]. In addition, the proximal ends of the caudal fin rays lengthen anteriorly, in parallel to the neural tube; these extremities eventually fuse altogether to form the dorsomedian and ventromedian rods. Even though the appendicular skeleton of *P. marinus* is extremely simple, the developmental pattern of its fin rays is consistent with the one found in derived taxa. Thus, proximodistal development of rays is a very conservative pattern and likely represents a plesiomorphic condition for vertebrates.

We showed that the fin rays of *P. marinus* are composed of layers of flat rectangular chondrocytes; the same morphology can be observed in the branchial arches [9,25]. On the other hand, median rods are formed by large, pentagonal cells; such cellular morphology was also found in the trabecula, subchordal bar and parachordals of ammocoetes [9]. Our results suggest that two different cellular morphologies are present among the skeletal elements of *P. marinus*, thereby supporting the hypothesis of Martin *et al.* [9]. Based on the

cellular morphological differences, Martin *et al.* [9] suggested that "type I" cartilage, consisting of stacked rectangular cells and described as "soft" tissue by Parker [94], possess a support function for gills and other tissues [9]; and "type II" cartilage, formed by larger polygonal cells and called "hard" tissue by Parker [94], would represent another type of tissue with an unknown function. Based on these descriptions, fin rays can be considered as a "type I" cartilage, whereas the median rods correspond to "type II" cartilage. However, even if the cellular morphology underlines an obvious difference between these types of cartilage in *P. marinus*, histological analysis showed that the cellular morphology of the cartilage were mostly the same for fin rays and median rods. In both cases, the cartilage is formed by piled-up chondrocytes surrounded by a peripheral extracellular matrix - very thin in the case of the median rods. The chondrocytes in the median rods are slightly larger, more globular and less regularly stacked; this observation represents the only noticeable difference between the two cartilages; our results therefore showed a clear delineation between the cartilage of the median rods and the cartilage of fin rays.

The skeleton of *Myxine glutinosa* also consists of two distinct types of cartilage that are slightly different from the ones observed in *P. marinus*. The matrix of "hard" cartilage consists of an extensive extracellular material surrounding the chondrocytes, whereas "soft" cartilage is highly cellular with thin filaments of matrix between cells [58,70]. The "soft" cartilage was observed in the fin rays of *M. glutinosa*. Thus, both *P. marinus* and *M. glutinosa* possess a "type I" cellular morphology in their median fins. The fin ray cartilage of *P. marinus* shows no morphological or ultrastructural similarity whatsoever with hyaline cartilage, which is the tissue normally found in the cartilaginous endoskeletal elements of fishes (e.g., fin rays, hypurals, radials) [51,110]. However, this cartilage does share similarities with the cartilaginous tissue composing the fin rays of the inshore hagfish (*Eptatretus burgeri*). The presence of stacked cuboid chondrocytes with very little extracellular matrix has been observed in histological sections of the caudal fin rays of *E. burgeri* [70]. Lamprey and hagfish cartilages are known to possess similar Col1A2 expression patterns [116]; however, a number of significant differences in terms of the amino acid composition indicate that these two cartilages do not represent the same

cartilaginous tissue [58]. However, the morphological and cellular similarities between the cartilages of *P. marinus, E. burgeri,* and *M. glutinosa* suggest a shared plesiomorphic condition consisting of a basic cartilage formed of stacked cuboid chondrocytes surrounded by a thin peripheral network of extracellular matrix.

Metamorphosis

We observed the modifications of the skeleton in two stages of metamorphosis (i.e., stage 4 and stage 5). Because the neurocranium is the first skeletal system to show signs of modification [7,17], it was expected that skeletal elements of this region would already be formed by stage 4. In fact, neurocranial and branchial elements were always well developed in stage 4 and stage 5 specimens, indicating that the development of these structures occurred in earlier stages of metamorphosis. Developmental patterns depend on the acquisition of structures that will quickly fulfil the functional requirements imposed by environmental and behavioural constraints [103]. Our results suggest that the development of the neurocranial and branchial elements are prioritized since their development and transformation is achieved before the buccal elements. However, the development of the feeding structures is normally prioritized over respiratory structures in osteichthyans [84]. The pharynx of the lampreys is a multifunctional system that serves for the respiration and osmoregulation [117]; thus, the transformation of the branchial skeleton may represent a priority during metamorphosis. Also, considering that there is a 4 to 10 month period of fasting between the onset of metamorphosis in ammocoetes and the beginning of feeding in adults [38,108], the development of the branchial skeleton is more relevant. In addition, because the buccal skeleton is not initially present in the ammocoete, the time required for its development may be longer.

The sequence of formation of buccal elements varies in terms of their relative timing. More specifically, four elements (i.e., anterior dorsal, posterior dorsal, anterior lateral,

posterior lateral) did not develop according to the same sequence in stage 4 and stage 5. Our results suggest a lack of developmental coordination among buccal elements and also among skeletal systems in *P. marinus*. In fact, a metamorphosing ammocoete can possess a large number of well-developed arcualia in addition to epitrematic and hypotrematic protuberances present along each of the branchial arches, whereas the pericardial cartilage is underdeveloped. For other specimens, the pericardial cartilage is well developed, but the epitrematic and hypotrematic protuberances are mostly absent. Specimens can even possess a well-developed pericardial cartilage, but few and weakly developed arcualia. Our observations strongly suggest that the skeletal systems (i.e., buccal, neurocranial, branchial, axial) develop independently in metamorphosing ammocoetes. As far as we know, asynchronous development among skeletal systems has never been documented in fishes. Ontogeny is a complex process that strongly depends on the timing of developmental events [118]. It was previously assumed that the temporal sequence of developmental events is constant within a species [119]. However, minor intraspecific variation in ossification sequences has been documented in the skull (i.e., *Danio rerio, Betta splendens*) [79,120] and the appendicular skeleton (i.e., *Salvelinus alpinus*) [77,121] of some osteichthyans. Grünbaum *et al.* [77] specifically stated that intraspecific developmental variation occurs in terms of timing. In fact, the timing of appearance and the timing of transitions of skeletal states (i.e., transition from cartilaginous to bony states) would better explain the developmental plasticity rather than the order of events within a sequence [77]. Thus, development can be variable intraspecifically and this variation mostly depends on the developmental timing. However, all of these investigations refer to osteichthyans; observation of intraspecific variation has never been observed in a basal group such as lampreys. This suggests that the asynchronous development may be a plesiomorphic condition for vertebrates.

We found that a staging based on the presence of skeletal structures is hardly possible because of (1) the asynchronous development among skeletal elements and among skeletal systems and (2) the fact that most of the specimens already possess all of their skeletal elements. In fact, specimens possessing all the typical traits of a stage-4 metamorphosing

ammocoete also possessed all of the adult skeletal elements, indicating that the transformation of the skeletal system occurs prior to the changes to the external morphology. Although most of skeletal elements were already present by stage 4, some morphological and developmental differences were noticeable between stage-4 and stage-5 specimens. Arcualia were found developing in the D1 and D2 regions in stage-5 specimens, whereas they were totally absent in these regions in stage-4 specimens. The general morphology of the branchial basket (i.e., elongated and compressed) in stage 5 was closer to the definitive condition of the adult, whereas the branchial basket in stage-4 specimens was more similar to the ammocoete condition. We can therefore infer that stage-5 specimens are effectively more advanced in their development based on the chondrogenesis of the branchial and axial skeletons. Overall, our results suggest that the classical staging by Youson and Potter [20] is relevant and easy to use in order to rapidly discriminate metamorphosing ammocoetes.

Vertebral Elements, Mediodorsal Vertebral Elements and Notochordal Cartilage (Dorsolateral Cartilage)

The presence of a notochord is a synapomorphy of chordates, while the presence of vertebral elements is considered as a vertebrate synapomorphy [32,122,123]. Development of the notochord is viewed as a secondary adaptation for a burrowing habit, as seen in *Amphioxus* [32]. In lower vertebrates such as the Petromyzontiformes, the notochord provides important internal support for their elongated body and represents the main component of the axial skeleton. The notochord of the Petromyzontiformes is composed of an inner core of vacuolated cells, covered by a thick perinotochordal fibrous sheath and an elastica externa [21,124]. The vertebral elements develop along the axial skeleton during metamorphosis [47]. We identified three types of elements along the axial skeleton of metamorphosing ammocoetes and adults: (1) arcualia, (2) mediodorsal vertebral elements and (3) notochordal cartilage/dorsolateral cartilage (also found in ammocoetes).

Arcualia develop anteroposteriorly in the branchial region. We detected a second center of development at the level of the second dorsal fin (i.e., D2-2), where arcualia are developing bidirectionnally. In many living [84,87,89,90,101] and fossil [72,74] taxa, the most common ossification pattern of the dorsal vertebral components occurs anteroposteriorly in the cranial region. The ossification pattern can occasionally occur bidirectionnally in the middle of the vertebral column, as seen in guppies (Poeciliidae) [74]. However, in certain taxa such as the ostariophysan actinopterygians, ossification occurs at two different locations; one is located at the third centrum of the Weberian apparatus and the other is present in the caudal region [74,110]. Although present in *P. marinus*, it is unclear whether the presence of two centers of formation for vertebral elements represents a plesiomorphic condition for vertebrates. In fact, the phylogenetic distribution for this condition is still poorly documented. However, the anteroposterior development of dorsal vertebral elements in the anteriormost region of the axial skeleton represents the most common pattern found in vertebrates and was also found in *P. marinus*, suggestive of a plesiomorphic condition.

The chondrogenesis of the vertebral elements begins during the metamorphosis [1,47]; we confirmed this condition because no arcualia were detected in ammocoetes. Thus, the chondrogenesis is still ongoing in adults because arcualia located in the D1 and C regions are still weakly developed for some adult specimens. Arcualia located in the predorsal, D1 and D2 regions are fairly constant in terms of size and morphology, whereas these aspects were variable for the arcualia located in the caudal region. Previous authors [1,14,125] have described the arcualia located in the trunk and caudal regions as small and irregular in their size and shape; however, except for the branchial and caudal regions, our observations suggest that the shape and size of arcualia are generally constant.

Two pairs of arcualia per myomere were observed on every specimen, placed anteriorly and posteriorly to the ventral root nerves of the posterior branches, except for the branchial region in which only one pair of arcualia is present. Wake [125] and Remane [126] hypothesized that the first arcualia located in the branchial region results from the

fusion of two adjacent arcualia, thus supporting the presence of two pairs of arcualia per segment in the branchial region of lampreys [125]. We think that this hypothesis is unlikely since progressive fusion of arcualia would have been noticed among metamorphosing ammocoetes. Even for metamorphosing specimens that possessed few arcualia in the branchial region (i.e., S3-8, S3-9), none showed signs of ongoing fusion.

Our observations confirm that the arcualia are not fused to the elastica externa, thus supporting the observations by Potter and Welsch [47] on *Geotria australis*. In teleosts, the elastic sheath of the notochord possesses perforations at the level of the neural arches; in early developmental stages, cartilaginous cells invade the fibrous sheath or the elastic sheath through these perforations [32,51]. Since the notochordal sheath of *P. marinus* does not develop an external cartilaginous ring and no perforations were observed on the elastica externa, fusion of the notochord and arcualia is unlikely to be observed.

The mediodorsal vertebral elements are present alongside the arcualia in the branchial region and were found in metamorphosing ammocoetes and adults. Even though the number of mediodorsal vertebral elements is variable among specimens, we determined that their development was occurring anteroposteriorly. We never observed mediodorsal elements along the first two arcualia of the branchial region *contra* observations by Tretjakoff [48]. Since the presence of mediodorsal elements is restricted to the branchial region only, no second center of development was observed. These elements were originally called "median cartilages" [1,14,50]; however, this term is incorrect because these elements are arranged on an axis that is medial and parallel to the arcualia, but they are never located on the top of the notochord (i.e., median position). Mediodorsal elements are unpaired elements as they are not bilaterally present along the notochord. We occasionally observed the presence of two mediodorsal elements alongside the same arcualia; this condition has never been documented before.

Mediodorsal vertebral elements are not serial homologues to arcualia because they are not in the same topographical relationship with the notochord; moreover, a mediodorsal element never bifurcates and does not possess a foramen. The homology between the

arcualia of the Petromyzontiformes and the neural arch of gnathostomes is uncertain. The term "basidorsal" has also been used to designate the arcualia of lampreys [50,125]; however, both terms are considered to be synonyms. Basidorsals/arcualia are bilaterally paired cartilages located on the dorsolateral side of the notochord that eventually fuse distally and ossify into neural arches or dorsal arcocentra [51,81,127], whereas arcualia of lampreys are paired cartilaginous elements that are also present on the dorsolateral side of the notochord, but they never fuse distally and never mineralize or ossify. The term arcualia may be slightly misused when designating the vertebral elements of lampreys. However, we cannot deny that the general morphology and the localisation of lamprey arcualia along the notochord strikingly resemble the condition of the neural arches; in addition, the anteroposterior development of arcualia located in the first center of development (i.e., branchial region) is similar to the pattern found in teleosts. The arcualia of the Petromyzontiformes may represent a precursor of the neural arch rather than a homologous form.

Løvtrup [123] considered that the Petromyzontiformes are more closely related to gnathostomes than to Myxiniformes because of the presence of a series of synapomorphies, including the arcualia. However, vertebral cartilages were found ventrally to the notochord in the caudal region of a species of hagfish (*Eptatretus burgeri*) [70,128]. The vertebral elements of *E. burgeri* are not paired and are not regularly arranged along the notochord; however, by studying the transcription factors involved in the differentiation of the sclerotomes in the early and late pharyngular stages (i.e., *Twist* and *Pax1/9* genes, respectively), these vertebral cartilages were found to be developmentally homologous with the ventral vertebral elements of the gnathostomes [70,128]. As far as we know, no such study exists for the vertebral elements of the Petromyzontiformes. Wake [125] reported that some ventral elements can be occasionally present in the caudal region of lampreys; however, no such structure has been observed in *P. marinus*. The only structure present ventrally to the notochord was the ventromedian rod of the caudal fin, which results from the fusion among the proximal parts of the hypochordal fin rays.

Goodrich [50] mentioned a fusion of arcualia in the caudal region of adults; he suggested that the last vertebral elements were fused altogether, thus resulting in the formation of a thin, continuous plate located dorsally to the notochord. We observed that the arcualia in the caudal region become reduced in size and irregular in shape, in addition to being bent posteriorly towards the notochord; however, we did not observe fusion among arcualia in this region. We observed the presence of a cartilaginous plate consisting of type II cartilage on cleared-and-stained specimens, dorsally to the notochord and along the entire length of the caudal region. Histological sections on an adult revealed the presence of two dorsolateral rods positioned dorsally to the notochord and encompassing both sides of the neural tube; such notochordal cartilage has never been described in lampreys before. The dorsolateral rods are fused to the ventral parts of the dorsomedian rod, thus forming a continuous arch encompassing the neural tube. Surprisingly, this notochordal cartilage was also observed in the posteriormost region of ammocoetes (i.e., small amounts of chondrocytes dorsally to the notochord in the posteriormost region of the caudal fin), indicating that chondrogenesis for this cartilage has occurred in earlier stages of development. However, this "cartilaginous plate" cannot be the result of the fusion of modified arcualia *contra* Goodrich's [32] interpretation. The cellular morphology of the dorsolateral cartilage clearly differs from that of arcualia. In fact, the cartilage found in the dorsolateral rods consists of stacked polygonal chondrocytes surrounded by very small amounts of extracellular matrix; such cartilage is similar to that of the median rods (i.e., dorsomedian and ventromedian rods). On the other hand, the chondrocytes forming the cartilage of arcualia are embedded in lacunae and surrounded by a network of territorial extracellular matrix of variable thickness.

Our observations of the arcualia cartilage suggest similarities with lamprin, which has been previously described in the piston, annular cartilage and neurocranial elements [52-54]. No subperichondrium zone was observed in the arcualia cartilage, whereas this zone is present in most lamprin-based cartilages; however, the absence of a zone of differentiating cells may be due to a functional or developmental constraint. Chondrocytes are organised in randomly arranged chondrones and are barely visible beneath the extracellular matrix,

making it difficult to compare chondrocyte morphologies between the peripheral and the central zones. However, histological sections of arcualia made by Potter and Welsch [47] indicate that morphology of the chondrocytes is rather similar in the peripheral and the central zones. The cellular composition of the arcualia cartilage has not yet been tested; thus, we cannot confirm for now that the major component of the extracellular matrix for this cartilage consists of lamprin. However, we can infer that the cartilaginous plate located in the posteriormost region of the caudal fin is not the result of arcualia fusion since their cellular morphologies clearly differ.

The presence of cartilaginous "demi-arches" in the posteriormost region of the caudal fin is also found in hagfishes (e.g., *E. burgeri, M. glutinosa, Bdellostoma* sp.) [70,128]. In every studied species, specimens have a median bar attached to the notochord by an intermediate cartilaginous nodule positioned at one discrete location. These "demi-arches" are present along each side of the neural tube with distal ends fusing with the median bar of the caudal fin. Based on Ota *et al.* [70], histological sections revealed that this peculiar structure was formed of stacked cuboid chondrocytes, which is the same cellular morphology that is found in the median rods of *P. marinus*. As previously mentioned, the notochord is an important organ for the stabilisation and undulatory swimming in the Petromyzontiformes, but also in the Myxiniformes. The presence of this notochordal cartilage in the caudal region might increase the stabilisation of the notochord - together with the posterior fusion of the rays, in order to provide a better swimming efficiency.

The notochord was found to synthesize perinotochordal proteoglycans, thus playing an important role in somite chondrogenesis in chordates [129]. A recent study by Jonansson [130] showed that gekkotan lizards possess notochordal cartilages (i.e., chordoid and chondroid tissues) that are likely produced by the notochord itself. This notochordal cartilage is composed of chordoid cells, found in intravertebral articulations, and of chondroid cells, present in mid-vertebral locations [130]. Studies have demonstrated similarities between cartilage and notochord at the level of the cellular structure and tissue function [131,132]; in fact, cartilage would share more characteristics with notochordal

tissue than osseous tissue (Brian K. Hall, pers. comm. 2013). Most of the notochord persists as chordoid tissue and can be viewed as a cartilage-like tissue that possesses the ability to produce cartilage (i.e., notochordal chondrogenesis) [130,133,134]. The notochordal sheath of *P. marinus* possesses a composition similar to that of cartilage, with the presence of cartilage-like proteoglycan [135] and type II collagen fibres [136]. Studies have reported strong expression patterns of Col1A2 in cartilaginous tissues of *P. marinus*, including the notochord and the notochordal sheath [116,137]. Based on these histological and histochemical analyses, the notochord of *P. marinus* could be considered as a cartilage-like tissue, which implies that it could also possess the ability to produce cartilage. However, since the chondrogenesis of the notochordal cartilage (i.e., dorsolateral rods) was not followed in our study, it is not clear whether the cartilage present dorsally on the notochord is the result of a notochordal chondrogenesis or the result of an extension of the dorsomedian rod.

Comparisons and Future Work

To our knowledge, our study is the first to find a direct relationship between the size and the skeletal development in ammocoetes. In this study, we achieved an extensive description of the patterning and morphology of the fin rays of a lamprey. The cellular morphology of the fin rays of *Petromyzon marinus* is greatly similar to that of the inshore hagfish (*Eptatretus burgeri*) [70]. The presence of an extremely simple cartilage that consists of stacked cuboid chondrocytes with very little peripheral extracellular matrix would likely represent a plesiomorphic condition shared by both species; more studies need to be done in order to clarify if this condition is shared by both taxa. Goodrich [32] had detected the presence of bifurcation of fin rays in adult lampreys; we observed the presence of both first and second order bifurcation in the median fins of *P. marinus*. Previous authors have suggested that the skeletal elements present in the median fins of lampreys should be called radials [8,61,109]. However, the fin rays of *P. marinus* are internally supporting the

fin from the base to the margin in addition to possess both first and second order bifurcation. Although more histochemical studies need to be done, we consider that the cartilaginous rods present in the median fins should be regarded as true rays rather than fin radials. We are the first to describe the patterning and the general morphology of arcualia in terms of body sections; our observations of the cellular morphology of arcualia corroborate those of Potter and Welsch [47]. The cartilage composing the arcualia differs from the hyaline cartilage, which is the most common type of cartilage found in teleosts [51]. However, the cellular morphology of the cartilage is likely similar to lamprin, a cartilage found in the piston, annular cartilage and neurocranial elements of lampreys [52-54]. We detected two centers of development for arcualia; as far as we know, this condition is only found in ostariophysan actinopterygians [110]. We are still not certain if arcualia are homologous to the neural arches; however, the anteroposterior development of the dorsal vertebral elements in the anteriormost region of the axial skeleton is the most common pattern in osteichthyans and was also found in *P. marinus*, suggestive of a plesiomorphic condition. We are the first to detect an asynchronous development among skeletal elements for a species of lamprey; we are also the first, to our knowledge, to mention an asynchronous development among skeletal systems.

Extensive histological and immunohistological studies need to be done on a wider variety of skeletal elements, including arcualia and fin rays, in order to determine their cellular composition and peculiarities. In addition, the development of the notochordal cartilage (i.e., dorsolateral rods) needs to be observed in earlier stages of development to confirm the existence of notochordal chondrogenesis in lampreys. Considering the phylogenetic importance of lampreys, all the information about its cartilage can be useful in order to comprehend the ancient origin of cartilage and its evolution.

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Tables

Table 1. Loadings of the first two principal components derived from a PCA based on the covariance matrix of eight $\log_{10}$-transformed morphometric traits upon 15 specimens of *Petromyzon marinus* (14 metamorphosing ammocoetes, 1 adult).

	PC1	PC2*
Eigenvalue	6.107	1.718
Explained variance (%)	71.383	20.082
Traits		
Mouth width (MW)	**-0.791**	**-0.563**
Mouth length (ML)	**0.430**	-0.333
Eye length (EL)	0.253	-0.370
Prenostril length (PNL)	0.240	**-0.556**
Snout length (SL)	0.185	-0.333
Postocular length (POL)	-0.169	0.104
Interocular distance (IL)	-0.054	-0.063
Branchial length (BL)	-0.048	0.012

Traits of greater contribution on each component are in **bold.**

*Number of statistically interpretable axes determined by the Kaiser-Gutman criterion.

Table 2. Development of the arcualia in 14 metamorphosing ammocoetes of *Petromyzon marinus* with respect to five body sections. Each body section is divided into three subregions (1, anterior; 2, middle; 3, posterior).

	Branchial			Predorsal			D1			D2			Caudal			TL
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	(mm)
S3-1	+++	++	+	0	0	0	0	0	0	0	0	0	0	0	0	125.6
S3-2	+++	++	+	0	0	0	0	0	0	0	0	0	0	0	0	128.6
S3-3	+++	+++	+	0	0	0	0	0	0	0	0	0	0	0	0	129.1
S3-4	+++	++	+	0	0	0	0	0	0	0	0	0	0	0	0	131.4
S3-5	+++	+++	++	0	0	0	0	0	0	0	0	0	0	0	0	131.5
S3-6	+++	++	+	0	0	0	0	0	0	0	0	0	0	0	0	132.8
S3-7	+++	++	+	0	0	0	0	0	0	0	0	0	0	0	0	133.0
S3-8	+++	++	0	0	0	0	0	0	0	0	0	0	0	0	0	136.6
S3-9	++	+	0	0	0	0	0	0	0	0	0	0	0	0	0	138.0
S3-10	+++	++	+	0	0	0	0	0	0	0	+	0	0	0	0	138.1
S3-11	+++	++	+	0	0	0	0	0	+	+	++	0	0	0	0	139.0
S3-12	+++	++	+	+	0	0	0	0	+	+	++	+	0	0	0	140.3
S3-13	+++	++	+	+	0	0	0	0	0	+	++	0	0	0	0	142.9
S3-14	+++	++	+	0	0	0	0	0	+	+	++	+	0	0	0	144.1

0 : Absent + : Weakly developed ++ : Moderately developed +++ : Well developed

Table 3. Development of the arcualia in 19 adults of *Petromyzon marinus* with respect to five body sections. Each body section is divided into three subregions (1, anterior; 2, middle; 3, posterior).

	Branchial			Predorsal			D1			D2			Caudal			TL
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	(mm)
S4-1	+++	++	++	+	+	++	++	++	++	++	+++	+	0	0	0	114.1
S4-2	+++	+++	++	+	+	++	+++	++	+++	+++	+++	+	0	0	0	119.0
S4-3	+++	++	++	+	+	+	+	++	++	++	++	+	0	0	0	119.4
S4-4	+++	++	++	+	+	++	++	++	+++	++	+++	+	0	0	0	119.6
S4-5	+++	++	+	0	0	+	+	+	+	+	++	+	0	0	0	119.9
S4-6	+++	+++	++	++	++	++	+++	+++	+++	+++	+++	++	+	0	0	120.7
S4-7	+++	+++	++	+	+	++	++	++	++	++	+++	+	+	+	0	123.9
S4-8	+++	++	++	+	+	+	++	++	+++	+++	+++	+	0	0	0	125.0
S4-9	+++	+++	++	++	++	++	+++	+++	+++	+++	+++	++	+	0	0	126.2
S4-10	+++	+++	++	++	++	++	++	++	++	++	+++	++	0	0	0	130.5
S4-11	+++	++	+	+	+	++	++	++	+++	+++	+++	+	0	0	0	130.8
S4-12	+++	+++	++	++	++	+++	+++	+++	+++	+++	+++	++	++	+	0	131.2
S4-13	+++	+++	++	++	++	+++	+++	+++	+++	+++	+++	+	0	0	0	131.7
S4-14	+++	+++	++	++	++	+++	+++	+++	+++	+++	+++	+	0	0	0	136.2
S4-15	+++	+++	++	++	++	++	+++	++	+++	+++	+++	+	0	0	0	138.7
S4-16	+++	++	++	+	+	+	++	++	++	++	++	+	0	0	0	140.0
S4-17	+++	++	+	+	+	+	+	++	++	++	++	+	0	0	0	140.6
S4-18	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	0	145.2
S4-19	+++	+++	++	++	++	++	++	++	++	++	+++	++	+	0	0	153.0

0: Absent +: Weakly developed ++: Moderately developed +++: Well developed

Figures

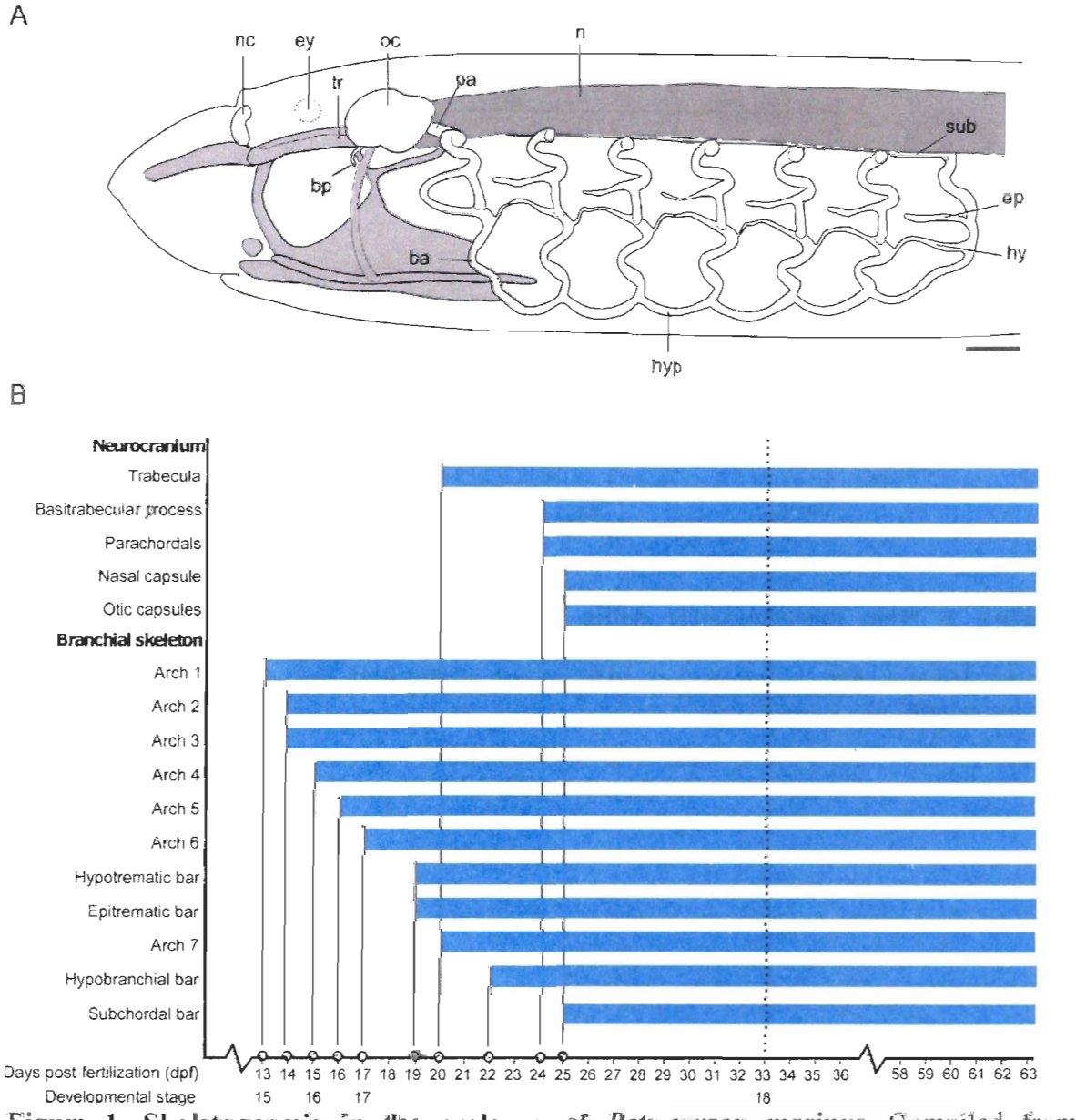


Figure 1. Skeletogenesis in the prolarva of *Petromyzon marinus*. Compiled from Morrison *et al.* [25], Yao *et al.* [12], Martin *et al.* [9] and Richardson *et al.* [5]. (A) General scheme of the neurocranial and branchial skeletons of the ammocoete. (B) Sequence of chondrogenesis of the skeletal elements, referring to the days post-fertilization (dpf) and the developmental stage based on Piavis [28]. White represents the cartilaginous elements of the skeleton, whereas light grey represents the mucocartilaginous elements. ba, branchial arch; bp, basitrabecular process; ep, epitrematic bar; ey, eye; hy, hypotrematic bar; hyb, hypobranchial bar; n, notochord; nc, nasal capsule; pa, parachordals; sub, subchordal bar; tr, trabecula; oc, otic capsule. Scale bar = 1 mm

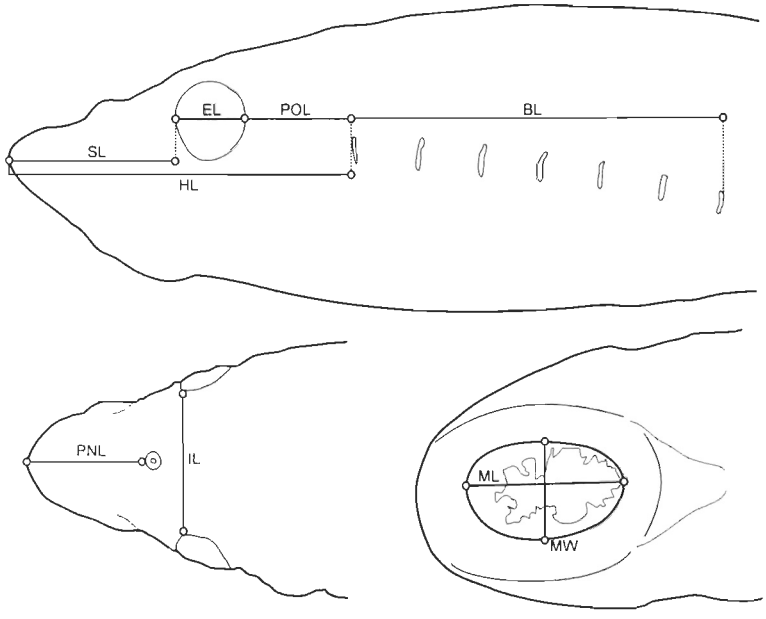


Figure 2. Nine morphometric traits measured on metamorphosing specimens of *Petromyzon marinus*. BL, branchial length; EL, eye length; HL, head length; IL, inter-ocular distance; ML, mouth length; MW, mouth width; PNL, prenostril length; POL, post-ocular length; SL, snout length.

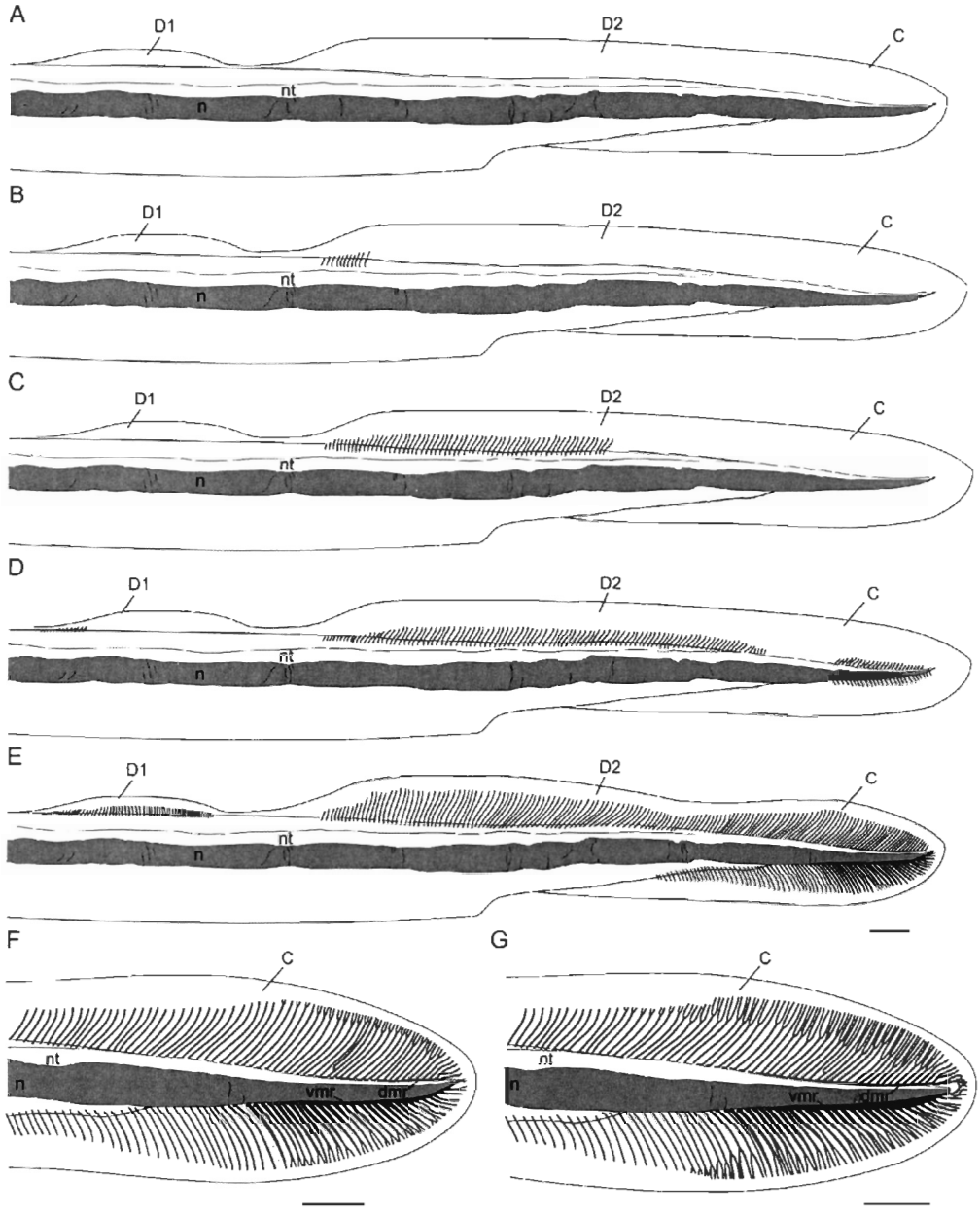


Figure 3. Developmental steps of median fins in *Petromyzon marinus* ammocoetes. Observations made on C&S specimens from the Sainte-Anne River population (n = 54) (A) Step 1, absence of fin rays, based on CMNFI 2013-0017-S1-03, 21.1 mm TL; (B) Step 2, based on CMNFI 2013-0017-S1-05, 32.3 mm TL; (C) Step 3, based on CMNFI 2013-0017-S1-07, 33.8 mm TL; (D) Step 4, based on CMNFI 2013-0017-S1-11, 38.7 mm TL; (E); Step 5, based on CMNFI 2013-0017-S1-28, 48.3 mm TL; (F) Step 6, based on CMNFI 2013-0017-S1-40, 69.2 mm TL and (G) Step 7, based on CMNFI 2013-0017-S1-41, 75.2 mm TL. dmr, dorsomedian rod; D1, first dorsal fin; D2, second dorsal fin; C, caudal fin; n, notochord; nt, neural tube; vmr, ventromedian rod. Scale bar = 1 mm.

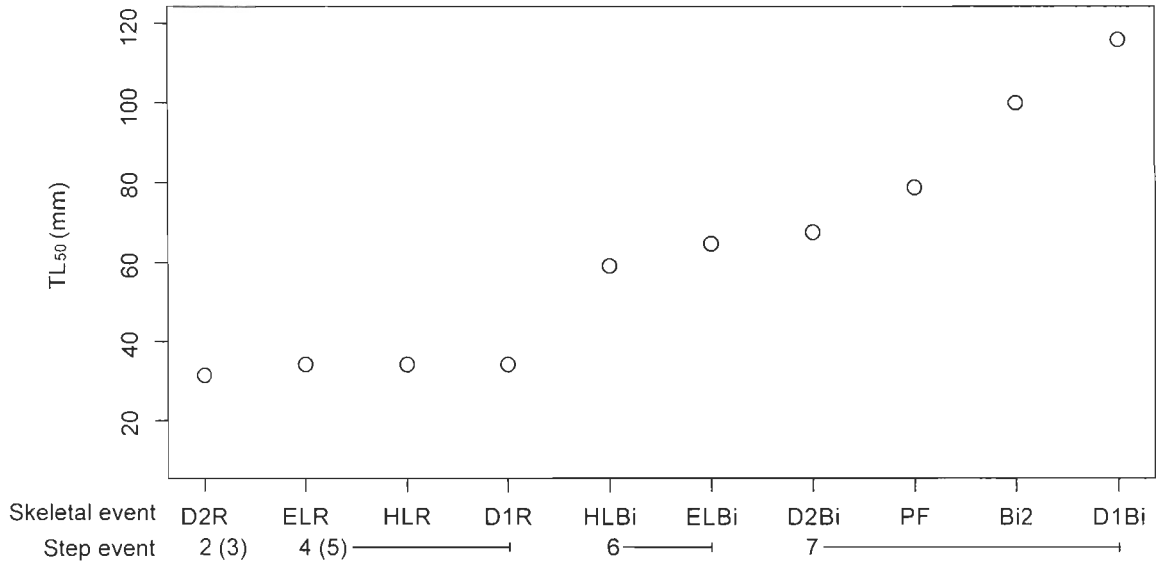


Figure 4. Sequence of developmental events in the median fins of *Petromyzon marinus* based on TL_{50}. Based on observations on C&S ammocoetes from the Sainte-Anne River population (n = 54); size ranges between 19 and 129 mm TL. Steps resulting from global observation are in parentheses. D2R: fin rays in D2; ELR: Fin rays in the epichordal lobe of the caudal fin; HLR: fin rays in the hypochordal lobe of the caudal fin; D1R: fin rays in D1; HLBi: bifurcation in hypochordal lobe; ELBi: bifurcation in epichordal lobe; D2Bi: bifurcation in D2; PF: posterior fusion; Bi2: second order bifurcation in D2 and caudal fin; D1Bi: bifurcation in D1.

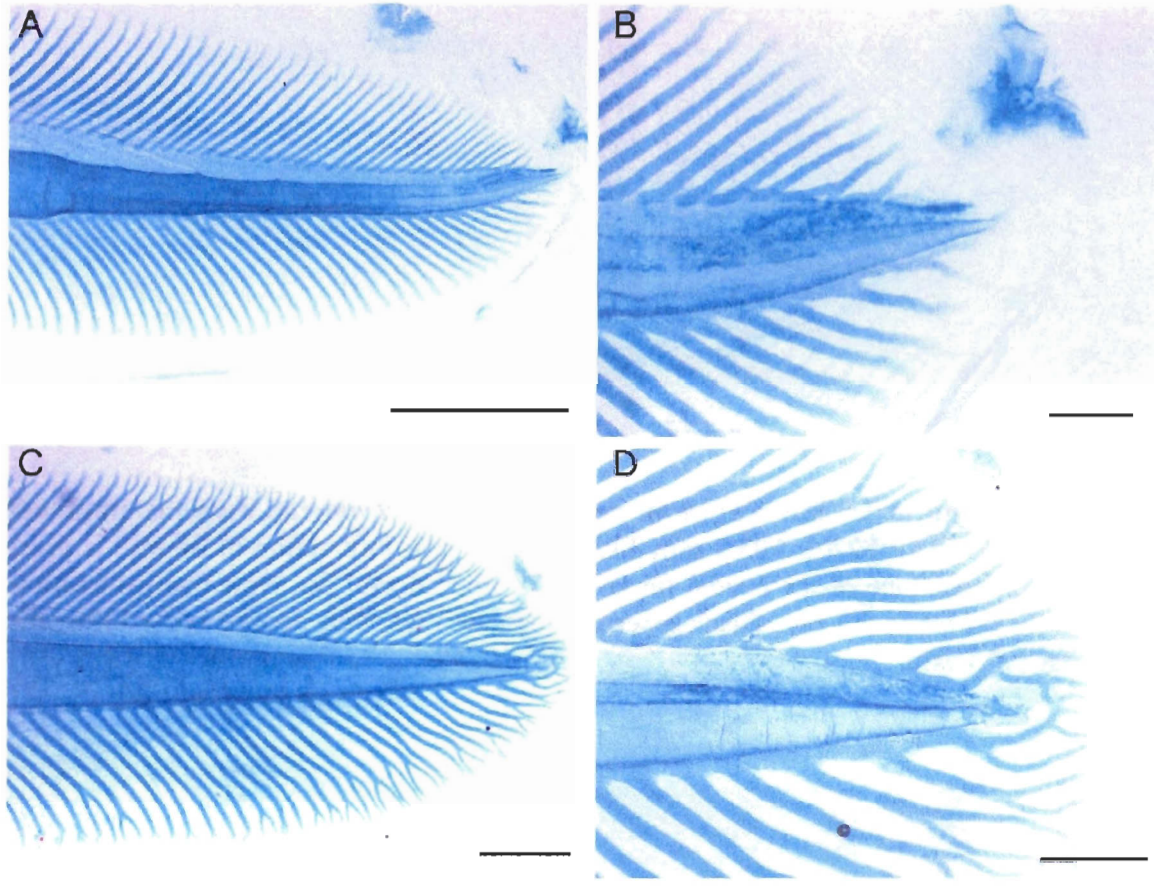


Figure 5. Developmental changes of the caudal fin in *Petromyzon marinus* ammocoetes. (A)-(B) Step 5 ammocoete (CMNFI 2013-0017-S1-36, 61.4 mm TL) with oval-shaped caudal fin. Note the absence of bifurcation, posterior fusion of fin rays and upward curve of the notochord. (C)-(D) Step 7 ammocoete (CMNFI 2013-0017-S1-41, 75.2 mm TL) with round caudal skeleton, presence of bifurcation, posterior fusion of fin rays and straight notochord. Scale bar for A and C = 1 mm; scale bar in B and D = 0.25 mm.

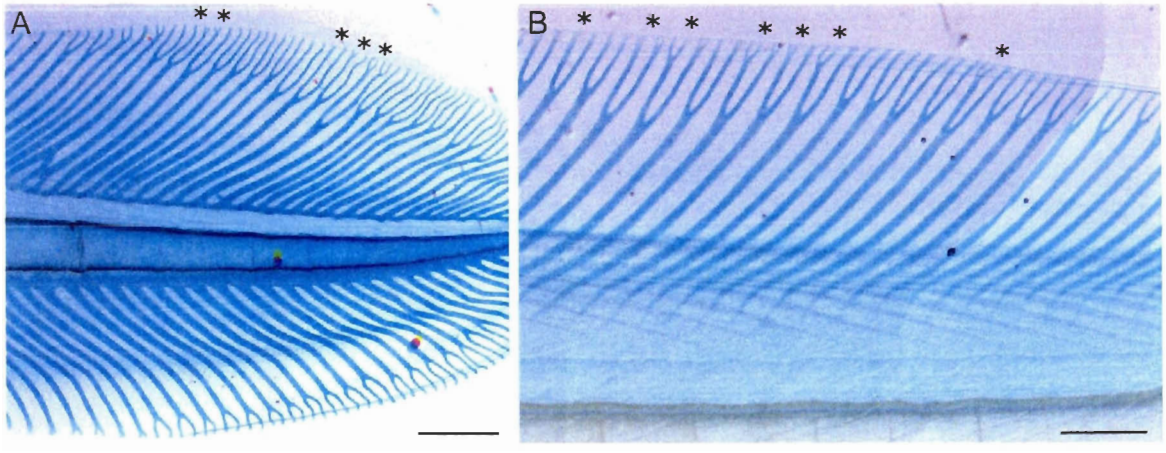


Figure 6. Second order bifurcation in median fins of *Petromyzon marinus* ammocoete (CMNFI 2013-0019-S2-02, 103.4 mm TL). Second order bifurcation of fin rays is present in (A) epichordal lobe of the caudal fin and (B) second dorsal fin. Note that bifurcation is not present on every fin ray. Black asterisks indicate rays with second order bifurcation. Scale bar = 0.1 mm.

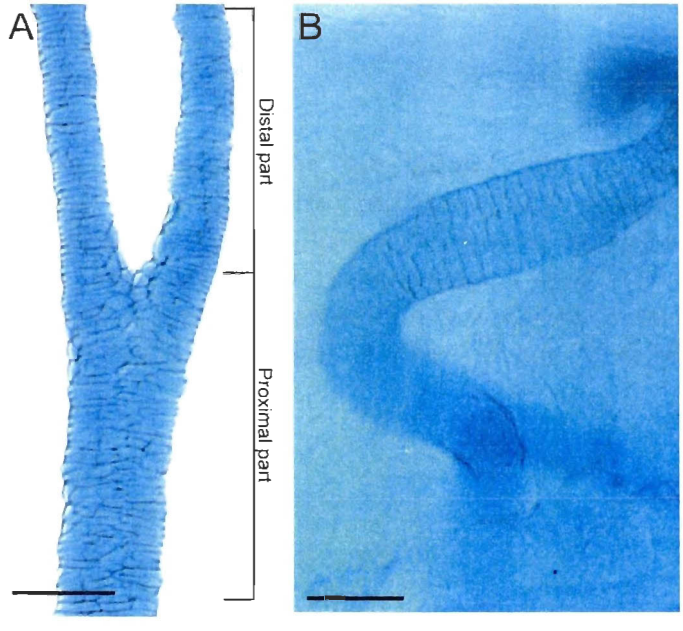


Figure 7. Comparative cellular morphology of a C&S *Petromyzon marinus* ammocoete (CMNFI 2013-0017-S1-41, 75.2 mm TL). Stacking of chondrocytes in (A) a caudal fin ray and (B) mid-section of branchial arch 7. Scale bar for A = 0.25 mm; Scale bar for B = 0.01 mm.

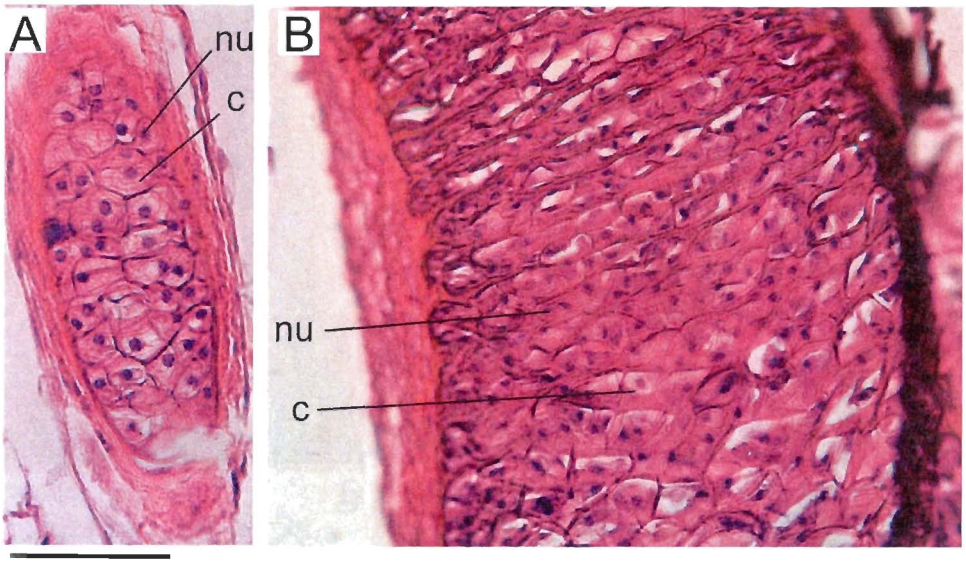


Figure 8. Transverse sections of a *Petromyzon marinus* ammocoete (AMPMH-01, 114.1 mm TL). Elongated and stacked chondrocytes are present in (A) a fin ray located in the posteriormost region of the caudal fin and (B) the mid-region of branchial arch 7. c, chondrocyte; nu, nucleus. Scale bar = 0.5 mm.

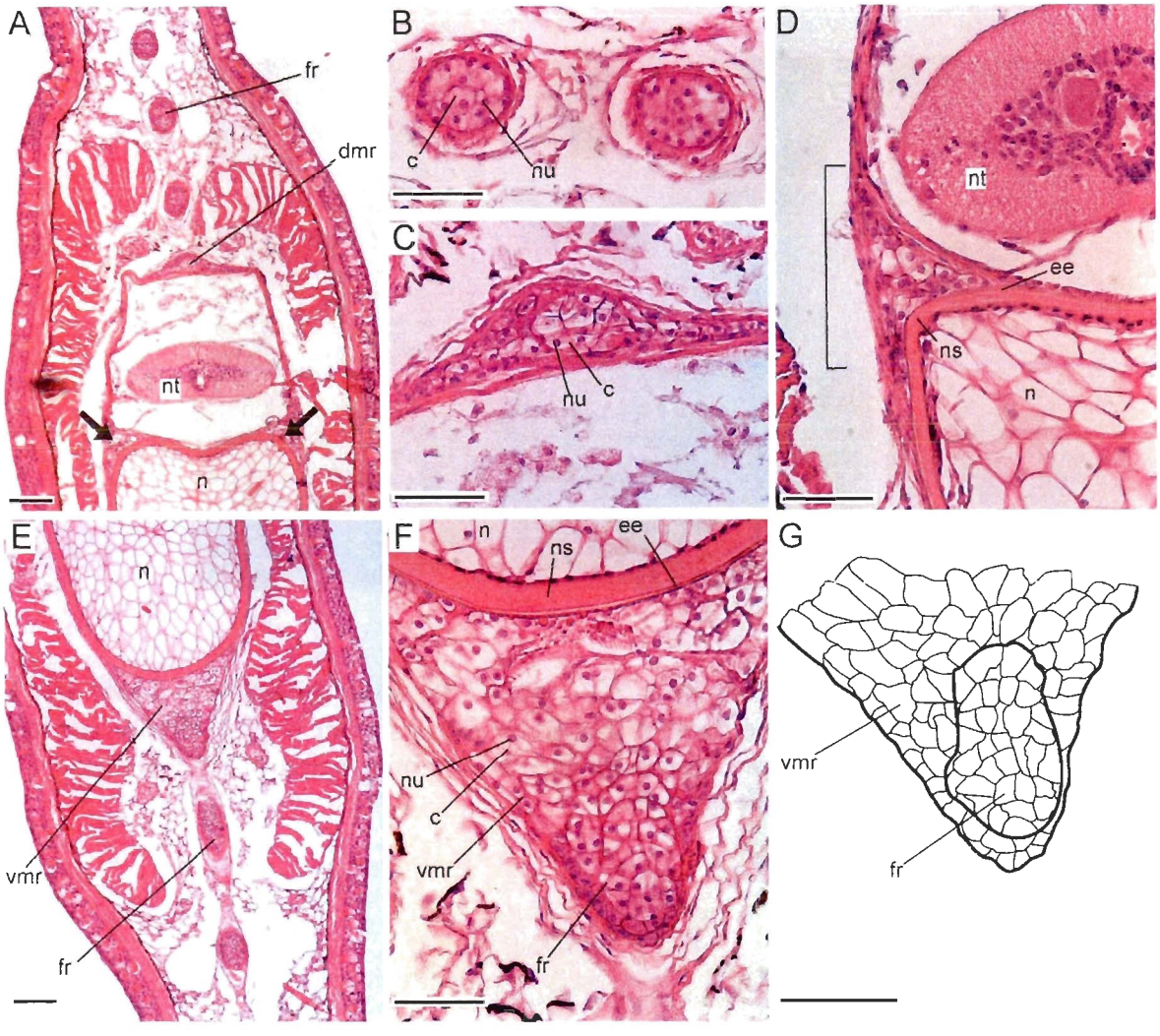


Figure 9. Transverse sections of a *Petromyzon marinus* ammocoete (AMPMH-01, 132.9 mm TL). Posteriormost region of the caudal fin. (A) Dorsal region of the caudal fin. (B) Chondrocytes in a fin ray, closely stacked and cuboid, whereas chondrocytes present in the dorsomedian rod are slightly more globular (C). (D) Chondrocytes located between the neural tube and the notochord, as indicated by black arrows in (A). Note the similarity of this structure to the shape and location of an adult arcualium. (E) Ventral region of the caudal fin, showing fin rays and ventromedian rod lying ventrally to the notochord (F) Ventromedian rod formed by larger globular chondrocytes, with well-delimited anchorage region for a fin ray (G). c, chondrocyte; dmr, dorsomedian rod; ee, elastic externa; fr, fin ray; n, notochord; ns, notochordal sheath; nt, neural tube; nu, nucleus; vmr, ventromedian rod. Scale bar for A and E = 0.1 mm; scale bar for B-D and F-G = 0.05 mm.

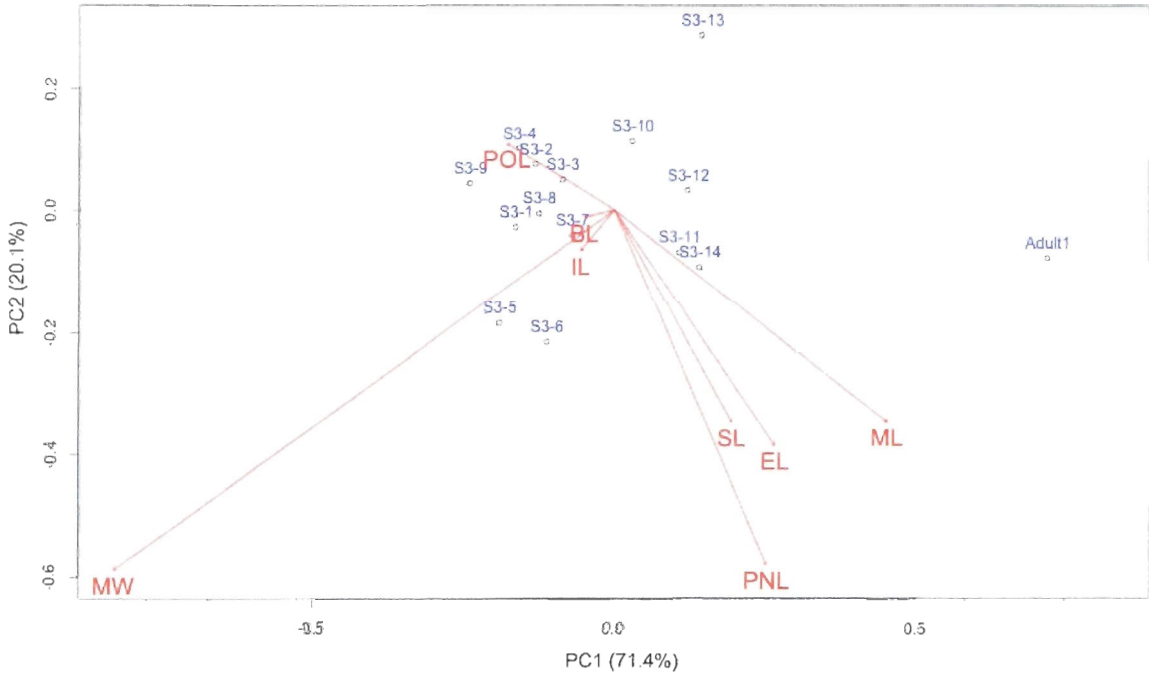


Figure 10. Covariance PCA of eight log_{10}-transformed morphometric traits of metamorphosing specimens of *Petromyzon marinus* (n = 14). Ordination graphic represents the factor loading plot of PC1 against PC2. Adult1 (S4-1, 114.1 mm TL) serves as reference for the adult condition.

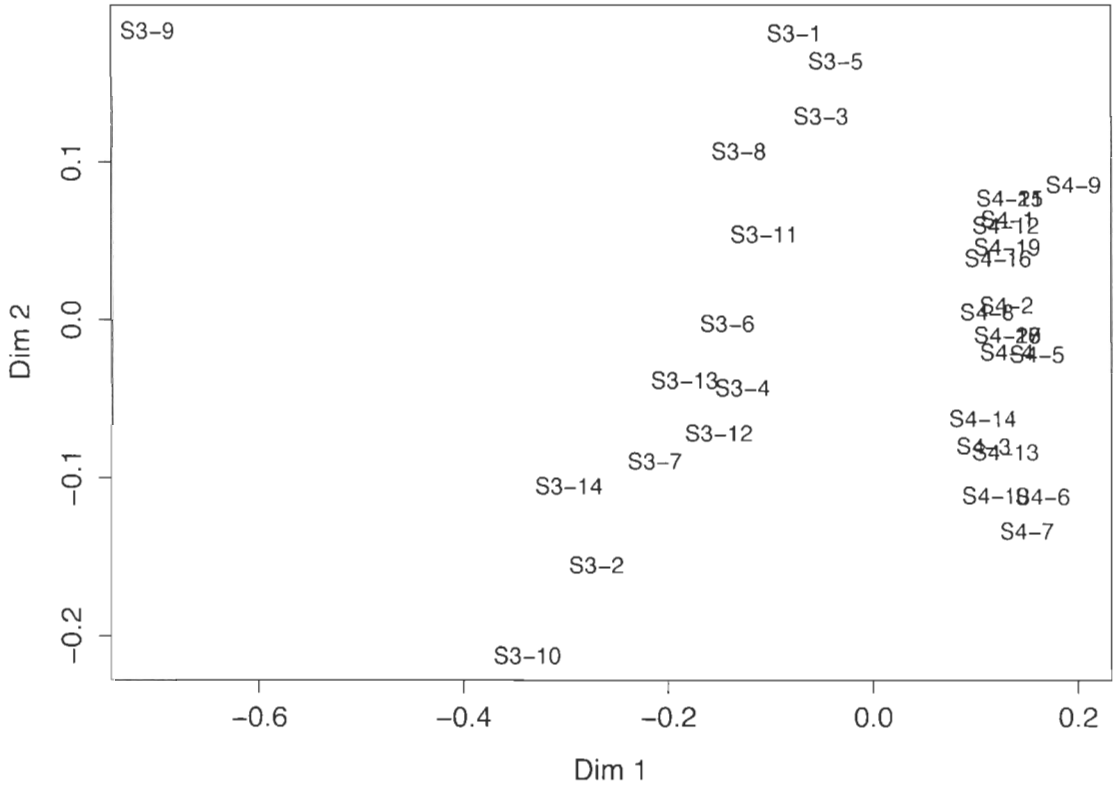


Figure 11. MDS analysis of metamorphosing ammocoetes (n = 14) and adults (n = 19) of *Petromyzon marinus*. Based on Dice distance matrix. S3-1 to S3-9 belong to stage 4 whereas S3-10 to S3-14 are stage 5 metamorphosing ammocoetes. S4-1 to S4-19 represent adults.

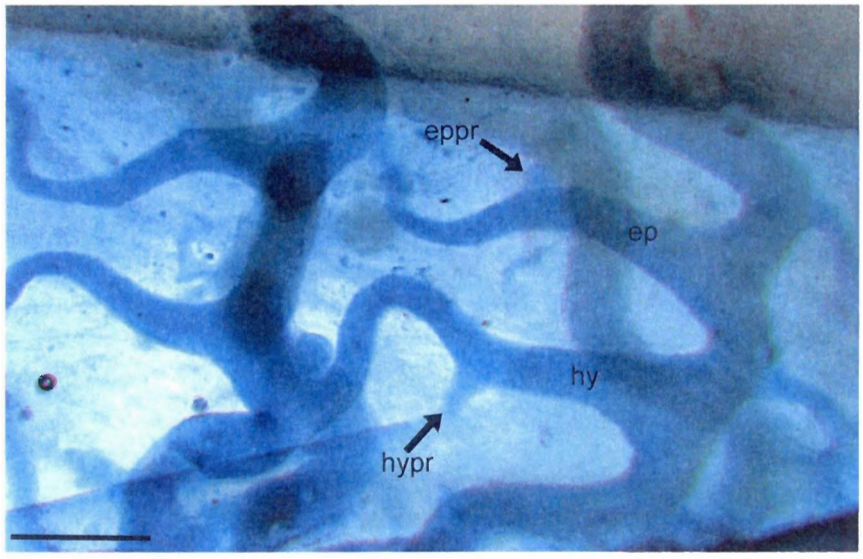


Figure 12. Developing cartilaginous protuberances during metamorphosis of *Petromyzon marinus*. Branchial arch 6 and 7 of a stage-5 ammocoete (S3-13, 142.9 mm TL). Black arrows point to the cartilaginous protuberances located on the epitrematic and hypotrematic bars. eppr, epitrematic protuberance; ep, epitrematic bar; hypr, hypotrematic protuberance; hy, hypotrematic bar. Scale bar = 1 mm.

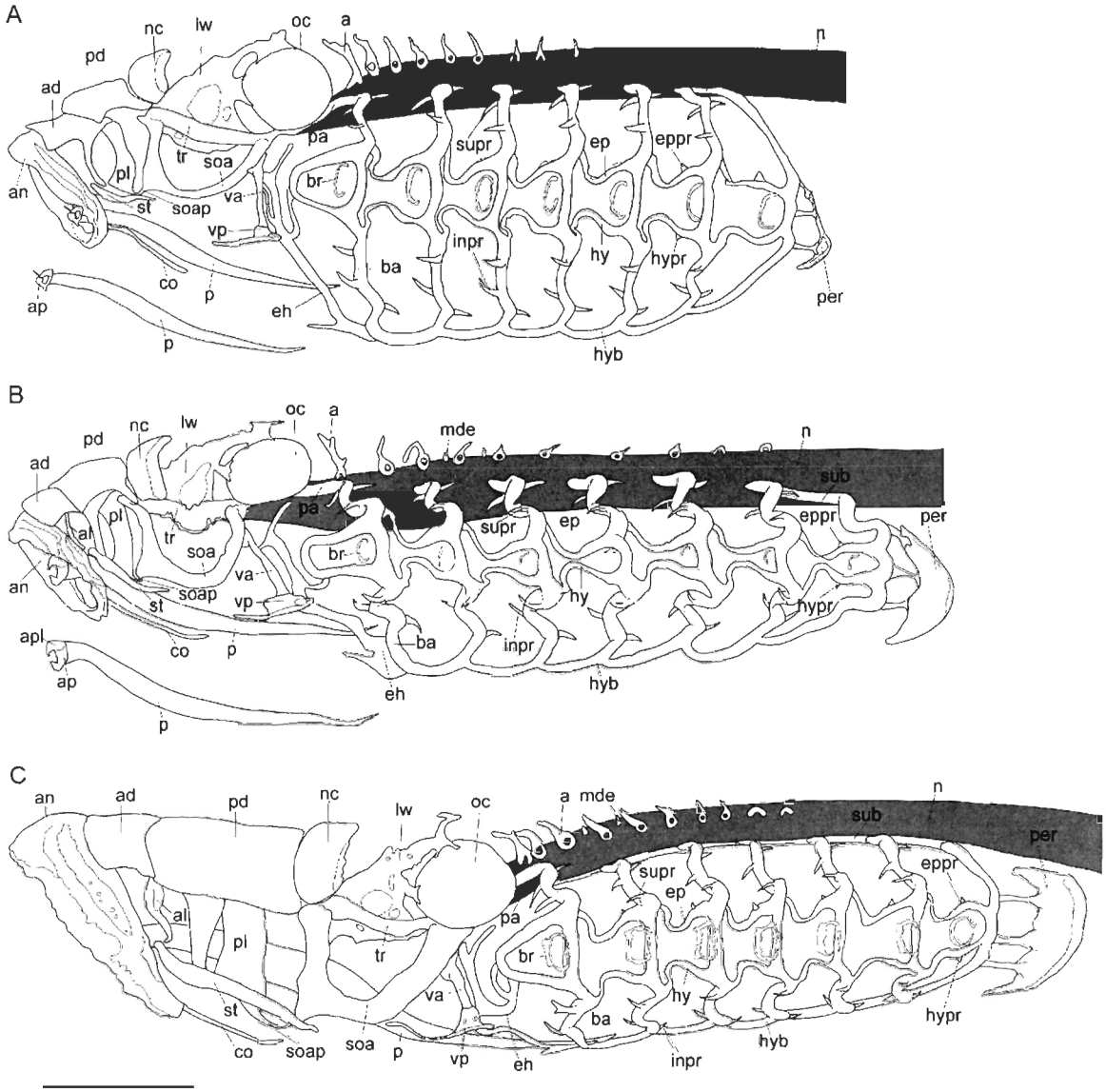


Figure 13. Development of the head, branchial and anterior trunk regions of *Petromyzon marinus*. (A) Stage-4 ammocoete (S3-1, 125.6 mm TL); (B) Stage-5 ammocoete (S3-10, 138.1 mm TL) and (C) adult skeleton (S4-1, 114.1 mm TL), apical cartilages not shown since not visible. a, arcualia; ad, anterior dorsal; al, anterior lateral; an, annular cartilage; ap, apical; apl, apical lateral; ba, branchial arch; br, branchial ring; co, copula; eh, extra hyal; ep, epitrematic bar; eppr, epitrematic protuberance; hy, hypotrematic bar; hyb, hypobranchial bar; hypr, hypotrematic protuberance; inpr, inferior process; lw, lateral wall of the neurocranium; mde, mediodorsal element; n, notochord; nc, nasal capsule; oc, otic capsule; p, piston; pa, parachordal; pd, posterior dorsal; per, pericardial cartilage; pl, posterior lateral plate; soa, subocular arch; soap, pillar of the subocular arch; st, stylet; sub, subchordal bar; supr, superior process; tr, trabecula; va, velar arch; vp, velar plate. Scale bar = 5 mm.

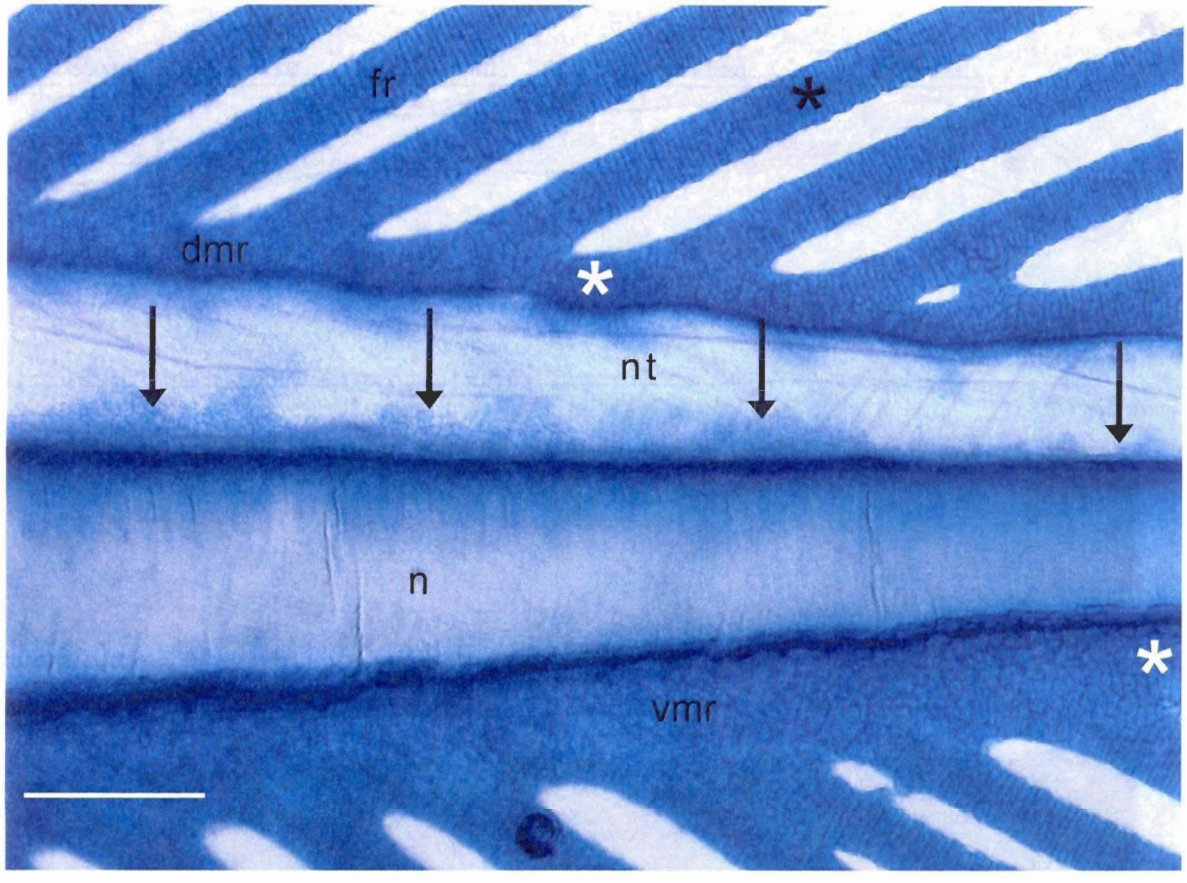


Figure 14. Two different cellular morphologies in the caudal region in *Petromyzon marinus*. Posterior region of the caudal fin of a metamorphosing ammocoete (S3-1, 125.6 mm TL). Large pentagonal cells present in the median rods of the caudal fin are shown by white asterisks. Regularly stacked cells with a rectangular shape forming the fin rays are indicated by black asterisks. Pentagonal chondrocytes lying dorsally to the notochord were also observed and are shown with black arrows. dmr, dorsomedian rod; fr, fin ray; n, notochord, nt, neural tube; vmr, ventromedian rod. Scale = 0.25 mm.

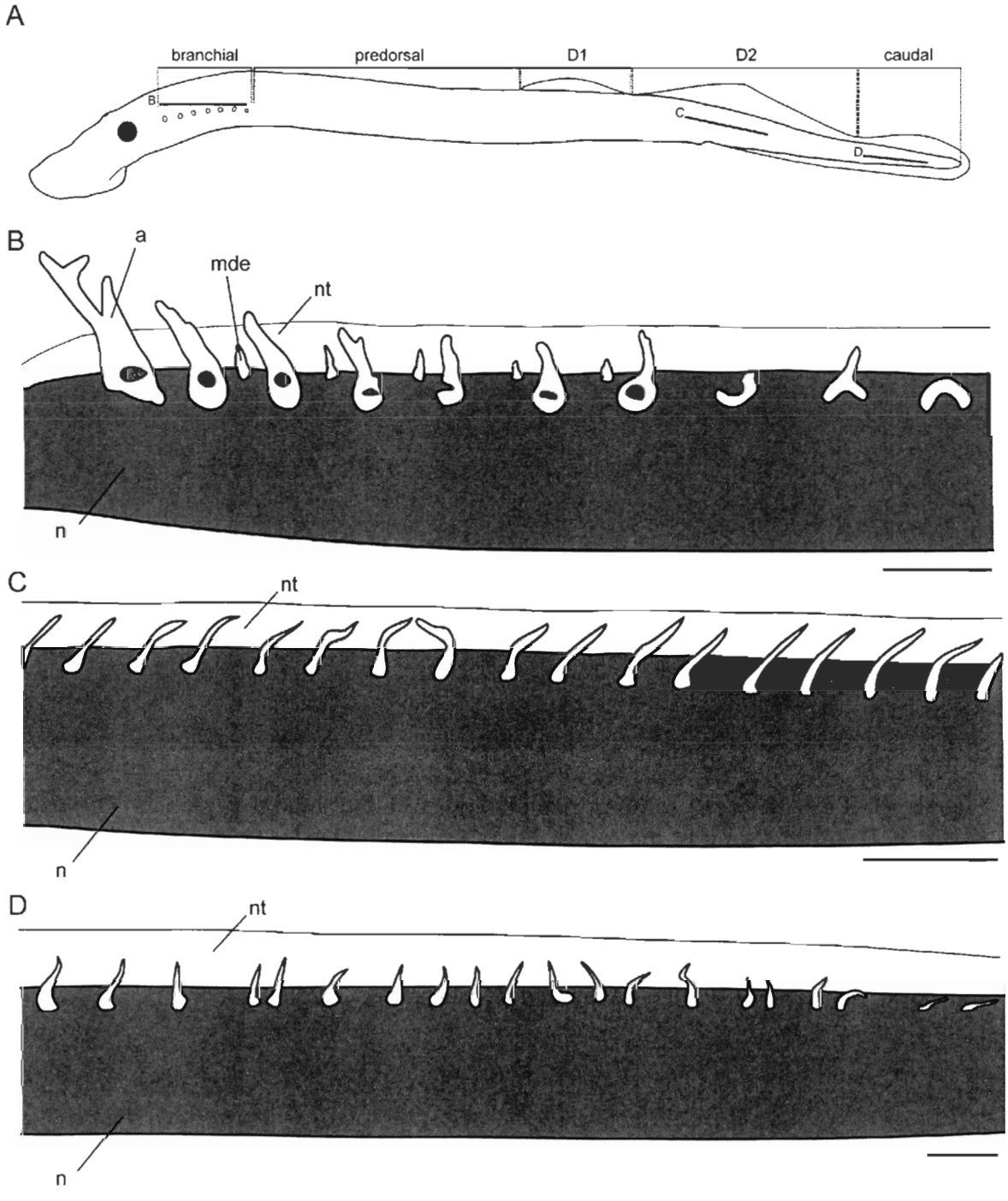


Figure 15. General morphology of the arcualia in *Petromyzon marinus*. Arcualia are assigned to five body sections (A). The scheme shows the morphology of arcualia located in the (B) branchial region, (C) D2 region and (D) caudal region. a, arcualia; mde, mediodorsal vertebral element; n, notochord; nt, neural tube. Scale bar in B: 1 mm ; Scale bar in C and D: 0.5 mm.

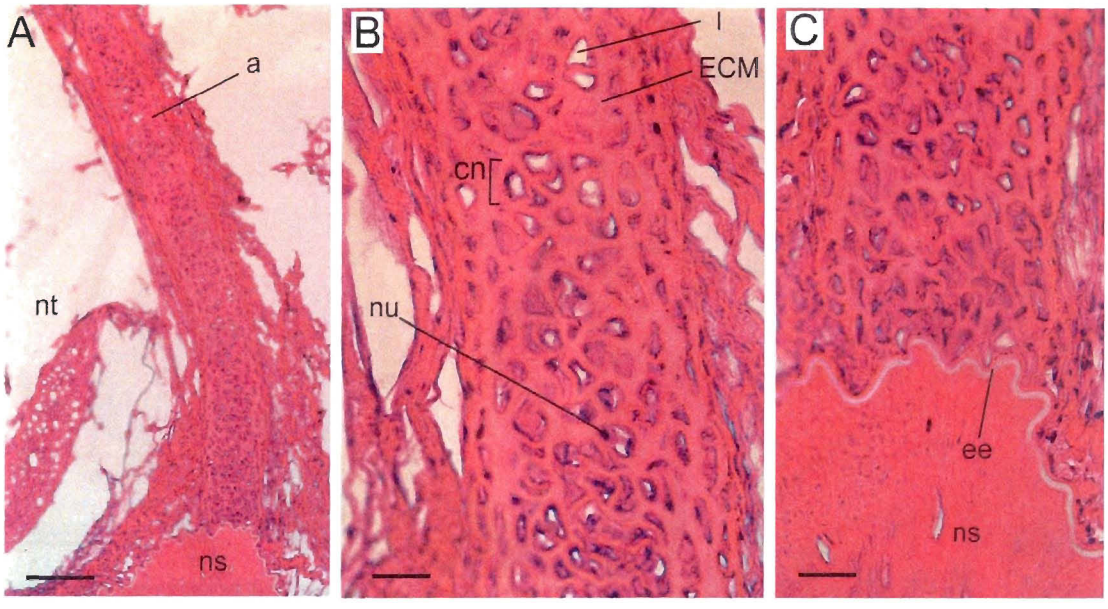


Figure 16. Transverse sections of arcualia of *Petromyzon marinus*. Beginning of D2 region of an adult (ADPMH-01, 270.5 mm TL). (A) General view, (B) mid-section and (C) proximal section of the arcualia. a, arcualia; cn, chondrone; ECM, extracellular matrix; ee, elastica externa; l, lacuna; ns, notochordal sheath; nt, neural tube ; nu, nucleus. Scale bar = 0.02 mm.

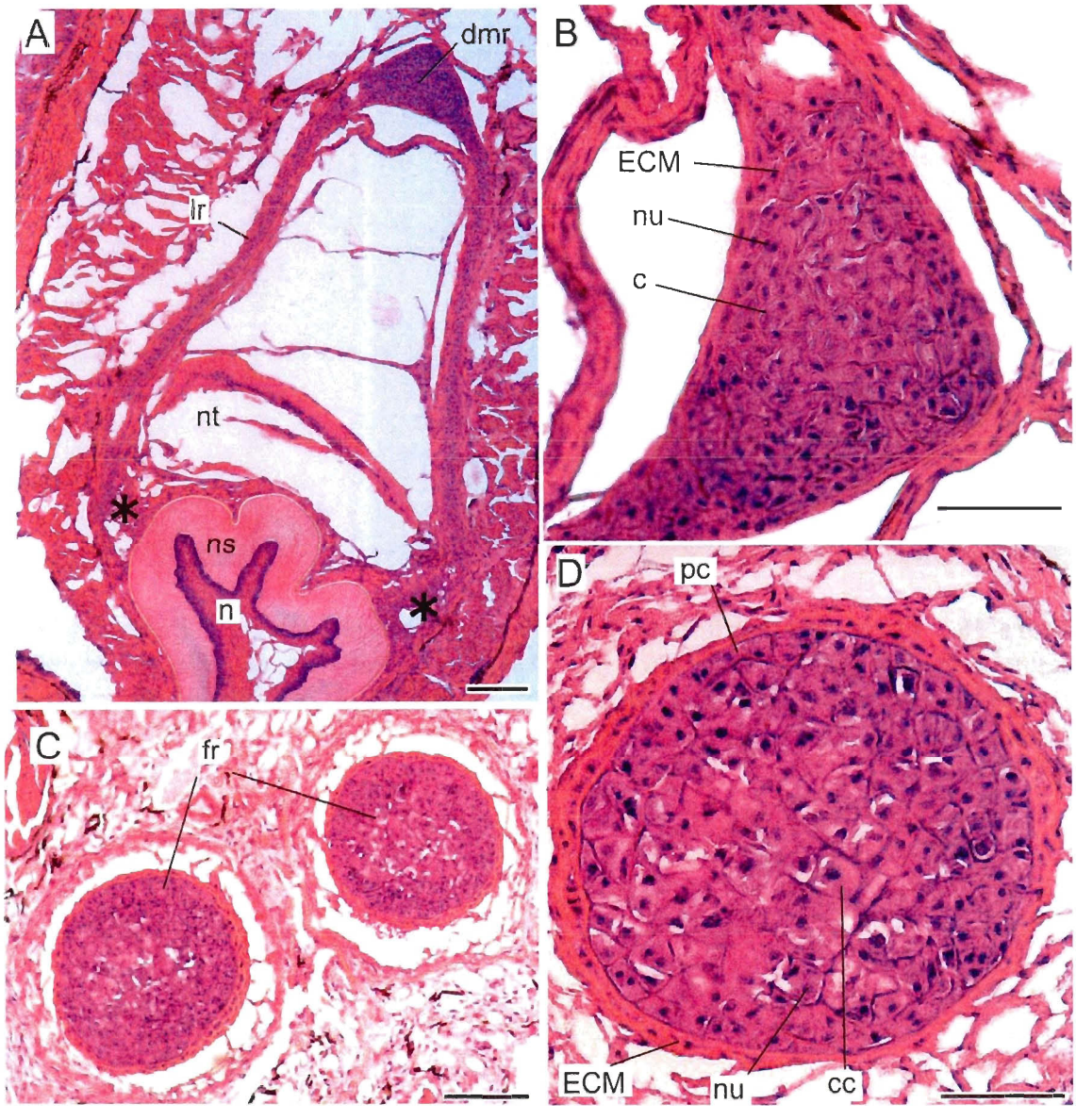
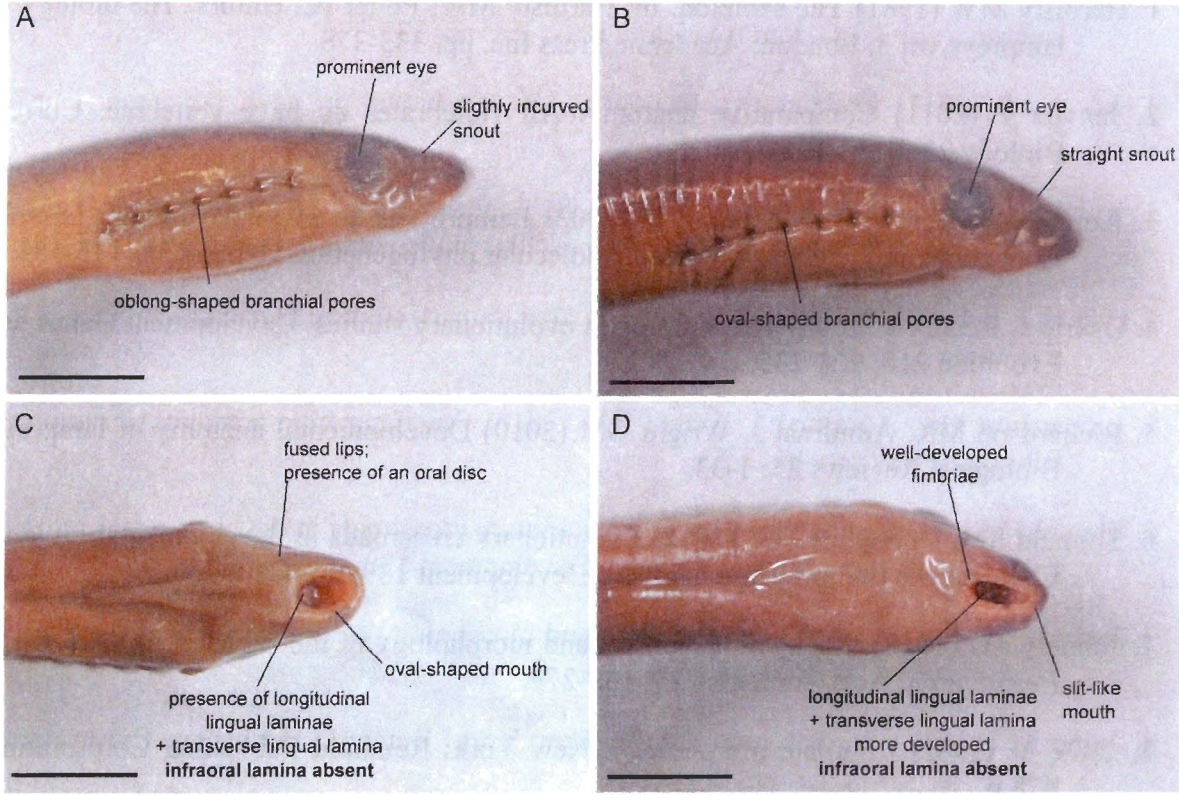


Figure 17. Transverse sections of the caudal fin of *Petromyzon marinus*. Posteriormost region of the caudal fin of an adult (ADPMH-01, 270.5 mm TL). (A) Cartilaginous dorsolateral rods are visible on each side of the neural tube in the dorsal region and reach the notochord (black asterisks). Note that no fusion is observed whatsoever with the external sheath of the notochord. (B) Dorsomedian rod composed of stacked cuboid-shaped chondrocytes. Territorial extracellular matrix is present between some groups of cells. (C) Regularly stacked chondrocytes with a rectangular shape are visible in fin rays, as extracellular matrix is present at the periphery only. (D) Chondrocytes located in the center of the rays are larger and more cuboid than those located in periphery. c, chondrocyte; cc, central chondrocyte; dmr, dorsomedian rod; ECM, extracellular matrix; fr, fin ray; lr, dorsolateral rod; n, notochord; ns, notochordal sheath; nt, neural tube; nu, nucleus; pc, peripheral chondrocytes. Scale bar for A and C: 0.1 mm; scale bar for B and D: 0.05 mm.

Appendix



Supplementary figure 1: Classical staging of metamorphosing ammocoetes based on the external morphology. (A) and (C) S3-1 to S3-9 specimens belong to stage 4 and possess oblong-shaped branchial pores, slightly incurved snout and an oval-shaped mouth with weakly-developed fimbriae. (B) and (D) S3-10 to S3-14 specimens are considered as stage 5 since they possess oval-shaped branchial pores, a fairly straight snout and a slit-like mouth that bears well-developed fimbriae. In both stages, the infraoral lamina was absent. Scale bar = 1 cm.

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CHAPITRE 2.
CONCLUSION

Nous avons observé le développement et la transformation des éléments squelettiques neurocrâniaux, branchiaux, buccaux, axiaux et appendiculaires pour trois stades de vie (i.e., ammocète, ammocète en métamorphose, adulte) chez la lamproie marine (*Petromyzon marinus*). Le squelette appendiculaire est le seul à se développer durant le stade ammocète. Notre étude est la première à trouver une relation directe entre la taille d'une ammocète et la progression de son développement squelettique. Cette relation a été confirmée par des calculs de TL_{50} effectués sur des matrices binaires contenant les données de présence d'éléments squelettiques et d'évènements développementaux (e.g., bifurcation, fusion postérieure des rayons) au niveau des nageoires médianes. Les ammocètes plus longues possèdent un nombre de rayons plus élevé avec des niveaux de complexité supérieurs (i.e., bifurcation de premier et second ordre). Avec un squelette appendiculaire mieux développé, les nageoires sont plus robustes, donnant potentiellement aux ammocètes une meilleure efficacité de nage ; les ammocètes sont ainsi capables de déplacements plus efficaces lors de leur migration occasionnelle [41]. Cette relation taille-dépendante a été confirmée pour les ammocètes provenant de la rivière Sainte-Anne ; cependant, nous ne savons pas si cette relation peut s'appliquer à l'ensemble des populations d'ammocètes de *P. marinus* ou même à d'autre espèces de lamproies. Une multitude de facteurs extrinsèques pourraient éventuellement influencer la taille et/ou le développement des ammocètes, tels que la température de l'eau [36,54,57], la disponibilité de la nourriture [36,54] et potentiellement la distribution tropique (i.e., hémisphère nord versus hémisphère sud). Des observations doivent être effectuées sur un meilleur échantillonnage de

populations et d'espèces différentes afin de confirmer si cette relation est inconditionnellement présente chez les Pétromyzontiformes.

Nous sommes les premiers à notre connaissance à décrire les évènements développementaux et la morphologie au niveau du squelette appendiculaire pour une espèce de lamproie. Grâce aux observations sur les spécimens colorés au bleu alcian et à un proxy de taille standardisé (TL_{50}), nous avons décrit sept étapes développementales se produisant au niveau des nageoires médianes des ammocètes. Nous avons également décrit la morphologie des rayons des nageoires au niveau macroscopique (i.e., coloration au bleu alcian) et microscopique (i.e., coupes histologiques avec coloration hématoxyline-éosine). Les seuls travaux effectués sur la description des nageoires médianes et leur support squelettique depuis le siècle dernier sont ceux de Tretjakoff [70] et Goodrich [1]. Nos résultats complètent leurs observations et schémas qui ont été maintes fois repris dans la littérature. Cependant, la fusion postérieure des rayons ainsi que la bifurcation de premier et second ordres devront tous les trois être incorporés dans les futurs schémas portant sur les nageoires médianes de *P. marinus*.

Notre étude est la première à décrire le patron développemental des éléments squelettiques des nageoires médianes chez une espèce de lamproie. Pour *P. marinus*, les rayons cartilagineux se développent tout d'abord antéropostérieurement dans la deuxième nageoire dorsale, puis bidirectionnellement dans la nageoire caudale et antéropostérieurement dans la première nageoire dorsale. Le développement bidirectionnel des éléments squelettiques au niveau de la nageoire caudale est le patron le plus commun chez les actinoptérygiens; ce patron est aussi présent chez un groupe aussi basal que *P. marinus*, indiquant potentiellement une condition plésiomorphe. Cependant, nous ne savons pas si ce patron est constant chez toutes les espèces de lamproies; ainsi, nous ne pouvons pas encore généraliser cette condition pour tous les Pétromyzontiformes.

La morphologie générale et cellulaire des rayons de *P. marinus* ressemble beaucoup à celle des rayons d'une espèce de myxine (*Eptatretus burgeri*) [9]. Cependant, malgré ces similarités, nous savons que le cartilage de la myxine est très différent du cartilage retrouvé

chez la lamproie; en effet, la composition en acides aminés diffère entre ces deux types de cartilages [133]. Toutefois, la présence d'un cartilage très simple composé de chondrocytes cuboïdes étroitement empilés et regroupés par un mince filet de matrice extracellulaire périphérique pourrait représenter une condition plésiomorphe partagée par ces deux espèces – éventuellement, par ces deux taxas (i.e., Pétromyzontiformes, Myxiniformes). Nos résultats sur la morphologie cellulaire impliquent que le cartilage retrouvé au niveau des rayons serait le même que celui des arcs branchiaux (i.e., type I) et serait composé de cellules cuboïdes/rectangulaires étroitement et régulièrement empilées [88,97]. Le cartilage retrouvé au niveau des barre médianes, composé de cellules pentagonales/globulaires dispersées de façon irrégulière (i.e., type II), serait le même que celui de la trabécule, des capsules otiques et de la barre subcordale [97].

Les rayons des nageoires des Pétromyzontiformes ont souvent été nommés « radiaux ». Un rayon est décrit comme un élément squelettique localisé dans la membrane qui offre un support interne à la nageoire [48,50,134], alors qu'un radial est un élément endochondral servant de lien entre les rayons et la colonne vertébrale [1,50]. Cependant, nous considérons que les éléments squelettiques présents dans les nageoires médianes de *P. marinus* devraient être considérés comme de véritables rayons et non comme simples radiaux. En effet, ces rayons sont bel et bien présents de la base à la marge de la nageoire, lui prodiguant ainsi un support interne, en plus de posséder une bifurcation de premier et second ordres. La bifurcation des rayons est retrouvée chez les taxons plus dérivés (e.g., dipneustes, actinoptérigiens) [48,50,134]; une étude sur *Cladoselache* et quelques bradyodontes a également montré que la bifurcation de premier ordre existe sur les radials appartenant aux nageoires paires. Cependant, les cas de bifurcation de second ordre au niveau des radiaux sont, à notre connaissance, inexistants dans la littérature. La bifurcation de second ordre, quoi que plus rarement présente, a été répertoriée chez plusieurs taxa ; en effet, la bifurcation de second ordre a été observée sur les lépidotriches de la nageoire caudale des poissons-zèbres (*Danio rerio*), ainsi que sur les rayons de certains batoïdes [135], d'actinoptérygiens fossile (e.g., *Cheirolepis canadensis*) [136] et de porolépiformes fossiles [137]. Pour les taxa fossiles, la bifurcation peut varier entre un 3^{e} et 5^{e} ordre. La

fonction de la bifurcation n'a jamais été décrite dans la littérature. Pour l'instant, nous émettons l'hypothèse que la présence de bifurcation permet un meilleur soutien au niveau de la partie distale de la membrane de la nageoire. Des études portant sur la détermination du rôle de la bifurcation dans l'efficacité de la nageoire devront être conduites chez plusieurs espèces de poissons et chez les lamproies.

Tous les systèmes squelettiques subissent des changements mineurs ou majeurs durant la métamorphose. Les éléments neurocrâniaux et buccaux étaient déjà présents et bien formés chez les ammocètes en stade 4 de métamorphose, suggérant que ces éléments se sont transformés lors des stades plus précoces de la métamorphose. D'un autre côté, les éléments squelettiques buccaux se développent après et possèdent un développement asynchrone. En effet, certain éléments (i.e., antérieur dorsal, postérieur dorsal, antérieur latéral, postérieur latéral) n'apparaissaient pas avec le même ordre d'un spécimen à l'autre et ce peu importe le stade de métamorphose. Cette asynchronie développementale a aussi été observée entre les systèmes squelettiques (e.g., les squelettes branchial, axial, buccal). La variation au niveau du timing développemental des séquences d'ossification a été étudiés pour certains ostéichthyens (e.g., *Danio rerio, Betta splendens*, *Salvelinus alpinus*) [116,118,138,139]. Les résultats de ces études démontrent que le développement intraspécifique peut être variable et que cette variation dépend majoritairement du timing développemental. Or, nos résultats impliquent que cette variation intraspécifique peut aussi être présente au niveau de groupes plus basaux tels que les Pétromyzontiformes, ce qui n'a jamais été documenté auparavant.

Nous avons comparé un staging basé sur la présence d'éléments squelettiques au staging classique basé sur l'apparence externe [58]. Cependant, nous n'avons pas été en mesure d'établir un staging clair basé sur la présence d'éléments squelettiques, principalement pour deux raisons : (1) les éléments squelettiques étaient déjà majoritairement présents pour les deux stades de métamorphose et (2) l'asynchronie développementale entre les éléments d'un même système (i.e., squelette buccal) et d'un système squelettique à l'autre. Les ammocètes appartenant au stade 5 possédaient des

arcualia au niveau de la région D1 et D2, ce qui n'était pas le cas des ammocètes en stade 4. Cette observation confirme que les ammocètes en stade 5 sont plus avancées dans leur développement axial. Nos résultats montrent que, somme toute, les ammocètes en stade 5 sont plus avancées dans leur développement, prouvant du même coup que le staging classique basé sur l'apparence externe de Youson et Potter [58] est somme toute fiable et cohérent. Cependant, nos observations ont été effectuées sur deux stades de métamorphose seulement; les sept stades devraient être étudiés afin d'obtenir un portrait plus précis du développement et de la transformation des éléments squelettiques durant la métamorphose, et ce sur un plus grand nombre de spécimens.

Notre étude est la première à décrire le patron développemental des arcualia pour deux stades de vie (i.e., ammocète, ammocète en métamorphose). Dans le cas de *P. marinus*, il existe deux centres de développement et chacun possède un patron différent; dans la région branchiale, les arcualia se forment antéropostérieurement alors que dans la région D2, les arcualia se développent bidirectionnellement. La présence de deux centres de développement pour les éléments vertébraux dorsaux est rare chez les téléostéens; en effet, cette condition a seulement été répertoriée chez les actinoptérygiens Ostariophysi [140]. Cependant, nous ne savons pas si l'acquisition d'un tel patron développemental au niveau des éléments vertébraux est une condition dérivée ou plésiomorphe. Nous avons également établis une description détaillée de la morphologie des arcualia pour cinq régions [i.e., branchiale (B), prédorsale (P), première nageoire dorsale (D1), deuxième nageoire dorsale (D2), nageoire caudale (C)] pour deux stades (i.e., ammocète en metamorphose, adultes). Les arcualia localisés dans la région branchiale sont toujours les plus complexes et les plus développés, ce qui corrobore ce qui a été précédemment décrit à ce sujet [64,101]. Les éléments vertébraux médiodorsaux, qui ont été appelés à tort « cartilages médians » [7,64], ont également été décrits dans notre étude. En effet, ces éléments sont disposés sur la notochorde sur un axe plus médian que les arcualia, mais jamais directement au sommet de la notochorde. Ces éléments sont présents dans la région branchiale seulement et possèdent tout une gamme de tailles et de formes. Nous avons également découvert que la morphologie et la taille des arcualia localisés dans les régions P, D1 et D2 étaient assez

constantes, alors que les mêmes aspects pour ces éléments avaient été initialement décrits comme étant variables [7,64,68]. Le cartilage des arcualia est composé de chondrocytes organisés aléatoirement en chondrones. Ces chondrocytes sont encastrés dans les lacunae d'une matrice extracellulaire territoriale à épaisseur variable. Ce cartilage ne ressemble en rien au cartilage hyalin, qui est le plus fréquemment retrouvés chez les taxons plus dérivés (e.g., chondrichtyens, ostéichthyens) [50]; cependant, ce cartilage présente de nombreuses similarités à la lamprine. Contrairement à ce qui a été observé au niveau du piston et du cartilage annulaire [103,104], le cartilage des arcualia ne possède pas de zone de différentiation cellulaire (i.e., subpérichondrium) ; cette absence peut être causée par une restriction développementale ou fonctionnelle. Pour l'instant, nous ne sommes pas totalement certains que le cartilage des arcualia soit majoritairement composé de protéines de lamprine. De nouvelles analyses histologiques et immunohistochimiques devront être effectuées afin d'éclaircir cette question.

Nous sommes les premiers à avoir observé des agglomérations de chondrocytes de types II dorsalement à la notochorde dans la région caudale. Ces plaques de cartilage étaient visibles sur les spécimens colorés au bleu alcian pour tous les stades étudiés; nous avons également été en mesure de confirmer leur présence en coupes histologiques. Ce cartilage, attaché dorsalement à la notochorde, s'étend distalement de chaque côté du tube neural dorsal. Ce cartilage dorsolatéral se fusionne avec la barre dorsomédiane, donnant l'aspect général d'un arc neural complet. À notre connaissance, ce cartilage notochordal n'a jamais été documenté chez les Pétromyzontiformes; cependant, une structure similaire (i.e., une forme d'arc neural) a été détectée dans la partie postérieure de la région caudale pour une espèce de myxine (*Eptatretus burgeri*) [9]. De récentes études ont démontré que la notochorde de la lamproie pouvait être considérée comme un type de cartilage particulier puisque du collagène [133] et des protéoglycans [67] y ont été détectés. Il a également été démontré que la notochorde est un type de cartilage particulier capable de produire du cartilage notochordal (i.e., chondrogenèse notochordale) chez plusieurs espèces de geckos [141]. Ainsi, il serait probable que la notochorde de *P. marinus* soit également capable d'effectuer une chondrogenèse notochordale, expliquant ainsi l'origine du développement

du cartilage présent dorsalement à la notochorde. Cependant, étant donné que la chondrogenèse de ce cartilage n'a pas été suivie, nous ne pouvons affirmer qu'il s'agisse bien de cartilage d'origine notochordale. De plus, ce cartilage possède exactement la même morphologie cellulaire que la barre dorsomédiane; or, il se pourrait que ce cartilage soit simplement le résultat d'une élongation ventrale de cette barre. De plus amples études devront être menées afin de découvrir l'origine du développement et la fonction exacte de ce cartilage.

Toutes nos observations ont été effectuées sur une seule espèce de lamproie (*Petromyzon marinus*); il n'est donc pas possible de généraliser nos observations sur l'ensemble des lamproies. D'autres études devront être menées afin de générer un portrait plus général et applicable à l'ensemble de ce taxon. Une autre limite de l'étude est le manque de techniques de colorations alternatives. En effet, l'utilisation de trichome de Masson aurait pu servir pour la détection de collagène et l'iodine de Verhoeff pour la détection d'élastine. L'utilisation d'une multitude de techniques de coloration combinées à une coloration immunohistochimique aurait pu apporter des informations supplémentaires sur la composition cellulaire des éléments squelettiques étudiés.

Les précédentes études qui se sont concentrées sur la composition cellulaire de certains éléments squelettiques chez les lamproies ont répertorié deux types de cartilage : un cartilage composé de protéines cyanogènes bromide-insolubles, détecté au niveau de la trabécule, des arcs branchiaux et du cartilage péricardial [132]; l'autre type est composé de protéines de lamprine et est présent dans le piston, le cartilage annulaire et les éléments neurocraniaux [52-54]. Cependant, deux autres types de cartilage complètement différents ont également été décrits pour les lamproies, basé cette fois sur la morphologie cellulaire (i.e., type I, type II). Nous ne savons pas si le cartilage de type I serait en réalité composé de protéines cyanogènes bromide-insolubles et le type II, de lamprine. Ces deux protéines ont précédemment été décrites comme ne possédant aucune fibre de collagène; cependant, une étude récente a montré que le collagène était exprimé dans certains éléments squelettiques chez *P. marinus* (e.g., arcs branchiaux, notochorde, membrane notochordale,

arcualia, rayons) [59]. Ces résultats viennent à l'encontre de tout ce qui a été décrit sur le cartilage des lamproies. Les éléments squelettiques pourraient être tous constitués totalement ou partiellement de fibres de collagène. Or, il existe une incohérence entre les types de cartilages décrits sur la base de leur composition en protéine et ceux décrits sur la base de la morphologie cellulaire. D'autres études histologiques doivent être menées sur un plus grand ensemble d'éléments squelettiques (e.g., piston, cartilage annulaire, trabécule, capsules otiques, arcs branchiaux, cartilage péricardial) afin d'obtenir une information concise et cohérente sur la composition et la morphologie cellulaire pour chaque élément. Ces informations pourront être utilisées afin de dresser un portrait général des particularités histologiques et histochimiques des types de cartilage présents chez les lamproies; ces informations pourraient nous permettre de comparer avec les cartilages présents chez les gnathostomes et ainsi mieux comprendre l'origine du cartilage et son évolution dans la phylogénie des vertébrés basaux.

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