Comparison of aspects of the physiology and morphology of diploid and triploid Atlantic salmon *Salmo salar*.

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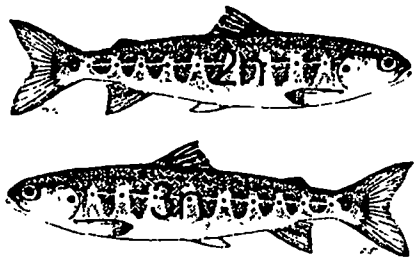

by

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Doctor of Philosophy

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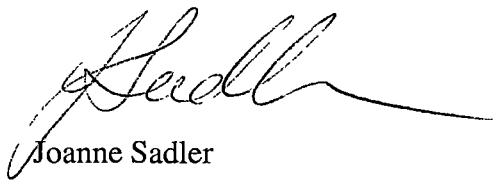

Joanne Sadler

THESIS ABSTRACT

High mortality rates under suboptimal culture conditions and the incidence of lower jaw deformity (LJD) are problems associated with the commercial production of all-female triploid Atlantic salmon (*Salmo salar*) within the Tasmanian Atlantic salmon industry. The current study investigates the primary and secondary haematological stress responses, the skeletal ontogeny and the incidence of skeletal deformity in fish from different population types (all-female diploids, all-female triploids, mixed sex diploids and mixed sex triploids), throughout development, up until 7 weeks post-sea water (SW) transfer.

Plasma cortisol (F) levels of rested freshwater (FW) parr (n = 10) were less than 10 $ng.ml^{-1}$ and were subsequently elevated in parr subject to 1 hour 20 minutes of confinement, regardless of sex or ploidy status. Rested all-female diploid SW smolt (n = 10) had significantly lower plasma F levels than rested all-female triploid, mixed sex diploid, and mixed sex triploid SW smolt. In all SW smolt, plasma F and plasma lactate levels were elevated following 1 and 3 hours of confinement, but were statistically similar to rested levels following 6 hours of confinement. In a subsequent experiment, plasma F levels of diploid and triploid SW smolt (n = 7) subject to 2 hours of confinement, decreased to rested levels within a 6 hour recovery period, post-treatment.

Haematological parameters were measured in SW smolt from different populations following 2.5 h confinement in aerated SW. Plasma cortisol levels, plasma lactate and glucose levels increased following confinement, irrespective of ploidy status. Total blood haemoglobin (Hb) was lower in all-female triploid fish compared to all-female diploids, however there was no difference between mixed sex groups. There was no difference in haematocrit (Hct), mean cell haemoglobin concentration (MCHC), whole blood adenosine triphosphate concentration [ATP], Hb:ATP and total plasma protein between diploid and triploid individuals. *In vitro* examination revealed the blood oxygen affinity (P_{50} values) and percentage oxygen saturation of Hb at various physiological pH levels were similar between diploid and triploid

smolt. Blood viscosity was dependant on Hct and shear rate, irrespective of ploidy status. These results show there is no difference in the primary or secondary stress responses of *S. salar* with ploidy status. Furthermore, blood oxygen carrying capacity and the extent of anaerobic metabolism did not differ with ploidy status under these experimental conditions.

The gross morphology and ossification of the lower jaw, cranium, branchial apparatus and fin skeleton throughout ontogeny is described for each population type. There appeared to be no differences in the skeletal ontogeny of normal diploid and triploid fish. Triploid Atlantic salmon were susceptible to significantly higher incidence of deformity post-first feeding compared to diploid fish. Short opercula were observed in up to 20% of fish from each population post-first feed, regardless of ploidy or sex status, where as gill filament deformity syndrome (GFD), occurred almost exclusively in triploid fish, at rates of up to 60%. Triploid fish with either normal gills or gills affected by GFD had a lower gill surface area index than diploid salmon. LJD was detected almost exclusively in triploid smolt cultured under either FW and SW conditions, following the time of SW transfer.

It is unlikely that the primary endocrine and secondary haematological stress responses or the aerobic capacity of triploid fish contribute to high mortality under suboptimal commercial conditions. The repercussions of decreased gill surface area on ionoregulation or respiration in triploid fish under conditions of hypoxia and/or exhaustive exercise are discussed.

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For Natalie Szabo

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LIST OF ABBREVIATIONS

2N	diploid
3N	triploid
4N	tetraploid
A	angular bone
Ac	auditory capsule
aCH	accessory caudal hypural
AcLL	auditory capsule lateral line
Adr	anal distal rad
AGD	Amoebic Gill Disease
Aj	articulation joint
Aos	anterior orbitosphenoid
Apr	anal primordial rays
Apxr	anal proximal radial
As	anal stays
Asr	anal rays
ATP-ase	Adenosine triphosphatase
ATU	accumulated temperature units (= rearing temperature in °C × accumulated number of hours or days)
Bb	basibranchials
bc	branchial chamber
Bh	basihyal
Bo	basioccipital
Br	branchiostegal rays
Bs	basisphenoid
Bt	basipterygium
Btp	basipterygium process
Cb	ceratobranchial
Ce	caudal epiurals
Ch	ceratohyal
CH	caudal hypural
Cl	cleithrum
Co	coracoid
Cpr	caudal primordial rays
cs	coracoid/ scapula
cspp	coracoid scapula cartilage posterior process
Csr	caudal soft rays
D	dentary bone
dD	deformed dentary
Ddr	dorsal distal radial
dmc	deformed Meckel's cartilage
dns	divided neural spine
DO	dissolved oxygen
dpgf	deformed primary gill filament
Dpr	dorsal primordial rays
Dpxr	dorsal proximal radial
Ds	dorsal stay
Dsr	dorsal soft rays
DT	dentary teeth
DW	distilled water
E	ethmoid
eb	ectethmoid bar
Eb	epibranchial
Ec	ethmoid cartilage
Ecp	ectopterygoid
Eg	entoglossus
Eh	epihyal

ENL	erythrocyte nucleus length
Enp	endopterygoid
Eo	epiotic
Ep	ethmoid plate
et	epiphysial tectum
Ex	exoccipital
F	frontal
fCb	fused caudal bones
fCe	fused caudal epiurals
FL	fork length
fp	fin plate cartilage
FT	all-female triploid
FW	freshwater
gc	germ cell
GFD	gill filament deformity syndrome
Gh	glossohyal
gi	gills
GMF	genetically modified fish
Gr	gill rakers
GSA	gill surface area
GT	glossohyal teeth
h	hour
Hb	haemoglobin
hb	hypobranchials
Hct	haematocrit
Hh	hypohyals
HhLo	hypohyal lower
HhUp	hypohyal upper
Hm	hyomandibular
Hmap	hyomandibular anterior process
hs	haemal spine
I	intercalar
Ib	isolated basibranchial
Ih	interhyal
Io	infraorbital bones
iO	interopercula
JD	jaw deformity
K	condition factor
K_2EDTA	potassium ethylenediaminetetracetate
L	litre
La	lachrymal
Le	lateral ethmoid
Lj	lower jaw
LJD	lower jaw deformity syndrome
Ll	lamellae
LP	lower pharyngeal
LPT	lower pharangeal teeth
M	maxilla
mc	Meckel's cartilage
MCH	mean cell haemoglobin
MCHC	mean cell haemoglobin concentration
MCR	metabolic clearance rate
MCV	mean cell volume
MD	mixed sex diploid
Mg	medial gular
mgf	missing gill filaments
min	minute
MLJD	mild lower jaw deformity
mlp	median longitudinal plane

mM.L	mmol.L
Mp	metapterygoid
mpf	months post-fertilisation
mq	metapterygoid-quadrate cartilage plate
MT	mixed sex triploid
Mt	maxilla teeth
Mtg	metapterygium
n	sample number
N	nasal
n50	hills cooperativity coefficient
NLL	nasal lateral line
no	notocord
ns	neural spine
NTP	nucleotide triphosphate
O	opercula
Oc	olfactory capsule
Oo	oogonia
P_{100}	partial pressure at oxygen saturation
P_{50}	oxygen affinity coefficient
Pa	parietal
pAsr	pre-anal soft ray
Pb	pharyngobranchial
Pc	parachordal
pClLo	post-cliethrum lower
pClUp	post-cleithrum upper
pCsr	precaudal soft rays
Pd	pectoral digitals
pDpxr	predorsal proximal rays
Pdr	pectoral distal radials
pDsr	predorsal soft rays
pgf	primary gill filament
pm	premaxilla and teeth
pO	preopercula
Po	prootic
pOC	perinucleolytic oocyte
Ppr	pectoral primordial rays
Ps	parasphenoid
Psr	pectoral soft rays
PT	palantine plus teeth
Pt	post-temporal
Pto	pterotic
Pts	pterosphenoid
Q	quadrate
Qpp	quadrate posterior process
Ra	retroarticular bone
RBC	red blood cell
RBCC	red blood cell count
RIA	radio immuno assay
s	stromal tissue
S	sclerotic
SALTAS	Salmon Enterprises of Tasmania Pty Ltd.
SC	spermatocyte
Sc	scapula
ScClp	scapula / cleithrum process
SCl	supra cleithrum
Sd	spermatid
SD	standard deviation
SE	standard error
Sg	spermatogonia

SGR	specific growth rate
sL	secondary lamellae
Sm	supramaxilla
smqf	symplectic and metapterygoid-quadrate plate fused
sO	subopercula
SO	short opercula
So	supraorbital cartılage
SoLL	supraorbital lateral line
Sp	sphenotic
sSy	short symplectıc
St	supratemporal
SW	sea water
Sy	symplectic
Syd	symplectic divided
Sz	spermatozoa
t	trabecula
TOT	total prevalence of deformity (TOT. DEF)
TWWt.	total wet weight
Uh	urohyal
Uj	upper jaw
Un	uroneural
UPT1	upper pharangeal teeth
UPT2	upper pharyngeal teeth
Vdr	pelvic digital radials
Vpr	pelvic prımordial rays
Vsr	pelvic soft rays
wpf	weeks post-fertilisation
wph	weeks post-hatching
ϕ	bohr factor
Δ	change in

Chapter 1:

GENERAL INTRODUCTION

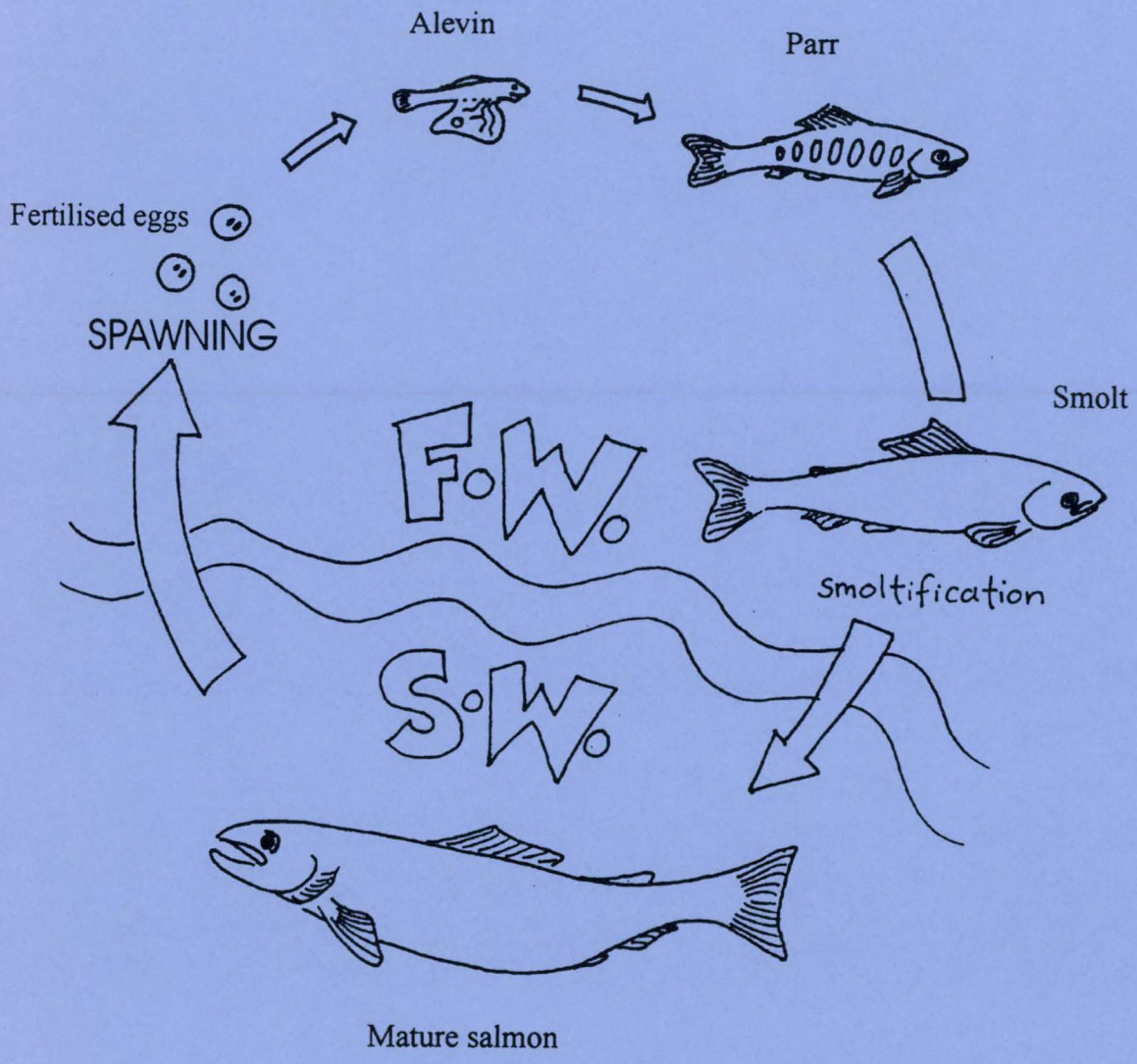


Figure 1. Life cycle of the Atlantic salmon (*Salmo salar*)

1. GENERAL INTRODUCTION

The species, *Salmo salar* (L.) (Atlantic salmon), of the Family Salmonidae, is native to the Atlantic coast of north America and Europe. Atlantic salmon are anadromous, that is their lifecycle is divided between a freshwater (FW) phase and a seawater (SW) phase (Bond, 1996). In the wild, sexually mature fish migrate to the river of their birth after several years at sea. The female fish lays negatively buoyant eggs in a small nest or 'redd' created within the riverbed and the eggs are immediately fertilised by the male, then buried under a light protective layer of gravel (Landau, 1992). Here, embryological development proceeds over a period of approximately 400° days (accumulated temperature units (ATU) = rearing temperature in °C × accumulated number of days or hours) post-fertilisation; subsequently embryos hatch as yolk sac larvae called 'alevins' (Fig.1) (Sedgwick, 1988). Alevins remain within the gravel bed until they absorb their yolk-sac and emerge into the water column as 'fry' ready for first feeding (≈ 400° days post-hatching). Fry develop into 'parr' when they reach a total wet weight (TWWt.) of approximately 1 g or more, at which stage the fish have developed characteristic vertical bar markings along their body length (Fig. 1). At approximately 10 months post-fertilisation, parr undergo a series of physiological changes known collectively as smoltification which prepare the fish for life under SW conditions (Hoar, 1988). In the wild, these changes occur progressively as the fish migrate down river towards estuarine conditions. Under culture conditions, fish are fed a high salt diet during this period of development to facilitate the increase in salt intake that would otherwise occur during seaward migration. Atlantic salmon that have completed smoltification are called smolt and are characterised by their silver body colouration and black fin extremities. Under culture conditions, smolt are tested for SW tolerance prior to being transferred to SW facilities using a SW challenge and subsequent measurement of blood osmolality.

Atlantic salmon were first introduced to Tasmanian waters in 1864 (Ovenden *et al.*, 1993). Despite repeated efforts a breeding population was not established at this

time. Three shipments of Philip River stock (Cobequid hatchery, Nova Scotia, Canada) were introduced to Australia during 1963 -1965 and were held at the federal government hatchery at Gaden, N.S.W. Despite high mortality, a small breeding population of these salmon was maintained and these fish were used to establish the Atlantic salmon aquaculture industry in Tasmania (Jungawalla, 1991, Ovenden *et al.*, 1993). Between, 1968 and 1999, legislation prevented importation of fresh salmonid products into Australia, precluding importation of further genetic stocks. This has implications for the genetic diversity of cultured Atlantic salmon in Australia.

The potential for successful farming of salmonids in Tasmania was recognised following the establishment of a small number of small-scale trout farms in the state during the 1970-80's (Jungawalla, 1991). By 1983, a plan for the development of an extensive industry based on Atlantic salmon was formulated by the Tasmanian Fisheries Development Authority (T.F.D.A). Salmon Enterprises of Tasmania (SALTAS) was formed in 1985 to provide a reliable source of salmon stock and to facilitate research and development in the new industry (Jungawalla, 1991). SALTAS was initially financed by the Tasmanian state government (51 %) and industry investors (49%). Under the establishing agreement SALTAS had sole production rights for smolt in the Tasmanian industry for ten years (1985 -1995). Since then farmers have been permitted to produce their own smolt; however, SALTAS continues to be the primary supplier of smolt to the industry in Tasmania.

The Atlantic salmon industry is currently one of the most important aquaculture industries in Australia, second to the pearl oyster industry and equal to the bluefin tuna industry, with a value of over 60 million dollars and annual production of 10,000 tonnes or 2 million smolt in 1999 (Brown *et al.*, 1997; *pers comm.* Harry King, Operations Manager, SALTAS Pty Ltd., Tasmania, 1999). The industry consists of ten companies with most of the leases located on the South East coast of Tasmania (Brown *et al.*, 1997). Salmonid production in the southern hemisphere has a marketing advantage in that the peak of the production cycle is out of phase with that of the northern hemisphere. Although the volume of Australian production is

very small compared to that of Chile, the main southern hemisphere competitor (100,000 tonnes pa = $340 million, Food and Agricultural Organisation (FAO), 1999), the Australian industry is geared to the production of a quality product, which currently fetches a premium price in the world market (Brown *et al.*, 1997). However, commercial production of this northern hemisphere species in Tasmania does present a unique challenge to the local producers.

The rate at which Atlantic salmon stocks reach sexual maturity differs according to environmental and genetic factors. The Philip River stock, from which the Tasmanian stock originated, is characteristically prone to high grilsing rates (*pers comm.* Brian Glebe, Atlantic Salmon Federation, Canada, 1999); that is the fish display early sexual maturation within one year of SW propagation. The warm water conditions in Tasmania further enhance the rate of maturation and extremely high grilsing rates of up to ≈ 97% are the norm. Other Atlantic salmon stocks generally become sexually mature between 12 and 36 months post-SW transfer (Johnstone *et al.*, 1978, 1991). Although high growth rates are beneficial to the industry, the high grilsing rate presents four main difficulties for the management of a quality harvest. Firstly, investment of energy during early sexual maturation is diverted from somatic growth to reproduction, resulting in diminished flesh quality (Johnstone *et al.*, 1978; Sedgwick, 1988). Secondly, grilsing males develop secondary sexual characteristics including dark skin tones and a hooked jaw or 'kype' (Johnstone *et al.*, 1978; Sedgwick, 1988), which are undesirable features for the market product. Third, sexual maturation increases susceptibility to bacterial and fungal invasion (Johnstone *et al.*, 1978). Lastly, Tasmanian Atlantic salmon reach a harvest size of 3.5 - 5.0 kg just 15 months after sea transfer (within 30 months post-fertilisation) with the result that the potential harvest period is severely truncated compared to the northern hemisphere competitors. This leads to potential problems with continuity of market supply.

To control the extent of early maturation and facilitate a year-round harvest, different populations with unique growth charcteristics are routinely cultured by the Tasmanian Industry to be harvested at different times of the year. These include

normal diploids, all-female diploids, all-female triploids and photo-manipulated fish. Normal diploid fish may only be harvested for approximately 8 months of the year and contribute approximately 75% of the total annual production (*pers comm.* Harry King, Operations Manager, SALTAS Pty Ltd., Tasmania, 1996). Since male salmon mature earlier than females, the production of all-female populations avoids precocious sexual maturation and unwanted secondary sexual characteristics associated with sexually maturing male fish (Johnstone, 1992; Johnstone *et al.*, 1978). In addition, photomanipulation of normal and all-female diploid populations is used to phase-shift the development of the fish, to further extend the potential annual harvest period. With the production of normal diploid fish, all-female diploids and photomanipulated fish, there remains 3 - 4 weeks of the year in which Tasmanian Atlantic salmon cannot be harvested. Year round harvest is facilitated by the production of sterile all-female triploids.

Triploid fish are sterile because they possess an extra set of chromosomes (3N) compared to normal diploids (2N). Triploidy may be induced experimentally by inter-ploidy crossbreeding of tetraploids (4N) and diploids (2N), or by causing the retention of the second polar body, which would normally be discarded during the 2nd meiotic division of embryological development (Johnstone, 1985). The latter is achieved either by chemical treatment, thermal shock or application of hydrostatic pressure shock (Johnstone *et al.* ,1991; Jungawalla, 1991). The third set of chromosomes in triploid fish disrupt the first meiotic division of gametogenesis (Lincoln and Scott, 1983, 1984) and, subsequently, results in testicular development and the production of functionally sterile sperm in male salmon (Lincoln and Scott, 1983; Benfey and Sutterlin, 1984d), but complete suppression of ovarian development in female fish (Lincoln and Scott, 1983, 1984; Solar *et al.*, 1984; Benfey and Sutterlin, 1984d). The production of all-female triploids avoids sexual maturation and the subsequent decrease in growth rate and flesh quality that ensues in sexually maturing diploids and male triploids. Although, all-female triploid fish contribute less than 10% of total production for the Tasmanian Atlantic salmon industry, they are an extremely important component of production as they facilitate harvest continuity throughout the year.

Triploid salmonids have been produced in Scotland, Ireland, Canada and Norway on a trial basis, where the production of sterile triploid populations has been proposed as potentially beneficial for the prevention of genetic dilution of wild stocks should reproductive interaction between wild fish and domestic escapees ever occur (Marine Laboratory Aberdeen (MLA) *et al.*, 1999). This issue is of particular concern in the advent of commercial production of genetically modified or transgenic fish (GMF's) (Devlin, 1999). However, this issue is not relevant to the Tasmanian industry, where there are no native or wild Atlantic salmon stocks.

Despite the advantages of triploid induction to the local industry, there appear to be a number of problems associated with the commercial production of all-female triploid fish. Triploid populations display a higher incidence of "pin-heading" (Lee and King, 1994), a condition characterised by the dramatic loss of body mass relative to head size following seawater transfer. In addition, previous reports within the Tasmanian industry, and from overseas, suggest triploid salmonids are more prone to stress than diploid fish, as indicated by altered behaviour and lower survival under suboptimal culture conditions such as high water temperatures, low dissolved oxygen levels, during SW transfer, and during confinement and handling (King and Lee, 1993; Johnson *et. al.*, 1986; Quillet *et. al.,* 1987; Quillet and Gaignon, 1990; Virtanen *et. al.*, 1990; Johnstone *et. al.*, 1991; Aliah *et. al.*, 1991; Jungawalla, 1991; Myers and Hershberger, 1991; Blanc *et. al.*, 1992; Simon *et. al.*, 1993; Ojolick *et. al.*, 1995; Benfey, 1996; McGeachy *et. al.*, 1996; O'flynn *et. al.*, 1997). Triploid Atlantic salmon also appear to be prone to a high incidence of lower jaw deformity syndrome (LJD) during the SW phase (Jungawalla, 1991; Hughes, 1993; King and Lee, 1993; Lee and King, 1994; McGeachy *et al.*, 1996, Benfey, 1999). LJD is characterised by the downward curvature of the lower jaw (Hughes, 1993) and may necessitate ram ventilation of the gills. Ram ventilation could affect respiratory efficiency in afflicted fish or may be a problem when fish are crowded and cannot swim, since they cannot irrigate the gills. Furthermore, LJD reduces the value of afflicted fish as they cannot be sold as whole carcass. The mechanisms which contribute to these observed differences between diploid and triploid populations are

unknown. The perception of altered physiology in triploid Atlantic salmon and the prevalence of skeletal deformities in triploid fish provide the focus for the present study.

1.1 TRIPLOID PHYSIOLOGY

It has been hypothesised that the physiology of triploid fish may differ to that of diploids and therefore triploid fish may have different environmental requirements to diploid fish (Benfey, 1999). Triploid fish differ from diploids in that they have larger but fewer cells at the tissue level (Swarup, 1959a; Small and Benfey, 1987), and are more heterozygous (Allendorf and Leary, 1984). It is possible that differences which occur in cellular morphology with ploidy status contribute to differential physiological tissue function, since decreased cell surface to volume ratio may alter cellular energetics, metabolic processes, diffusion rates and the distribution of molecular receptors at the cell surface (reviewed by Benfey, 1999). Furthermore, the increased heterozygosity of triploid fish may result in differential phenotypic expression at the molecular (Allen *et al.*, 1982; Seeb *et al.*, 1988) or tissue level.

Previous studies have examined various aspects of physiology of triploid fish to determine whether differences in physiological function occur with ploidy status and these studies have culminated in a considerable knowledge base of triploid physiology (reviewed by Benfey, 1999). Despite higher mortality of triploids during SW propagation, the osmoregulatory ability of triploid salmonids appears to be similar to that of diploids (Jungawalla, 1991; Johnson *et al.*, 1986). The immunocompetance and response to vaccination of triploid fish is similar to that of diploids, although triploids have been shown to be susceptible to higher mortality following infection with *Vibrio* sp. or bacterial gill disease (reviewed by Benfey, 1999). Furthermore, there appears to be no difference in dietary energy intake, utilisation, partitioning and nitrogen balance between diploid and triploid fish (reviewed by Benfey, 1999).

It is however unclear whether triploids display reduced respiratory efficiency or aerobic capacity compared to diploids, particularly since critical swimming speed and the time to exhaustion at constant swimming speed is similar between diploid and triploid fish, although tail beat frequency at a given swimming speed has been shown to be higher in triploids (reviewed by Benfey, 1999). There is conflicting evidence regarding possible differences in blood haemoglobin levels, oxygen consumption rates and opercula abduction rates between diploid and triploid fish (reviewed by Benfey, 1999). Some studies of Atlantic salmon (Benfey and Sutterlin, 1984a; Graham *et al.*, 1985), other salmonids (coho salmon *Oncorhynchus kisutch*: Small and Randall, 1989; rainbow trout *Oncorhynchus mykiss*: Yamamoto and Iida, 1994) and non- salmonids (white crappies *Pomoxis annularis*: Parsons, 1993) indicate triploids may have lower blood haemoglobin content, whereas other studies indicate blood haemoglobin is similar between diploids and triploids (brook trout *Salvelinus fontinalis*: Stillwell and Benfey, 1994, 1996a; Grass carp *Ctenopharyngodon idella* × bighead carp *Hypophthalmichys nobilis* hybrids: Barker *et al.*, 1983; ginbuna *Carassius auratus langsdorfi*: Sezaki *et al.*, 1983, 1991; ayu *Plecoglossus altevelis*: Aliah *et al.*, 1991). Oxygen consumption rates of diploid and triploid fish have been reported to be similar in some studies (Atlantic salmon: Benfey and Sutterlin 1984b; rainbow trout: Oliva-Teles and Kaushik, 1987a, 1990a, 1990b; Yamamoto and Iida, 1994b; threespine stickleback: Swarup, 1959b; ayu: Aliah *et al.*, 1991; ginbuna: Sezaki *et al.*, 1991; white crappies: Parsons, 1993), whereas, one study indicated that triploid rainbow trout derived from retention of the second polar body, rather than interploidy cross breeding, had higher oxygen consumption rates than diploids (Oliva-Teles and Kaushik, 1987b). Another study showed triploid rainbow trout display signs of respiratory distress at higher oxygen saturation levels than diploids (Yamamoto and Iida, 1994). Opercula abduction rates were higher in triploids in some studies (Atlantic salmon: King and Lee, 1993; ginbuna: Sezaki *et al.*, 1991;), but similar between diploids and triploids in others (brook trout: Stillwell and Benfey, 1994, 1996a; ayu: Aliah *et al.*, 1991). The *in vivo* blood oxygen affinity, blood pH and nucleotide triphosphate (NTP) levels, all of which affect blood oxygen carrying capacity, were found to be similar between diploid and triploid Atlantic salmon under pre-stress conditions, although the blood

oxygen carrying capacity of triploid Atlantic salmon was two thirds that of diploids due to reduced haemoglobin levels and reduced haemoglobin oxygen loading (Graham *et al.*, 1985).

In view of what is known, it remains unclear whether differential physiological responses contribute to the perceived problems associated with the culture of triploid salmon in Tasmania since there is a lack of information regarding the physiological responses of these fish. For example, stress responses have been known to affect physiological function or even cause mortality in diploid salmonids under suboptimal conditions such as extended aerobic activity (Graham *et al.,* 1982; Wood *et al.,* 1983; Barton and Iwama, 1991; Ferguson and Tufts, 1992). Whether the primary endocrine stress response or secondary stress responses contribute to differential mortality in triploid fish is unknown. Prior to the current study, only one other study has compared the primary endocrine (plasma cortisol), haematocrit and plasma glucose stress response of diploid and triploid salmonids, following 5 minutes handling stress in brook trout (Biron and Benfey, 1994). The stress responses of triploid Atlantic salmon following periods of confinement, particularly in seawater, are unknown and are relevent to the husbandry of this species. The present study examined the primary endocrine stress response to confinement in fish from all-female diploid, all-female triploid, mixed sex diploid and mixed sex triploid populations, during both the FW parr and SW smolt phases of the life cycle, to determine whether the stress response differed with ploidy or sex status. Experimental protocols were applied to these four populations, so that the potential confounding influence of gender could be differentiated from that arising from ploidy status alone.

In addition to the above, it is unclear whether asphyxiation or other physiological responses contribute to the differential mortality observed in triploid salmon under conditions of high oxygen demand / low oxygen availability. The haematological stress responses of triploid fish, including changes in blood oxygen carrying capacity which may occur with changes in blood plasma pH, under stressful suboptimal husbandry conditions are unknown, but may affect the aerobic capacity of the fish.

Furthermore, potential differences in blood viscosity, according to differences in erythrocyte cell size and number, have yet to be examined for diploid and triploid fish. This may be relevant since increased blood viscosity may contribute to resistance in peripheral blood circulation and thereby affect respiratory efficiency in terms of the effort required to circulate blood around the body. In this study, the secondary haematological responses to confinement in fish from all-female triploid, all-female diploid, mixed sex triploid and mixed sex diploid populations were examined during the SW smolt phase to determine if aerobic capacity following stress differed according to sex or ploidy status. Changes in blood oxygen affinity and blood oxygen saturation with blood plasma pH were examined in diploid and triploid fish to determine if blood oxygen carrying capacity differed with ploidy status. Changes in blood viscosity with haematocrit (red blood cell volume per unit blood) were examined in diploid and triploid fish to determine if an intra-specific increase in erythrocyte size affects blood viscosity. Examining the extent of possible differences in physiological responses between fish from different population types is a prerequisite for optimising management protocols in culture.

1.2 SKELETAL DEFORMITY IN TRIPLOID ATLANTIC SALMON

Previous reports of LJD in Tasmanian Atlantic salmon have described the morphology of the deformity and have indicated the prevalence of LJD is up to 25 - 30% in triploid populations under seawater conditions (Jungawalla, 1991; Hughes, 1992; King and Lee, 1993; Lee and King, 1994). Reports of LJD in Atlantic salmon from overseas indicate that the deformity may occur in diploid fish (Bruno, 1990; Quigley, 1995) and under freshwater conditions just prior to SW transfer (Goicoechea *et al.*, 1999). No study has examined the prevalence of LJD during early development in freshwater, nor compared the prevalence of deformity between diploid and triploid populations under controlled experimental conditions. The development of diploid and triploid fish has previously been compared in terms of the temporal pattern of hatching and first feeding, growth, sexual maturation and muscle development (Happe *et al.*, 1988; Quillet *et al.*, 1988; Johnstone *et al.*, 1991; Jungawalla, 1991; Yamashita, 1993; McGeachy *et al.*, 1994; MLA *et al.*, 1999), but

no study has compared the morphology of skeletal development between diploid and triploid salmon, although some studies indicate several morphological differences and abnormalities in triploid fish (reviewed by Benfey, 1999), including differences in pelvic fin shape and length in triploid and diploid tench *Tinca tinca* (Flajshans *et al.*, 1993) and facial deformities in triploid bighead carp *H. nobilis* (Tave, 1993). On the basis of differences in cell morphology with ploidy status, one might expect skeletal development to differ between diploid and triploid fish since the processes which determine skeletal morphology and mineralisation may differ with cell morphology (reviewed by Hall and Miyake,1995; Kimmel *et al.*, 1998). In addition, triploids are more heterozygous than diploids (Allendorf and Leary, 1986), and this may result in greater variation of skeletal phenotypic expression.

This study examined the temporal pattern of morphology and ossification throughout the skeletal development of fish from all-female triploid, all-female diploid, mixed sex triploid and mixed sex diploid populations, maintained under standardised conditions, to determine whether there were differences in normal skeletal development with ploidy or sex status. The comparison of skeletal development between diploid and triploid fish is completely novel and offers the unique opportunity to examine the possible effects of intra-specific differences in cell size and heterozygosity on normal skeletal development. This study provides a knowledge base upon which we may describe abnormal skeletal development. The temporal onset and prevalence of different types of skeletal deformity, including LJD, was examined for each population, throughout development up until 8 weeks post-SW transfer to ascertain whether deformity prevalence differed with ploidy or sex status. Examining the incidence of deformity throughout ontogeny of the different population types is fundamental to determining the possible mechanisms by which these deformities occur, and thereby facilitates the improvement of management procedures and harvest quality.

Chapter 2:

GENERAL MATERIALS AND METHODS:
Population production, quality assurance, growth and mortality

2. GENERAL MATERIALS AND METHODS: Population production, quality assurance, growth and mortality

2.1 ABSTRACT:

Four different populations of Atlantic salmon were produced using standard commercial techniques; all-female diploids, all-female triploids, mixed sex diploids and mixed sex triploids. These populations were maintained under standardised conditions throughout development. Ploidy status of fish was determined by measuring mean erythrocyte nucleus length (ENL) and the sex status of fish was determined histologically.

Triploidy rates for the all-female triploid population and mixed sex triploid population were 96% and 100%, respectively. The male: female sex ratio was 1: 1.4 for the mixed sex diploids, 1: 1.2 for the mixed sex triploids and 0:1 for the all-female populations. The ovaries of female triploids consisted predominantly of oogonia arranged in lamellae with an occasional previtellogenic oocyte within the stroma, whereas female diploids had ovaries which consisted predominantly of previtellogenic oocytes within the lamellae. Male triploids displayed similar testicular development to male diploids.

Specific growth rate (SGR) for the period of freshwater (FW) development prior to seawater (SW) transfer, was similar between diploid and triploid populations. At the time of SW transfer populations were divided into two groups, one of which was transferred to SW facilities (SW smolt) and the other group remained in FW facilities (FW smolt). The SGR of FW smolt was significantly higher than that of SW smolt. The SGR of SW smolt did not differ with ploidy or sex status. Diploid FW smolt had higher SGR values than triploid FW smolt. Triploid SW smolt were subject to higher mortality rates than diploid SW smolt.

2.2 INTRODUCTION

As discussed in Chapter 1, the aim of the present study was to examine specific physiological responses and skeletal morphology of Atlantic salmon according to ploidy and sex status. The objective was to ellucidate possible mechanisms that may contribute to the perceived problems associated with the commercial production of all-female triploids and thereby facilitate improvement of current production practices. Experimental protocols were applied to four different populations, three of which are produced commercially (mixed sex diploid, all-female diploid and all-female triploid populations) and a fourth (mixed sex triploid population), which was produced specifically for this study. The present chapter outlines population production techniques, husbandry practices and techniques used to determine fish ploidy status and population sex ratio.

The use of hormones to control the sex of developing Atlantic salmon is an effective technique for producing all-female populations. The administration of oestradiol in the diet of male fry from the time of first feeding, has induced ovarian development and growth comparable to that of normal control females (Johnstone and Youngson, 1984). However, such a method of producing all-female stock may not be economically viable (Johnstone *et al.*, 1978; Jungawalla, 1991) and there are implications regarding market acceptability of a product directly exposed to steroids (Johnstone *et al.*, 1991; Jungawalla, 1991). Alternatively, sex reversal by steroids may be utilised indirectly to achieve the same results. Oral administration of 17 ∝-methyldihydrotestosterone to genotypic females at first feeding induces masculinisation of female brood fish and results in the production of milt in female genotype fish (Johnstone *et al.*, 1978). The milt of these 'sex-reversed' or 'masculinised' females can be used to fertilise the eggs of normal females to produce an all-female (100%) diploid stock. The production of all-female populations to avoid the unwanted secondary sexual characteristics of male salmon and to delay sexual maturity has been applied very successfully in commercial production (Johnstone *et. al.*, 1991; Jungawalla, 1991).

Alternatively, complete suppression of gonadal development in fish may be achieved by means of auto-immune, hormonal, chemical, irradiation or triploidy induction techniques. Most of these methods are not considered to be commercially viable because of market resistance, time and monetary constraints (Jungawalla, 1991). However, triploidy induction remains a reliable and commercially viable technique for the production of sterile stocks (Pepper, 1991; Johnstone *et al.*, 1991; Jungawalla, 1991). The possible development of testes capable of producing non-viable sperm (Lincoln and Scott, 1983; Benfey and Sutterlin, 1984) and the undesirable secondary sexual characteristics of sexually maturing male triploids can be eliminated by the production of all female triploids in which gonad development is completely suppressed (Lincoln and Scott, 1983, 1984; Solar *et al.*, 1984; Benfey and Sutterlin, 1984). All-female triploid salmonid populations may be produced commercially by subjecting the fertilised eggs of an all-female diploid population to either a thermal or hydrostatic pressure shock treatment (Benfey and Sutterlin, 1984c; Johnstone, 1985; 1989). Other techniques of triploidy induction in fish such as chemical treatments (Shelton *et al.*, 1986; Johnstone *et al.*, 1989), or inter-ploidy cross-breeding of tetraploid and diploid fish (Chourrout *et al.*, 1986; Myers and Hershberger, 1991), have not been favourably received in salmonid aquaculture either because of the perceived stigma associated with the use of chemicals on food products or non-viability on a commercial-scale.

The effectiveness of triploid induction treatment can be measured in terms of triploid yield which is influenced by triploid induction rate (percentage triploids) and the number of triploid fish which survive at hatching. Previous studies indicate that the effectiveness of thermal and pressure triploid induction treatments may vary according to egg quality, the magnitude and duration of the incubation temperature or pressure shock to which the eggs are exposed, and the time post-fertilisation that the shock treatment is applied (Benfey and Sutterlin, 1984c; Teskeredzic *et al.*, 1993). Optimisation of both thermal and pressure triploid induction protocols for Atlantic salmon (Benfey and Sutterlin, 1984c) and coho salmon *Oncorhynchus kisutch* (Teskeredzic *et al.*, 1993) eggs has resulted in a similar triploidy rate (100 %) and survival to hatching (up to 80 - 98 %) irrespective of treatment. In spite of this, it

has been previously suggested that in salmonids thermal shock is not as reliable as application of hydrostatic pressure because of variable triploid yields either due to decreased triploidy induction rates and / or higher mortality post-treatment (Benfey *et al.*, 1988; Johnstone, 1989; Boulanger, 1991). For example, in a study by Solar *et al.* (1984), rainbow trout eggs were subjected to different heat shock treatments, two of which resulted in either 100% triploidy with 50% survival at hatching or 30 % triploidy with similar survival to controls (93 %). Notwithstanding the confounding influence of suboptimal treatment protocols, the variability in triploid yields with heat treatment may be caused by differences in heat sensitivity between eggs and egg batches according to egg quality and size (Johnstone, 1989; Teskeredzic *et al.*, 1993).

To assess the effectiveness of the triploid induction treatment, the ploidy status of fish may be determined by a variety of techniques including: particle sizing using either a coulter counter (Benfey and Sutterlin, 1984c; Johnson *et al.*, 1984) or direct measurement of erythrocyte nucleus dimensions from blood smears (Wolters *et al.*, 1982; Benfey *et al.*, 1984; Johnstone and Lincoln, 1986; Thomas and Morrison, 1995); measuring erythrocyte DNA content either by flow cytometry (Allen, 1983; Benfey *et al.*, 1984; Johnson *et al.*, 1984; Seeb *et al.*, 1988) or microdensitometry (Gervai *et al.*, 1980, Johnstone, 1985; Shelton *et al.*, 1986); karyological assessment (Quillet and Gaignon, 1990); and protein isozyme analysis (Allen *et al.*, 1982; Seeb *et al.*, 1988). When available, flow cytometry is considered to be the technique of choice for both accuracy and relative ease of application (Benfey *et al.*, 1984; Johnson *et al.*, 1984); however, measurement of erythrocyte nucleus length (ENL) from blood smears is considered the next most reliable technique (Benfey *et al.*, 1984; Johnson *et al.*, 1984). Comparison of mean ENL of diploid and triploid individuals provides a conservative estimate of ploidy status (Thomas and Morrison, 1995). In addition, the simplicity of this technique is advantageous for the purposes of field work as it does not require specialised equipment.

The four populations used in this study were produced using standard commercial husbandry techniques and were maintained under standardised conditions. All-

female populations were produced by fertilising eggs from normal females with milt from masculinised genotypic females. Triploid populations were produced using a hydrostatic pressure treatment. Ploidy status was assessed using the particle sizing method by measuring mean ENL. The fertility rate, embryonic abnormality, egg recovery, growth and survival of each population during development was also examined.

2.3 MATERIALS AND METHODS

2.3.1 Fish Production

Four populations of Atlantic salmon; 'normal' = mixed sex diploids, all-female diploids, all-female triploids and mixed sex triploids, were produced for the purposes of this study at the SALTAS Wayatinah Hatchery, Wayatinah, Tasmania, during May 1996.

Populations were unrelated in that they were not derived from the same broodfish. Each population was produced by fertilising eggs pooled from 15 - 18 females with sperm pooled from 7 - 12 males and it was assumed that the number of broodfish used was sufficient to preclude significant genotypic variation between populations within this strain of fish. Development of fish from each population was described in accumulated temperature units (ATU = ° hours, or ° days) from the time of fertilisation.

Brood stock maintenance

Brood fish, comprising normal diploid males, normal diploid females and masculinised genotypic female diploids, were maintained from October 1994 to March 1996, in floating sea cages located in the Derwent estuary, Dover, Tasmania. Here they were subject to ambient water temperatures ranging between 9 - 18° C for 17-18 months. Sea cage nets were changed periodically to avoid the deleterious

effects of bio-fouling upon water flow and oxygen availability. Broodstock were fed with GIBSONS salmon growers diet at least twice daily. Male diploid, female diploid and sex-reversed genotypic female broodstock were stocked communally within a single cage and each type was distinguished by freeze-branding demarcation. Maturing brood fish were selected from each group and transported to FW facilities at the SALTAS Wayatinah hatchery in March, 1996, 2 months prior to spawning. They were maintained in D-ended raceway tanks (1 m depth × 3 m width × 13 m length), supplied by a flow-through water system with FW from the Upper Derwent river. Broodstock were subject to ambient water temperatures (1 - 5° C) and light conditions and were not fed during the FW phase prior to spawning.

Masculinisation of genotypic female broodstock

Sex reversal or 'masculinisation' of genotypic female broodstock was induced at first feeding by the oral administration of 17∝-methyldihydrotestosterone to all-female fry (1.0 mg.kg feed^{-1}) for 800° days. Masculinised fish were subject to normal growout conditions until maturity. Verification of masculinisation required dissection of the gonads of mature fish. Hermaphroditic development, or the presence of immature oocytes in the testes of a mature masculinised fish were the criteria used to confirm that a fish was a genotypic female (*pers comm.* Harry King, Operations manager, SALTAS, Tasmania, 1996). The testes of masculinised genotypic females also displayed assymetrical development, were often lobulated, and the sperm ducts were often absent (Bye and Lincoln, 1986). The latter however was considered an unreliable diagnostic feature for differentiating masculinised fish because normal genotypic males treated with methyltestosterone may have improperly developed sperm ducts and in some cases masculinised genotypic females freely express milt, indicating functional sperm ducts (*pers comm.* Harry King, Operations manager, SALTAS, Tasmania, 1996).

Stripping procedures

Prior to spawning, Broodstock were examined every 3 - 4 days and were sorted according to spawning history (lst or 2nd reproductive season), sex and maturity.

Mature females and males were checked for ovulation and spermiation, respectively, by application of gentle pressure to the abdomen. Ovulated and spermiated fish were either anaesthetised in a 25 ppm bath of Benzocaine (maiden fish to be returned to the sea cages for a second spawning season) or they were euthanased by a swift blow to the head.

Eggs were then stripped from ovulated females and collected in a stainless steel mesh strainer and the ovarian fluid was drained. Care was taken not to contaminate eggs with water during the stripping process as exposure to water results in the closure of the micropyle (precluding sperm access) and hardening of the egg chorion. Five to ten females were stripped at a time and the eggs from each fish were mixed evenly between 5-8 spawning bowls. Milt was stripped directly from each male (n = 3 - 6) into a spawning bowl. Each population consisted of a pooled batch of fertilised eggs, obtained by crossing a total of 15 - 18 females with 7 - 12 males, as specified below.

Testes from masculinised genotypic female broodstock were dissected and washed in 'milt extender' solution (Appendix A), an isotonic solution routinely used to dilute salmon milt. Testes that did not have the diagnostic features of genotypic females (described above) were discarded. The remaining testes were then macerated with a scalpel blade in a sieve. A small volume (10 - 20 ml) of milt was collected from each genotypic female and stored in tissue culture bottles at 2 - 4° C until it was required for fertilisation in the same day. Milt motility was assessed using a light microscope and a subjective motility scale (*pers comm.* Harry King, Operations manager, SALTAS, Tasmania). Milt samples which demonstrated greatest motility were selected for fertilisation.

Fertilisation

Normal mixed sex diploid population

The milt of 8 males was used to fertilise the eggs of 16 females to produce a normal, mixed sex diploid population. Immediately after stripping, the milt and eggs were mixed by hand. One to two litres of FW was added and the eggs were left to stand for 2 minutes to allow fertilisation to occur. The fertilised eggs were gently transferred to 50 L bins containing FW at ambient temperature (8° C). Fertilised eggs were left to stand for 1 - 2 hours to water-harden, after which time water was exchanged twice at 30 minute intervals and the eggs were periodically stirred by hand to avoid clumping.

All-female diploid population

The all-female diploid population was produced by fertilisation of eggs from 15 normal females with the milt of 7 masculinised genotypic females. Approximately 10 ml of milt from masculinised female fish was diluted with 10 - 20 ml of 'milt extender' solution (Appendix A). This volume of milt was sufficient to fertilise a bowl of approximately 10,000 eggs. Milt and eggs were mixed by hand and 500 ml of 'milt activator' solution was added. The addition of 'milt activator', a solution isotonic with ovarian fluid (Appendix A), enhanced the motility and consequently the ability of milt from genotypic females to fertilise eggs (Scott and Baynes, 1980; Levanduski and Cloud, 1988; Perchec *et al.*, 1995). The fertilised eggs were allowed to stand for 2 minutes before transfer to a 50 L bin of FW at 8° C for water hardening.

Triploidy induction

Triploidy was induced by the application of hydrostatic pressure to fertilised eggs. Water temperature and the time of pressure treatment post-fertilisation are critical to successful triploidy induction, as the time at which the second polar body is extruded from the egg following fertilisation, is very brief. The time of fertilisation was

defined as the instant at which the eggs were exposed to milt. Once fertilised, eggs were held at a constant temperature of 10° C (± 0.2) for 30 mins (300° mins). The eggs were transferred to a 6L hydrostatic pressure vessel and pressure was increased to 9500 psi over 90 secs before treatment commenced. Eggs were held at a constant pressure for 4 mins, after which pressure was released over a period of 60 s.

All-female triploid population

Milt was collected from 12 masculinised genotypic females and was used to fertilise eggs from 18 all-female diploids, as previously described, and the fertilised eggs were subject to the hydrostatic pressure treatment as described above.

Mixed sex triploid population

Milt was collected by catheter from 12 normal diploid males and stored in tissue culture bottles at 4° C, as described for storage of milt from genotypic females. This milt was used to fertilise the eggs from 15 females and triploidy was induced by hydrostatic pressure as described previously.

Fertility determination

Three samples of 250 eggs from each population batch were weighed, the total weight of each batch determined and the total number of eggs calculated. In addition, 100 eggs from each batch were held for 120° hours and were then cleared in a solution of methanol, glacial acetic acid and water (1:1:1). Within one minute 2 - 4 cells of the developing embryo were visible to the naked eye in fertile eggs, whereas the absence of cellular division indicated that eggs had not been fertilised. Fertility was expressed as a percentage of the total number of eggs present.

2.3.2 Husbandry: Fresh water (FW) phase

Fertilised eggs from each population were incubated independently under standard commercial conditions. Fertilised eggs were maintained in darkness in an upweller incubator for approximately 400° days (≈ 2 months) post-fertilisation. An upweller incubator consisted of a plastic cylinder (diameter = 40 cm) with a perforated stainless steel floor and a guttered rim at the top. Incubators were supplied by a hatchery flow-through water system in which incoming water was heated to 8° C and filtered to 25 μm. The water inlet was situated at the base of each incubator and produced an upward water flow that percolated gently through the eggs without disturbing them. Outflowing water spilled over the upper rim of the incubator into the collar gutter and drained into a main outflow pipe.

Eggs were treated *in situ* with an antifungal bath of malachite green (2 ppm) for 1 hour every 2nd day from 3 days post-fertilisation. After 1 week, the treatment was increased to 4 ppm. Treatment continued up until one week prior to hatch (≈ 400° days).

Fertilised eggs remained undisturbed for 250° days post-fertilisation, after which time they were subjected to a mechanical shock treatment to eliminate the less hardy embryos and those displaying retarded development (standard industry practice). Shock treatment involved the physical transfer of eggs from one container to another. Subsequently, dead eggs (opaque and white in appearance) were separated by automated "Egg pickers", quantified and discarded. Egg recovery rates post-mechanical shock were expressed as the number of live eggs recovered as a percentage of the original number of eggs fertilised. A sample of 250 eggs from each population was checked for abnormal embryonic development and expressed as a percentage of live eggs recovered.
Prior to hatching (≈ 400° days), unhatched embryos were transferred from the upweller incubators to trough incubators. A trough incubator consisted of a perforated box which contained an artificial substrate of "bio-balls". A perforated

tray inside each box supported the unhatched embryos on top of the artificial substrate. A number of incubators were placed adjacent to one another within a trough supplied with fresh water filtered to 100 μm, heated to 8° C and subject to unidirectional water flow. Upon hatching, the alevins dropped through the tray perforations to settle in the substrate below, where they were supported in an upright position. The alevins remained in this environment for approximately 6 weeks, during which time they utilised endogenous nutrient reserves contained in their yolk sac. Alevins emerged from the incubation substrate as fry (a process called "swim up") with less than 10% of their yolk sac remaining, at approximately 650° days.

Fry were then transferred from the trough incubators to larval hatchery tanks. Each population was divided between two of eight tanks during the FW growout phase (between 772 - 3118° days). Initially the fry were maintained in 1 m^3 square tanks (1m ×1m ×1m) supplied by the hatchery flow-through system (8° C, filtered to 100 μm), at a water flow greater than 1 $L.s^{-1}$. Water drained from a central outlet at the bottom of each tank. Artificial lighting set to ambient photoperiod was placed strategically over larval tanks to encourage activity in the fry and prevent crowding on the bottom of each tank. A crumble starter-feed (GIBSONS + NRM New Zealand Pacific Start Crumbles) was introduced at this stage and was dispersed by mechanised belt feeders, at a constant rate during the photophase.

Once fry had reached a total wet weight of 1.0 - 1.5 g, (Oct. 1996), they were transferred to 4 m^3 square tanks (2m × 2m × 1m) outside the hatchery (two for each population). Shade cloth covered the tanks to reduce ambient light and fish losses due to jumping out. The tanks were supplied by a flow-through water supply extracted from the Upper Derwent River and coursely filtered with a drum screen filter. An inflow pipe was positioned within each tank to generate a unidirectional water flow throughout the depth of the tank. A central drain at the bottom of each tank, guarded by perforated steel mesh, facilitated removal of wastes with water outflow. During the fry and parr stages of the lifecycle, fish were fed GIBSONS salmon starter diet (14% lipid, 50% protein), using automated belt feeders to maximise feeding opportunity. A diet with increased salt content (10% NaCl) was

introduced during smoltification at 12 months post-fertilisation (June, 1997), for the period up until SW transfer.

Populations of parr were "thinned" during January, 1997, to a stocking density of 0.5 $kg.m^{-3}$. The thinning process of populations involved random sampling of fish, irrespective of size, until the required biomass for each population was attained. Random sampling retained the different size classes in each population. Parr populations were "thinned" again during June, 1997, just prior to smoltification, to maintain stocking density at 25 $kg.m^{-3}$.

Smolt were ready for SW transfer at a weight of $\approx$ 80g, by October, 1997 (17 months post-fertilisation). Each population was divided into two groups. One group from each population was transferred to SW facilities at the School of Aquaculture Aquatic centre, University of Tasmania, Launceston. The remaining fish from each population were maintained in FW at the SALTAS Hatchery. The latter are henceforth referred to as FW smolt. FW smolt from each population were communally stocked in a 44 m^3 circular tank at a stocking density of < 2 $kg.m^{-3}$. They were subject to ambient water temperatures, ambient photoperiod, and were fed to satiation with GIBSONS salmon starter diet (14% lipid, 50% protein).

2.3.3 Husbandry: Seawater (SW) phase

During the SW phase, each population was maintained in one of four 2000 L tanks in a closed recirculating SW system. Water temperature (12.5 $\pm$ 0.5 °C), water flow rates (18 $L.min^{-1}$), salinity (33 $\pm$ 1 ppt) and stocking density (15 $kg.m^{-3}$) were consistent between tanks. Nitrite and Ammonia levels were monitored daily. Water quality was maintained by a biofilter, protein skimmer and regular water changes. Photoperiod simulated natural daylight hours of 11 hrs light : 13 hrs darkness. The smolt were fed GIBSONS salmon grower diet (22% Lipid, 45% Protein) to satiation twice daily (the equivalent of 1% body weight per day).

2.3.4 Population Triploidy Assessment

Population triploidy rates were determined immediately prior to SW transfer in FW parr (TWWt. ≈ 80g, 4202° days) by sampling 100 individuals from each triploid population and 50 individuals from the mixed sex diploid population. The mean erythrocyte nucleus length (ENL) data collected from the mixed sex diploid population included ENL values for female diploid fish (≈ 50% according to population sex ratio rseults). Even if it cannot be assumed that the ENL values of male and female diploids were the same, the mixed sex diploid population represented a conservative comparative group for both mixed sex and all-female triploid populations. Individuals were randomly sampled from each tank, anaesthetised in a 0.1% Benzocaine bath and killed by a blow to the head. Blood was sampled by caudal venesection and smeared onto glass slides. Blood smears were air dried, fixed in 70% ethanol and stained with haematoxylin. ENL was measured in 10 randomly selected red blood cells from each fish using an image analysis program (Cue-2 Image Analysis). Mean ENL values were determined for each fish and frequency histograms of mean ENL values from each triploid population were compared to those of the diploid population. The maximum mean ENL value observed for diploid fish represented the critical mean ENL value (Thomas and Morrison, 1995). Any triploid fish with a mean ENL value equal to, or less than the critical mean ENL value, was considered to be a possible diploid.

In addition to population triploidy assessment, the triploidy status of individual fish sampled for each experiment in Chapters 3, 4 and 5 was established in a similar fashion by determining the mean ENL from 10 erythrocytes in each fish.

2.3.5 Population Sex Ratio Assessment

Population sex ratios were determined histologically in FW parr (TWWt.≈ 80 g, n > 160), sampled at 4202° days, immediately prior to SW transfer. Individuals were randomly sampled from each tank, anaesthetised in a 0.1% Benzocaine bath and

killed by a blow to the head. Gonad tissue from each fish was excised, fixed in Bouin's fixative for 48 hours, and transferred to 70% ethanol prior to embedding in paraffin wax. Sections (5 μm) were stained in Haematoxylin and Eosin, mounted in DPX and examined using a compound light microscope for presence of oogonia or oocytes in ovarian tissue samples, and spermatogonia or spermatocytes in samples of testes. The sex of individuals affected by the various skeletal deformities reported in Chapter 3 was determined in the same manner.

2.3.6 Growth Assessment

The average total wet weight (TWWt), fork length (FL) and condition factor ($K = (TWWt. / FL^3) \times 100$) of fish randomly sampled from each population was measured at various stages of development (Table 1). For stages at which populations were held in two replicate tanks (between 772 - 3118° days), numbers sampled were divided evenly between tanks and data were pooled to obtain population means. Fish were anaesthetised in a 0.1% Benzocaine bath prior to growth measurement.

Specific growth rates were determined for each population between successive sampling times, throughout development, using population means and the following formula: Specific Growth Rate (SGR) = $100 \times \ln((W_f / W_o) / t)$, where W_f = final total wet weight (g), W_o = initial total wet weight (g) and t = time (calender days) between initial and final weight check. For the period between 772 - 3118° days, data for duplicate tanks A and B were pooled for each population.

2.3.7 Survival during SW phase

Mortalities following SW transfer and during SW growout were recorded daily. Cumulative % mortality was determined using the following formula: Cumulative % mortality = $(M / N_o) \times 100$, where M = total number of accumulated mortalities in the period (days) post-SW transfer and N_o = initial number of fish at SW transfer.

Table 1. Developmental stages and total number of fish sampled for growth determination during development of four populations of Atlantic salmon; all-female triploids (FT), mixed sex triploids (MT), mixed sex diploids (MD), all-female diploids (FD). Accumulated temperature units (ATU) represents development post-fertilisation.

Date	Developmental Stage	Weeks (post-fertilisation)	ATU (°days)	FT (n)	MT (n)	MD (n)	FD (n)
May 1996	Fertilisation		0				
July 1996	Hatching						
	ALEVINS	8	470	20	20	20	20
		9	528	20	20	20	20
		10	583	20	20	20	20
August 1996		11	642	20	20	20	20
		12	706	50	50	50	50
	Swim up and First Feeding						
	FRY	13	772	20	20	20	20
		14	843	20	20	20	20
September 1996		15	913	100	100	100	100
October 1996		19	1510	100	100	100	100
January 1997	PARR	31	2350	200	200	200	200
	Populations Thinned						
April 1997		45	3118	200	200	200	200
June 1997	Populations Thinned						
September 1997			4026	30	30	30	30
October 1997	SMOLT		4202	100	100	100	100
	Sea transfer						
November 1997	SW SMOLT		4810	100	100	100	100
	FW SMOLT		4928	100	100	100	100

2.3.8 Statistics

Each growth parameter (TWWt., FL., K) was compared, both between populations within each sampling time, and between sampling times within each population, by one way ANOVA analysis ($\propto = 0.05$). Each growth parameter was compared between populations according to ploidy (p) and sex status (s) throughout development by 3-way nested ANOVA analysis ($\propto = 0.05$). Prior to analysis, values were tested for normal distribution and homogeneity of variance within treatments using Shapiro-Wilk and Bartlett's tests, respectively. An arcsin transformation was used on data when required to conform to ANOVA assumptions. Alternatively, when ANOVA assumptions could not be satisfied, a non parametric Wilcoxon / Kruskal-Wallis test was used ($\propto = 0.05$). Statistical analysis was carried out using JMP software (version 3.1.6.2).

2.4 RESULTS

2.4.1 Fertility and embryo viability

Fertility of all-female populations did not differ significantly from those of mixed sex populations ($P > 0.05$, Table 2). The fertility of the all-female triploids (86.0%), was similar to that of the mixed sex triploids (84.5%), whereas the fertility of the all-female diploids (92.3%) was markedly higher than that of the mixed sex diploids (78.2%, Table 2).

There was no significant effect of ploidy or sex status on egg recovery following the mechanical shock treatment applied at 250° days ($P < 0.05$, Table 2). The proportion of live eggs recovered was higher for the mixed sex triploids (86.7%) than for either the all-female diploid population (78.9%) or the mixed sex diploid population (49.2%), whereas the all-female triploids had a higher egg recovery rate (66%) than the mixed sex diploids only (Table 2).

In addition, the rates of deformity (all-female diploids = 6.4%, all-female triploids = 3.6%, mixed sex diploids = 4.8% and mixed sex triploids = 5.2%, Table 2) detected during early embryonic development (250° days) were low and did not differ significantly with population sex or ploidy status ($P < 0.05$).

Table 2. Percentage fertility, egg recovery after mechanical shocking at 250° days, embryonic deformity, triploidy rate and population sex ratios of four Atlantic salmon populations; all-female diploids, all-female triploids, mixed sex diploids and mixed sex triploids. n denotes sample size.

Population	Fertility % (n)	Egg recovery %	Embryonic deformity % (n)	Triploids % (n)	Sex ratio male : female (n)
all-female diploids	92.3 (100)	78.9	6.4 (250)		0 : 1 (167)
all-female triploids	86 (100)	66	3.6 (250)	96 (99)	0 : 1 (199)
mixed sex diploids	78.2 (100)	49.2	4.8 (250)	0 (50)	1 : 1.4 (186)
mixed sex triploids	84.5 (100)	86.7	5.2 (250)	100 (100)	1 : 1.2 (174)

2.4.2 Population Triploidy Assessment

Mean erythrocyte nucleus length (ENL) ranged between 5.1 - 6.9 μm in mixed sex diploids, between 7.2 - 10.2 μm in mixed sex triploids and between 5.4 - 11.4 μm in all-female triploids (Fig. 1). An overlap of the normal distribution of mean ENL values of diploid and triploid fish is to be expected, consequently a conservative approach was taken in determining a critical mean ENL value for distinguishing between diploid and triploid fish (Thomas and Morrison, 1995). All fish sampled with a mean ENL value greater than the maximum diploid mean ENL (> 6.9 μm) were assumed to be triploids. Fish from the all-female triploid population with a mean ENL value within the diploid range (≤ 6.9 μm), may have been diploid fish and were treated as such. Of the fish sampled, 96% of all female triploids were considered to be triploids based on the above criterion (Table 2), likewise, 100% of the mixed sex triploid fish were considered to be triploids. One fish sampled from the all-female triploid population had a mean ENL of 11.4 (Fig. 1b) and it is possible that this individual was a tetra ploid. However, the latter cannot be verified without further sampling from a tetraploid population.

In subsequent experiments (Chapters 3, 4 and 5), Triploid individuals with a mean ENL value < 6.9 μm were considered to be possible diploid individuals and were excluded from data sets. Triploid assessment pertaining to experimental protocols are provided in the relevant chapters.

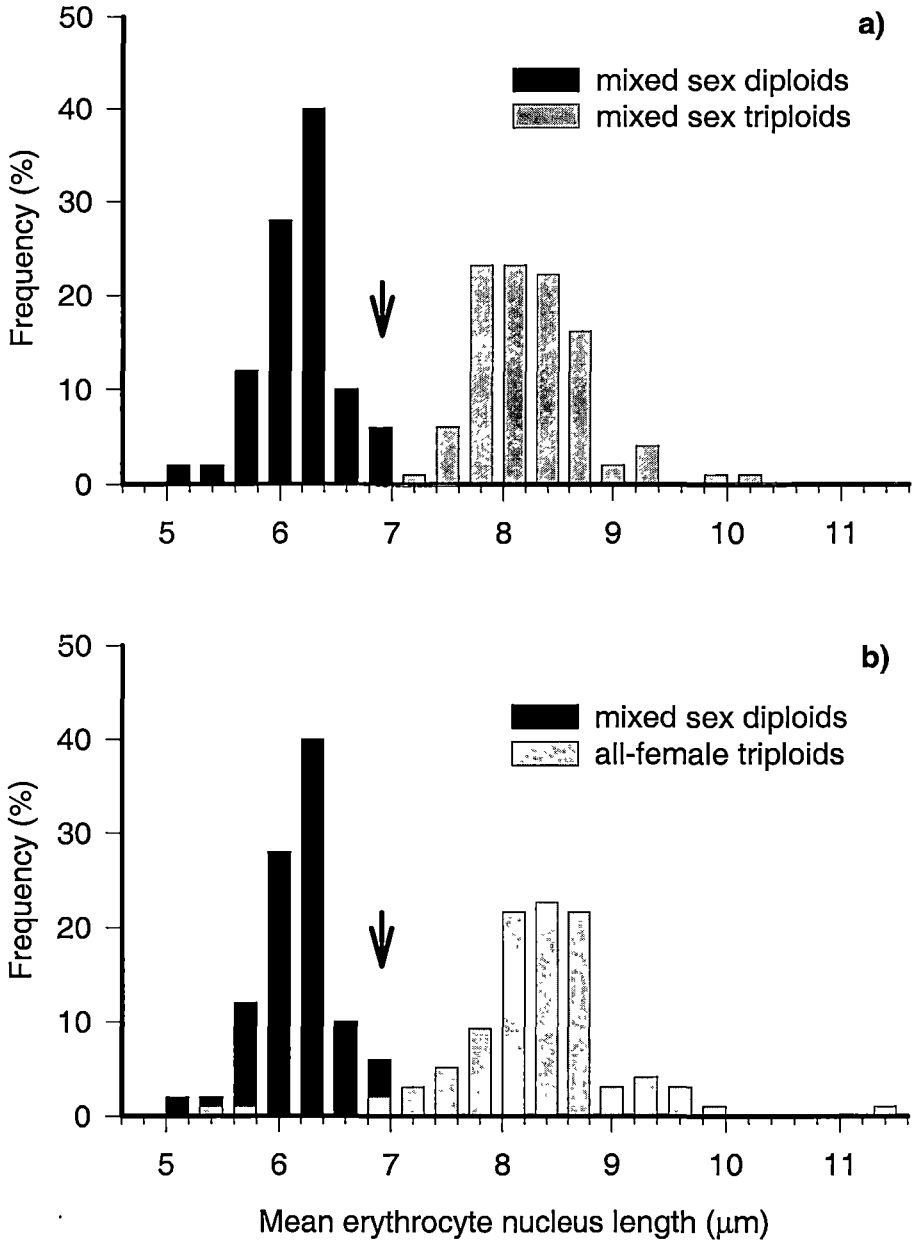


Figure 1. Percentage frequency histograms of individual mean erythrocyte nucleus length of a) mixed sex diploid (n = 186) and mixed sex triploid (n = 174), and b) mixed sex diploid (n = 186) and all-female triploid (n = 197) Atlantic salmon smolt, at 4202° days. Arrow indicates maximum mean ENL value for diploid fish (= critical ENL value).

2.4.3 Population Sex Ratio Assessment

Microscopic examination of the gonad structure confirmed the population sex ratios were 0 male: 1 female for the all-female diploids and all-female triploids; 1 male: 1.4 females for the mixed sex diploids and 1 male: 1.2 females for the mixed sex triploids (Table 2).

The ovarian tissue of juvenile diploid female Atlantic salmon consisted predominantly of synchronously developing pre-vitellogenic oocytes in the early perinucleolus stage (Nagahama, 1983). Oocytes were arranged in lamellae of relatively sparse stromal tissue in diploid individuals (Fig. 2a). Ovarian lamellae of juvenile triploid females consisted of a higher proportion of stromal tissue surrounding germ cells, oogonia, and the occasional perinucleolus oocyte (Fig. 2b). Juvenile diploid male Atlantic salmon were distinguished by the lobular arrangement of spermatogonia within the immature testes (Fig. 3a). A few of the male diploid individuals displayed differentiated spermatocytes, spermatid cells and spermatozoa within adjacent sperm ducts (Fig. 3b). The testes of triploid males, were very similar in appearance to immature diploid male testes, comprising of undifferentiated spermatogonia (Fig. 3c).

Figure 2. Ovarian tissue of an immature a) diploid female (mag × 100), b) triploid female (mag × 100) and c) triploid female (max × 400) Atlantic salmon FW parr at 4202° days. Abbreviations: Ll = lamellae, s = stromal tissue, gc = germ cell, Oo = oogonia, pOC = perinucleolitic oocyte. Sections stained with Haematoxylin and Eosin. Scale bar = 100 μm.

a)

b)

c)

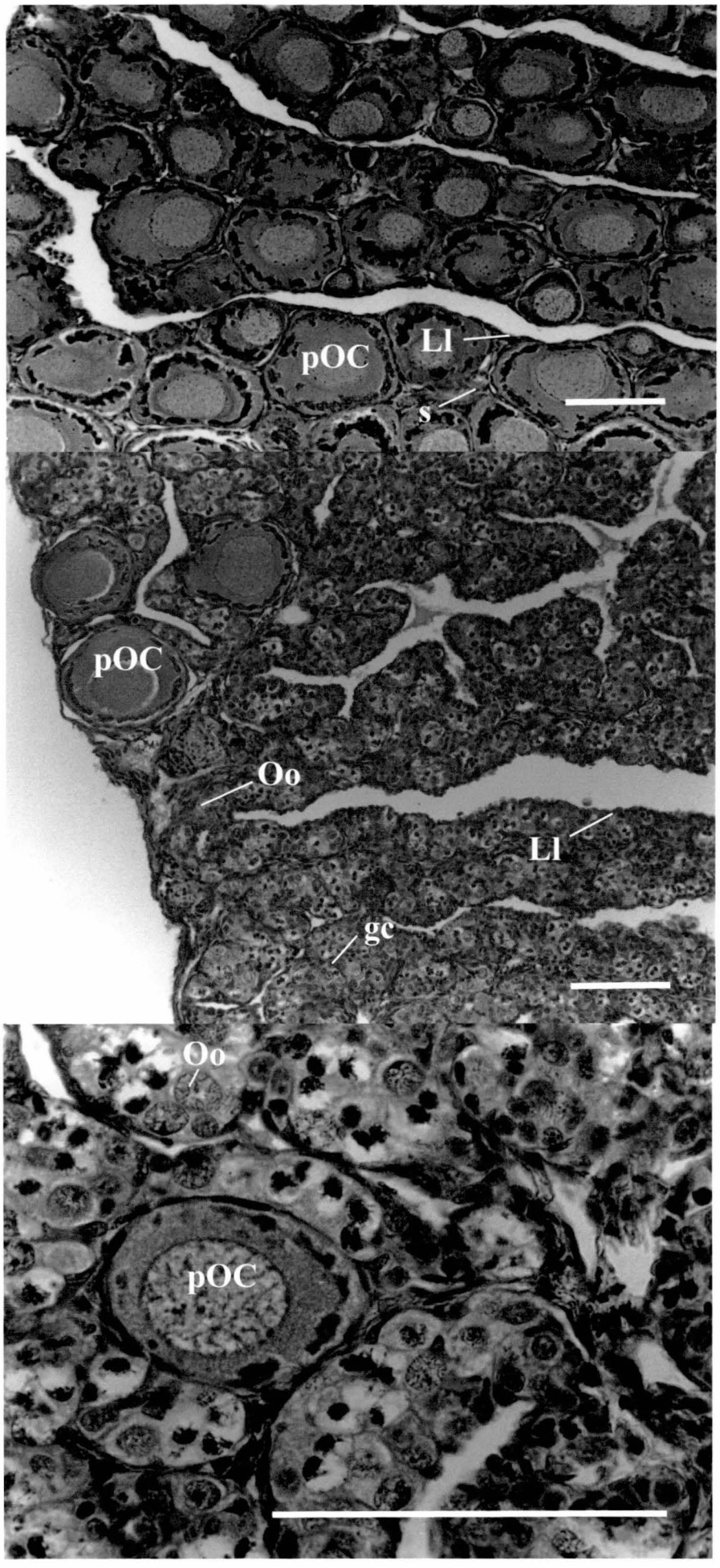
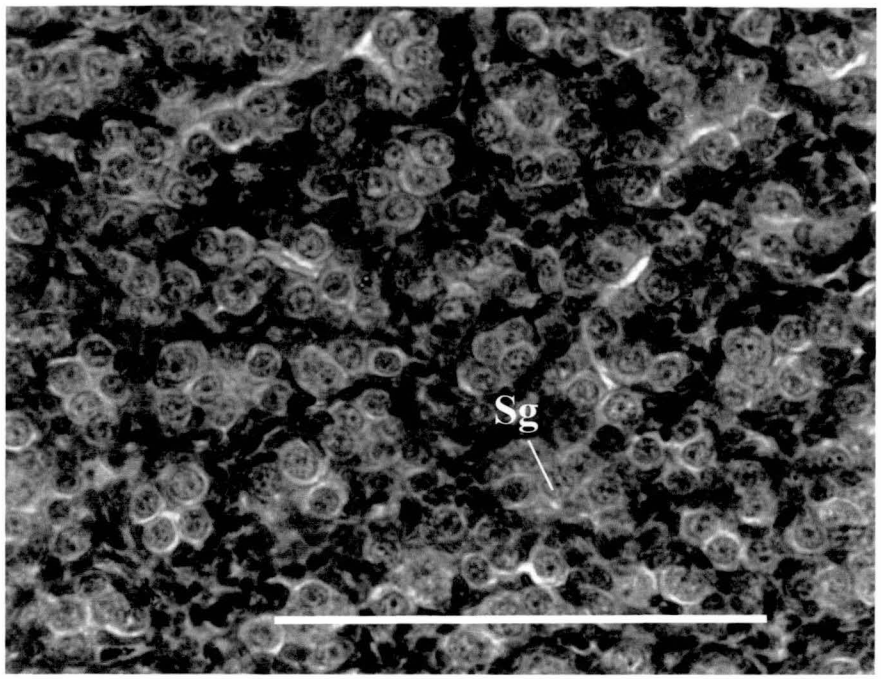


Figure 3. Testes of a) an immature diploid male (mag × 400), b) precociously mature diploid male (mag × 400) and c) triploid male (mag × 400) Atlantic salmon FW parr at 4202° days. Abbreviations: gc = germ cell, Sg = spermatogonia, SC = spermatocytes, Sz = spermatozoa. Sections stained with Haematoxylin and Eosin. Scale bar = 100 μm.

a)



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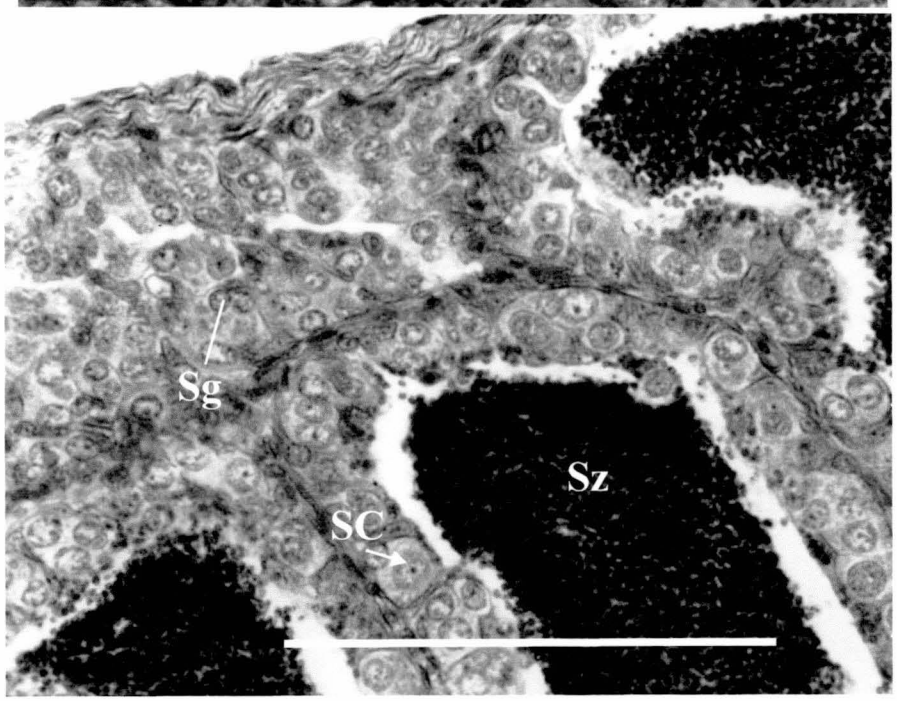
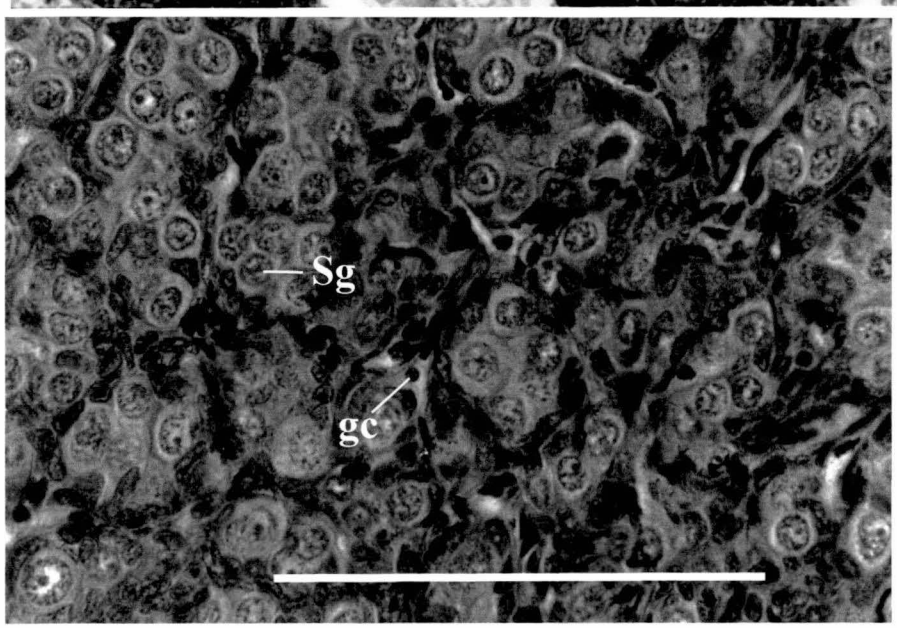


c)



2.4.4 Growth

Total wet weight increased during development in all population types (Fig. 4). Diploid populations had a significantly higher TWWt. than triploids during the early fry stages (913 - 2350° days, $P < 0.05$, Table A, Appendix B). Triploid populations had significantly higher TWWt. than diploids at 3118° days ($P < 0.05$, Table A, Appendix B). Ploidy status had no significant effect on weight at 4026° days and 4202° days ($P > 0.05$). Population sex ratio was associated with a significant difference in TWWt. at 3118° days and 4202° days ($P < 0.05$, Table A, Appendix B), such that mixed sex populations had a higher weight than all-female populations. At the smolt stage (> 4500° days), under both FW and SW conditons, diploid fish had a significantly higher TWWt. than triploid fish ($P < 0.05$, Fig. 5, Table A, Appendix B). Weight was significantly higher in FW smolt than SW smolt at the termination of the experiment, irrespective of population type ($P < 0.05$, Fig. 4).

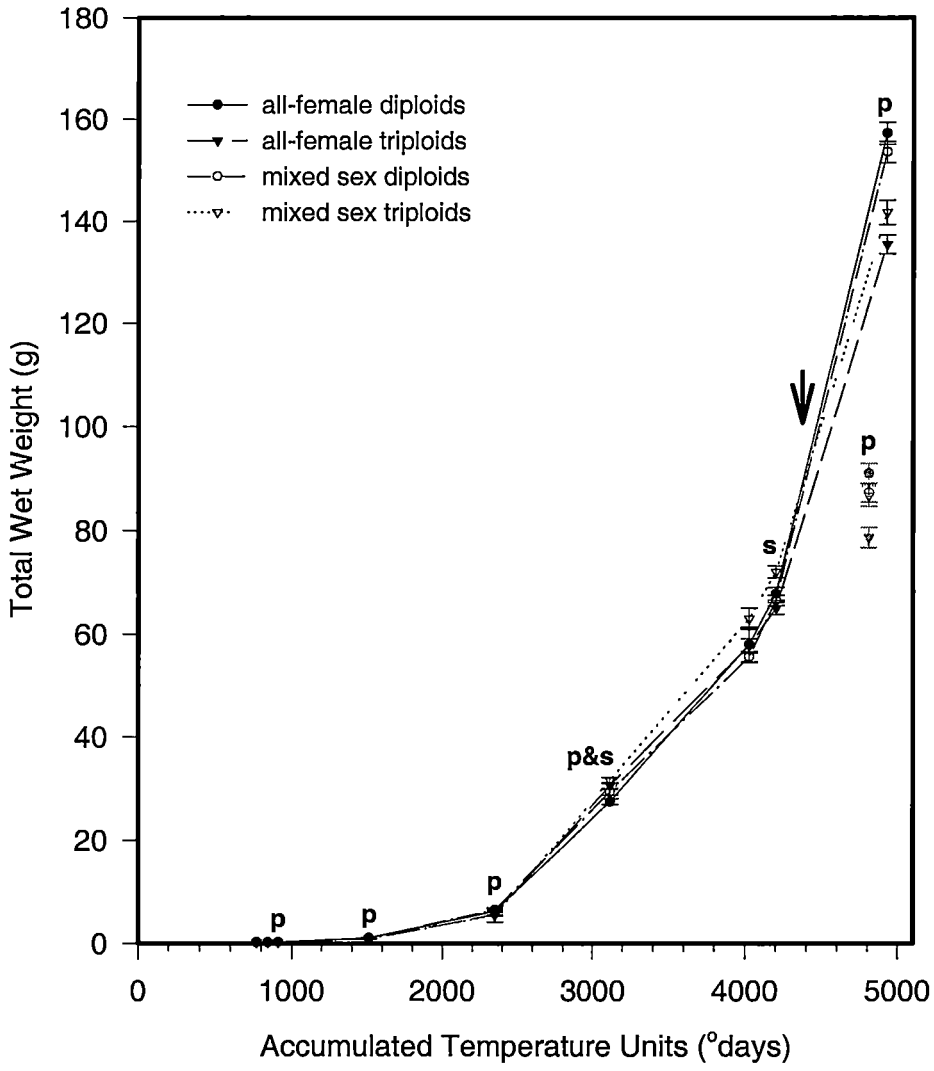


Figure 4. Mean (± SE) total wet weight of four Atlantic salmon populations; all-female diploids, all-female triploids, mixed sex diploids and mixed sex triploids, through development in freshwater (FW) and seawater (SW). Accumulated temperature units (ATU = ° days) represent development post-fertilisation. Black open and closed symbols denote parr and smolt held in FW. Grey symbols denote smolt held in SW. Arrow indicates time of SW transfer. Superscripts indicate significant difference between diploid and triploid populations (p) or between all-female and mixed sex populations (s) at each developmental stage. See Table A (Appendix B) for confirmation of values. Sample numbers n = 20 - 200 as per Table 2.

Fork length (FL) increased throughout development in all populations (Fig. 5). The effect of ploidy on FL values at any one stage was inconsistent throughout development. Ploidy had no effect on FL between 843 - 913° days ($P > 0.05$). Diploids had a significantly greater length than triploids from 1510 - 2350° days, but were significantly shorter than triploids from 3118 - 4202° days ($P < 0.05$, Table B, Appendix B). In both SW and FW conditions, diploid smolt (> 4500° days) were significantly longer than their triploid counterparts ($P < 0.05$, Fig. 5, Table B, Appendix B). All-female populations had significantly higher FL values than mixed sex populations at 913° days and mixed sex populations had a higher FL values than all-female populations at 3118 and 4202° days ($P < 0.05$, Table B, Appendix B).

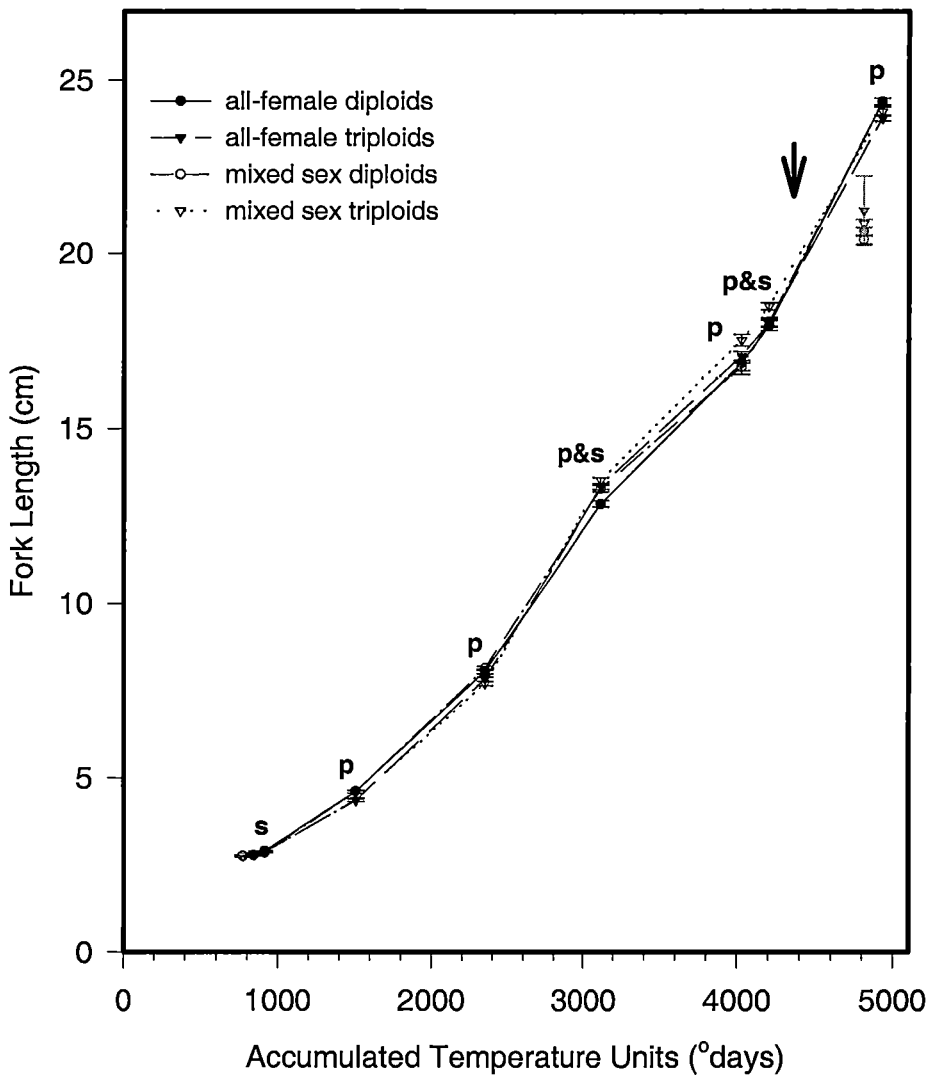


Figure 5. Mean (± SE) fork length of four Atlantic salmon populations; all-female diploids, all-female triploids, mixed sex diploids and mixed sex triploids, during development in freshwater (FW) and seawater (SW). Accumulated temperature units (ATU = ° days) represent development post-fertilisation. Black open and closed symbols denote parr and smolt held in FW. Grey symbols denote smolt held in SW. Arrow indicates time of SW transfer. Superscripts indicate significant difference between diploid and triploid populations (p) or between all-female and mixed sex populations (s) at each developmental stage. See Table B (Appendix B) for confirmation of values. Sample numbers n = 20 - 200 as per Table 2.

In all populations, condition factor (K) increased significantly from first feeding up until 3118° days ($P < 0.05$, Fig. 6). This was followed by a significant decrease in K during smoltification (3118 - 4500° days) prior to SW transfer ($P < 0.05$). Condition factor decreased further still in smolt either transferred to SW, or maintained in FW (> 4500° days, $P < 0.05$, Fig. 6). The mean K values of SW smolt were significantly lower than respective values of FW smolt at the termination of the experiment ($P < 0.05$).

The effect of ploidy status or population sex ratio on K values varied with development (Fig. 6). Ploidy status and population sex ratio had no significant effect on K values just after first feeding (772 - 843° days) and prior to SW transfer (4026 - 4202° days, $P > 0.05$). Diploids had significantly higher K values than triploids prior to smoltification (843 - 2350° days) and at the smolt stage, both in FW and SW (> 4500° days, $P < 0.05$). Population sex ratio effected K values post first feeding until smoltification (1510 - 3118° days, $P < 0.05$). At 2350° days mixed sex populations had higher mean K values than all-female populations, while the reverse was true at other stages ($P < 0.05$, Fig. 6).

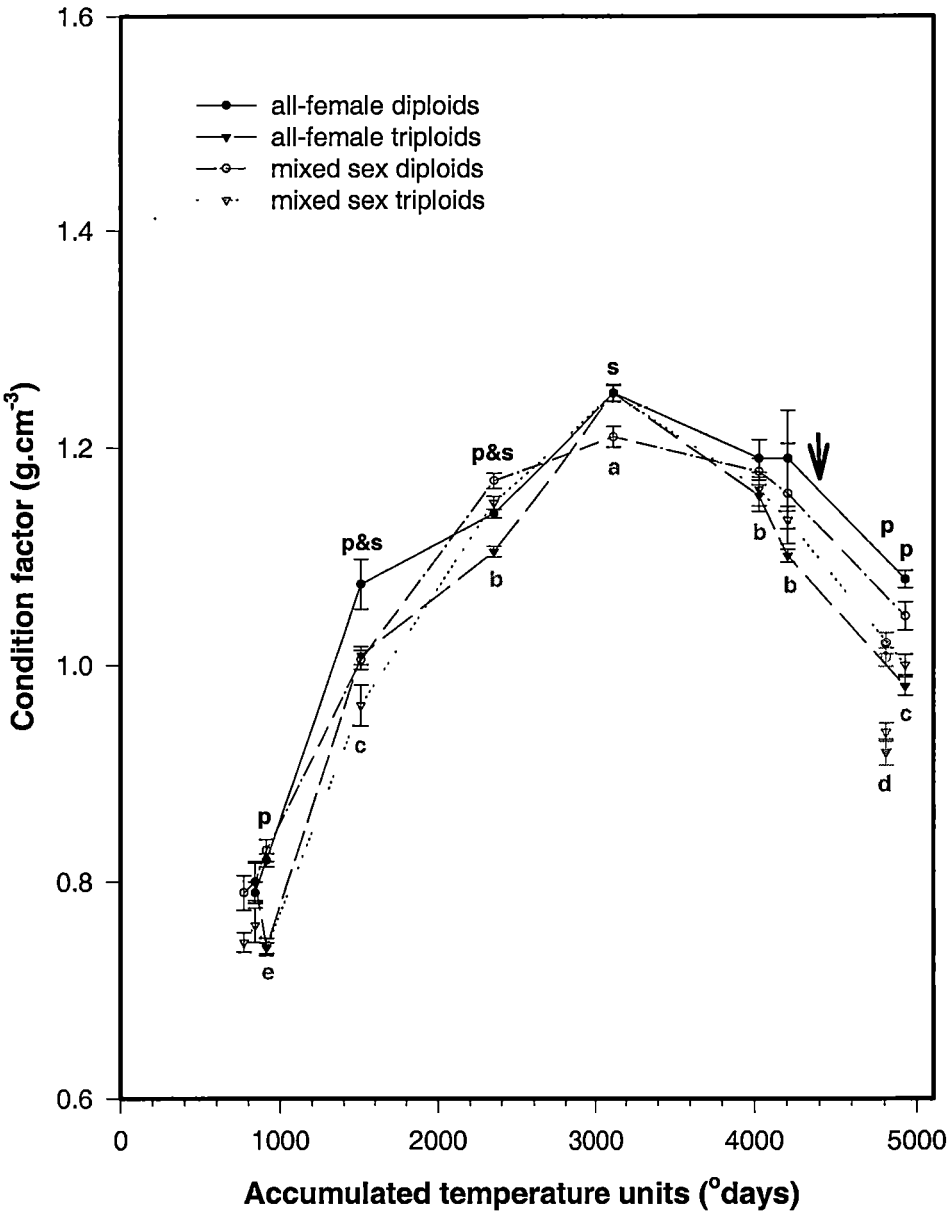


Figure 6. Mean (± SE) condition factor of four Atlantic salmon populations; all-female diploids, all-female triploids, mixed sex diploids and mixed sex triploids, during development in FW (FW) and SW (SW). Accumulated temperature units (ATU = ° days) represent development post-fertilisation. Black open and closed symbols denote parr and smolt held in FW. Grey symbols denote smolt held in SW. Arrow indicates time of SW transfer. Superscripts indicate significant difference between diploid and triploid populations (p) or between all-female and mixed sex populations (s) at each developmental stage. Different grey superscripts denote significant differences between developmental stages. Sample numbers n = 20 - 200, as per Table 2.

Specific Growth Rate (SGR)

Specific growth rates for each population during the early FW phase (772 - 4202° days, data for Tanks A and B pooled), ranged between 1.449 - 1.489 %.day^{-1} and did not differ with ploidy or sex status ($P > 0.05$, Table 3). In addition, population SGR values for SW smolt during the SW phase (4202 - 4810° days) ranged between 0.041 - 0.063 %.day^{-1} and did not vary with ploidy or sex status ($P > 0.05$, Table 3). Diploid smolt which were maintained under FW conditions (FW smolt) had significantly higher SGR values (0.175 - 0.176 %.day^{-1}) than triploid FW smolt (0.143 - 0.152 %.day^{-1}) for the period 4202 - 4928° days ($P < 0.05$, Table 3).

Population SGR values of FW smolt were higher than respective values of SW smolt ($P < 0.05$, Table 3). Both FW smolt and SW smolt had significantly lower population SGR values than FW parr ($P < 0.05$, Table 3).

Table 3. Specific growth rates (SGR) of four Atlantic salmon populations; all-female diploids, all-female triploids, mixed sex diploids and mixed sex triploids during development in FW (772 - 4202° days, data pooled for tanks A and B), for the SW smolt stage (4202 - 4810° days) and the FW smolt stage (4202 - 4928° days).

Population	SGR (%.day^{-1})		
	FW parr (772 - 4202° days)	SW smolt (4202 - 4810° days)	FW smolt (4202 - 4928° days)
all-female diploids	1.489	0.063	0.175
all-female triploids	1.449	0.041	0.152
mixed sex diploids	1.454	0.059	0.176
mixed sex triploids	1.483	0.062	0.143

2.4.5 Survival during SW phase

The cumulative % mortality of Atlantic salmon smolt post-SW transfer was significantly higher in triploid populations than respective diploid populations ($P < 0.05$, Fig. 7). A flexi-bacter infection was observed co-incident with the increase in mortality after 36 days in SW (Fig. 7).

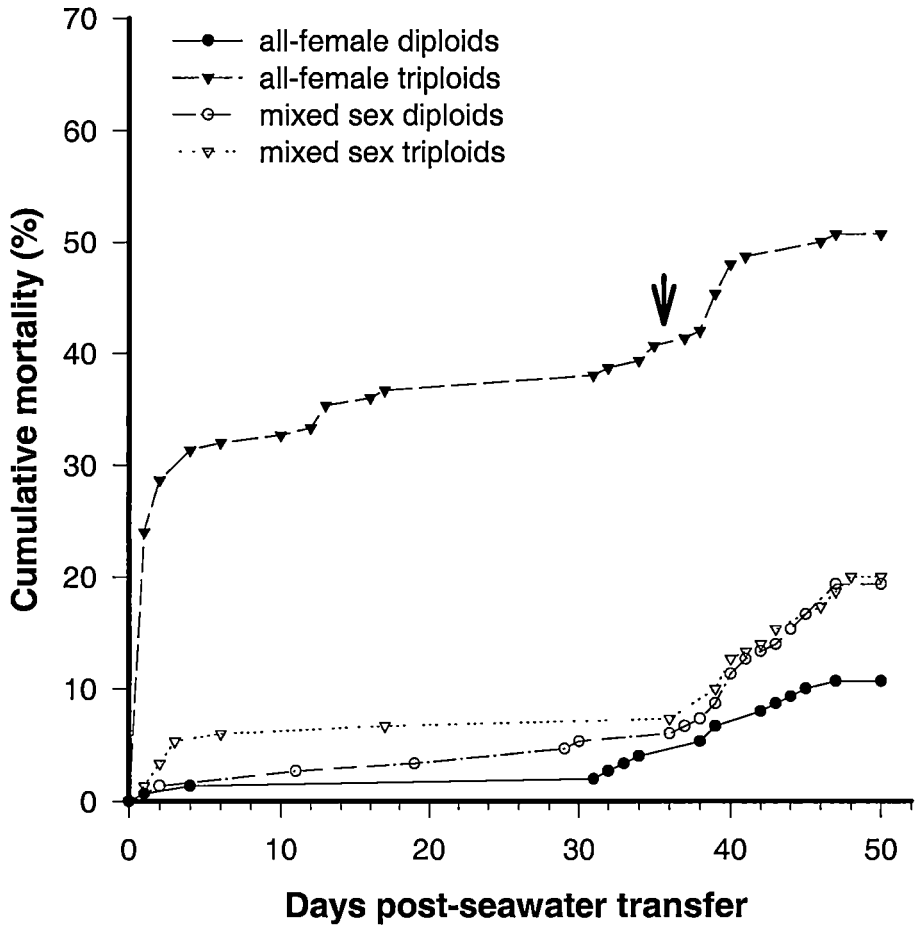


Figure 7. Cumulative percentage mortality within four populations of Atlantic salmon; all-female diploid, all-female triploid, mixed sex diploid and mixed sex triploid SW smolt, from the time of initial SW transfer until termination of the SW phase. Flexi-bacter infection was co-incident with the increase in mortality after 36 days post-SW transfer, indicated by an arrow.

2.5 DISCUSSION

2.5.1 Genotypic female milt

In the present study, the use of milt from genotypic females did not appear to have deleterious effects on fertility compared to the use of milt from normal male Atlantic salmon. In addition, egg recovery post-mechanical shock treatment and the the rate of embryonic deformity of all-female populations were similar to those of mixed sex populations, indicating there was little difference in embryonic viability irrespective of milt derivation. These results are supported by egg recovery and deformity rates observed for other all-female and mixed sex diploid populations produced by SALTAS in 1996 (unpublished data). In a study which examined the characteristics of milt from genotypic female rainbow trout, *Onchorhynchus mykiss*, the embryonic yield of eggs fertilised with milt from genotypically female fish was affected by the volume of milt used. However, at sufficiently high volumes (10 ml milt / 10, 000 ova), the embryonic yield was equivalent to that of milt from normal males (Cobcroft, 1996). The results of the present study indicate that the volume of milt from masculinised female Atlantic salmon used (10 - 20 ml milt / 10,000 ova) was sufficient such that fertilisation and embryo viability were not compromised.

2.5.2 Triploidy Assessment

The percentage of fish that were considered to be triploids was high, 96 % and 100 % in all-female and mixed sex triploid populations, respectively, confirming the desired ploidy status of these populations. Triploidy rates of 100 % have been previously recorded in Atlantic salmon (Benfey and Sutterlin, 1984c; Jungawalla, 1991) and other salmonids (brook trout *Salvelinus fontinalis*: Boulanger, 1991; coho salmon: Teskeredzic, 1993; rainbow trout: Chourrout, 1984) exposed to pressure treament. Similar triploidy rates (100 %) have also been achieved by thermal induction in Atlantic salmon (Benfey and Sutterlin, 1984c; Johnstone, 1985; Quillet and Gaignon, 1990; Galbreath *et al.*, 1994) and other salmonids (coho salmon:

Teskeredzic, 1993; Atlantic salmon × brown trout *Salmo trutta* hybrids: Galbreath and Thorgaard, 1994; rainbow trout: Lincoln and Scott, 1983; Solar *et al.,* 1984). However, triploidy rates may vary according to the triploid induction protocol, species and egg batches (Johnstone, 1989). Different thermal triploid induction protocols have resulted in much lower triploidy rates in Atlantic salmon (0 - 24 %, Benfey and Sutterlin, 1984c; 2 - 30 %, Johnstone, 1985; 3 - 31 %, Quillet and Gaignon, 1990), rainbow trout (45 ± 12 %, Thorgaard *et al.*, 1981; 50 %, Lincoln and Scott, 1983; 18 %, Solar *et al.*, 1984) and coho salmon (3 - 25 %, Teskeredzic *et al.,* 1993). Similarly, different pressure treatments have resulted in much lower triploidy rates in chum salmon *Oncorhynchus keta* (10 ± 17 %, Benfey *et al.,* 1988) and coho salmon (0 - 17.8 %, Teskeredzic *et al.,* 1993). The high triploidy rates observed in the current study and other studies, coupled with high egg survival and low deformity rates, characterise optimal triploid induction treatments. Other all-female triploid populations produced by SALTAS in 1996 had a mean (± SE) egg recovery rate (74.9 ± 6.2 %, n = 3) and deformity rate (3.7 ± 1.3 %, n = 3) similar to those of all-female diploid populations produced in the same year (egg recovery: 75.6 ± 2.4 %, n = 11; deformity rates 4.0 ± 0.6 %, n = 9). By contrast, Solar *et al.* (1984) found that although a 10 min heat treatment of 28° C applied 40 mins after fertilisation of rainbow trout eggs resulted in 100% triploidy, survivorship was 50 % that of controls and the rate of deformities (data not given) was higher than in controls. Thorgaard *et al.* (1981) also observed higher incidence of deformity in rainbow trout subject to thermal triploid induction (data not given) which may be attributed to either the heat shock itself or associated with triploidy.

2.5.3 Gonadal Development

In this study oogenesis was retarded in juvenile female triploid Atlantic salmon at 17 months post-fertilisation (MPF) compared with diploid females of the same age. Development of a few oocytes in female triploid ovaries has been previously recorded in both non salmonid species; flounder (*Platichthys flesus*, Purdom, 1972), plaice (*Plueronectes platessa*, Purdom, 1972; Lincoln, 1981a), carp (*Cyprinus*

carpio, Gervai *et al.*, 1980), channel catfish (*Ictalurus punctatus*, Wolters *et al.*, 1982), European catfish (*Silurus glanis*, Krasznai and Marian, 1986), and salmonids; Atlantic salmon (18 MPF, Benfey and Sutterlin, 1984d), coho salmon (30 MPF, Johnson *et al.*, 1986) and brook trout (Benfey, 1998). Benfey and Sutterlin (1984d) found that oocytes of mature female triploid Atlantic salmon individuals did not develop beyond the cortical alveolus stage, with the exception of a single triploid individual, which displayed asynchronous oogenesis, and ovulated eggs of various sizes. Other studies have indicated that oogenesis is completely inhibited, as evidenced by the absence of oocytes in the ovaries in female triploid rainbow trout (Thorgaard and Gall, 1979; 5 MPF, Lincoln and Scott, 1983; 23 MPF, Lincoln and Scott, 1984; 8 MPF, Solar *et al.*, 1984; 4.8 MPF, Krisfalusi and Cloud, 1996) and coho salmon (6.6 MPF, Piferrer *et al.*, 1994), which may in part be related to the relatively young age of the fish sampled.

The microscopical appearance of testes of juvenile diploid and triploid Atlantic salmon was very similar; however, samples were taken too early in development to determine whether spermatogenesis was suppressed in triploid individuals. Some previous studies confirm that male triploid testes are similar to those of diploid counterparts (plaice: Lincoln, 1981b; rainbow trout: 5 MPF, Lincoln and Scott 1983; 23 MPF, Lincoln and Scott, 1984; 8 MPF, Solar *et al.*, 1984) and in some cases have reported production of functionally sterile spermatozoa (Lincoln, 1981b; Lincoln and Scott, 1983, 1984). In contrast, other authors report an increase in the quantity of connective tissue (Wolters *et al.*, 1982; Gervai *et al.*, 1980), signs of spermatocyte degeneration (Swarup, 1957: reviewed by Lincoln and Scott, 1984), and an increase in size of spermatogonia (Pifferer *et al.*, 1994) or spermatozoa cells (Lincoln and Scott, 1984) in male triploids.

The lack of spermatogenesis and oogenesis in male and female triploid fish is the result of the disruption of the first meiotic prophase of gametogenesis caused by the third set of chromosomes present in triploid cells (Benfey, 1999). However, a small percentage of germ cells do progress beyond the meiotic block, as indicated by the production of functionally sterile spermatozoa in some male triploids and the

presence of post-mitotic oocytes in some female triploid fish (Benfey, 1991). The appearance of developed oocytes in triploid female fish may be due to the random segregation of chromosomes during meiosis (Pifferer *et al.*, 1994). Alternatively, recent studies suggest that post-meiotic growth of oocytes in female triploid fish may be inhibited by the lack of sufficient estradiol production by maturing oocytes, and subsequently a lack of hepatic vitellogenin production (Benfey *et al.*, 1990). Estradiol and testosterone levels in female triploid rainbow trout, were found to be negligible compared to diploid counterparts (Lincoln and Scott, 1984). In coho salmon, vitellogenin was undetectable in female triploid fish prior to sexual maturation, but was present in female diploid fish (Johnson *et al.*, 1986), which may indicate that estradiol was not present in sufficient quantities for vitellogenin production in triploid fish. Whereas endogenous 17β-estradiol treatment during the period of gonadal differentiation failed to induce oocyte development in juvenile triploid rainbow trout by 145 DPF (Krisfalusi and Cloud, 1996), vitellogenesis was induced in immature diploid and triploid coho salmon by the weekly injection of 17β-estradiol (Benfey *et al.*, 1990). These findings support the suggestion that post-meiotic development of oocytes in triploid fish may be substrate limited (Benfey and Sutterlin, 1984d).

2.5.4 Growth

Differences existed in growth between diploids and triploids during development. The growth of triploids was slightly retarded during early development post-first feeding, as reflected in the lower TWWt., FL and K values up to 2350° days, but was higher in triploids prior to smoltification. Overall, the specific growth rates calculated during the period of FW development prior to the time of sea transfer, were similar for each population, regardless of ploidy status. Some studies have indicated that the growth of triploid salmonids during early FW development may be lower (Atlantic salmon, Jungawalla, 1991; rainbow trout: Myers and Hershberger, 1991) or higher (Atlantic salmon: Galbreath *et al.*, 1994) than that of diploids. The weight of evidence indicates diploid and triploid Atlantic salmon (Johnstone, *et al.*, 1991; Jungawalla, 1991; Sutterlin and Collier, 1991; Galbreath and Thorgaard,

1994; McGeachy *et al.*, 1995; O'Flynn *et al.*, 1997), and rainbow trout (Quillet *et al.*, 1987; Myers and Hershberger, 1991) display similar growth during the FW phase. The current study is robust, in that all populations were maintained under standardised culture conditions (tanks, stocking density, water temperature, water flowrates, feeding regime), and supports the latter findings, whereas some previous reports describe the relative growth and survival of triploid populations under variable commercial conditions and may not reflect the relative growth of diploid and triploid fish.

The dramatic decrease in SGR of Atlantic salmon SW smolt following SW transfer may indicate that SW culture conditions were physiologically demanding and may reflect the hypo-osmoregulatory status of the fish. This is supported by the higher growth of smolt under FW conditions compared to SW conditions. However, growth rate may have been influenced by factors aside from the osmoregulatory status of the fish, which varied between SW and FW culture conditions, such as holding facilities, stocking density, feeding regime and the occurrence of a flexi-bacter infection in fish under SW conditions.

There was no consistent effect of ploidy or sex status on the growth of smolt under SW conditions. Other populations of Atlantic salmon produced by SALTAS during 1996, which were subsequently transferred to commercial sea pens in September, 1997, showed differential growth rates, such that diploid populations had higher SGR values and lower food conversion ratios (FCR) than triploid populations over the period of SW growout (17 - 18 months) (Mick Hortle, Operations Manager, Tassal, Tasmania, unpublished data). Poor growth of triploid salmonids in SW has been previously recorded in Atlantic salmon (Boeuf *et al.*, 1994; Johnstone, 1996; McCarthy *et al.*, 1996; Galbreath and Thorgaard, 1995) and rainbow trout (Ojolick *et al.*, 1995). Other studies have shown that growth of triploid Atlantic salmon in SW is greater than that of diploids (O'Flynn *et al.*, 1997) or similar to that of diploids up until the period of sexual maturation in diploids (Johnstone *et al.*, 1989; Jungawalla, 1991; Galbreath and Thorgaard, 1995; McGeachy *et al.*, 1996). Decreased growth in triploid salmonids during earlier development is usually

compensated for by increased growth concomittent with the period of sexual maturation in diploids (Atlantic salmon: Sutterlin and Collier, 1991; Johnstone, 1996; brook trout: Boulanger, 1991; rainbow trout: Quillet *et al.*, 1987). This study was terminated early in the SW phase, and we may have precluded possible compensation of growth later in the SW phase.

Caution is required when making comparisons of relative growth performance between different studies due to differences in acclimation procedures for SW transfer, population sex ratio, stages of development, sexual maturity and culture conditions. In some studies diploid and triploid populations were cultured under communal conditions. The performance of triploids under such conditions is usually impaired by interactions with the more aggressive diploid individuals (McCarthy *et al.*, 1996).

2.5.5 Survival

In the present study triploid smolt were subject to higher mortality following SW transfer than diploid smolt, which is in agreement with previous reports of Atlantic salmon populations upon SW transfer and during the SW phase both under local conditions (Jungawalla, 1991; Mick Hortle, Operations manager, Tassal, Tasmania, unpublished data, 1999) and elsewhere (Boeuf *et al.*, 1994; Galbreath and Thorgaard, 1995; McCarthy *et al.*, 1996; McGeachy *et al.*, 1996; O'Flynn *et al.*, 1997). This observation has also been made for rainbow trout (Quillet *et al.*, 1987; Ojolick *et al.*, 1995). An increase in cumulative mortality during the SW phase coincided with a flexibacter infection in all populations. Ojolick *et al.* (1995) found a correlation between ploidy status and the number of lesions observed in rainbow trout infected with *Vibrio* spp. following transfer to SW. No such observations were made in this study; however, the latter suggests a higher susceptibility to disease in triploid salmonids. In contrast, other reports have shown that the immuno-competance of triploid salmonids is similar to that of diploids (reviewed by Benfey, 1999).

2.5.6 Summary

The ploidy and sex status of four different populations of Atlantic salmon, was confirmed and provided quality assurance in support of the use of these populations for experiments described in Chapters 3, 4, 5 and 6. Generally, growth of diploid and triploid populations was similar for the period of FW development prior to SW transfer. Growth of fish from all populations decreased following the time of SW transfer, such that growth of smolt retained in FW was significantly higher than those transferred to SW. Diploid FW smolt were subject to higher growth rates than triploid FW smolt. Triploids were subject to higher mortality than diploids following transfer to sea water.

Chapter 3:

PHYSIOLOGICAL STRESS RESPONSES OF DIPLOID AND TRIPLOID ATLANTIC SALMON.

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3. STRESS RESPONSES OF DIPLOID AND TRIPLOID ATLANTIC SALMON

3.1 ABSTRACT

Juvenile Atlantic salmon from four different populations (all-female diploids, all-female triploids, mixed sex diploids and mixed sex triploids) were subjected to confinement either before (FW parr) or after (SW smolt) transfer to sea water. Confinement elevated plasma cortisol and plasma lactate from pre-stress control levels in all populations, at both the FW parr and SW smolt stages, irrespective of ploidy status. Both before and after confinement, plasma cortisol levels in SW smolt (6 - 174 $ng.ml^{-1}$) were higher than those in FW parr (4 - 58 $ng.ml^{-1}$) which possibly reflected the physiological challenge of acclimation to SW. Cortisol levels in SW smolt differed according to sex status, such that mixed sex populations had higher cortisol levels than all-female populations. The duration of confinement (1, 3 or 6 h) affected the magnitude of the plasma cortisol and lactate responses in SW smolt. Plasma cortisol levels in diploid and triploid SW smolt subjected to 2 h of confinement decreased to pre-stress levels within 6 h post-confinement. Plasma lactate levels did not fully recover to pre-stress levels for the duration of the experiment (> 48 h).

These results indicate no difference exists in primary and secondary stress responses of Atlantic salmon of differing ploidy status. Thus, it is unlikely that differences in mortality rates between diploid and triploid populations under commercial conditions, can be attributed to differences in their physiological responses to stress.

3.2 INTRODUCTION

As discussed in Chapter 1, the performance of all-female triploid salmonids under intensive aquacultural conditions has been variable, both in Australia and elsewhere (Happe *et al.*, 1988; Johnstone, 1991; Jungawalla, 1991; Galbreath *et al.*, 1994; Galbreath and Thorgaard,1995; McGeachy *et al.*, 1995; Benfey, 1996). Many reports suggest that triploid populations show higher mortality than diploid populations (Johnson *et al.*, 1986; Quillet *et al.*, 1987; Quillet and Gaignon, 1990; Virtanen *et al.*, 1990; Johnstone *et al.*, 1991; Aliah *et al.*, 1991; Jungawalla, 1991; Myers and Hershberger, 1991; Blanc *et al.*, 1992; King and Lee, 1993; Simon *et al.*, 1993; Ojolick *et al.*, 1995; Benfey, 1996; McGeachy *et al.*, 1996; O'Flynn *et al.*, 1997) and this has been attributed to a differential response to stress. The higher mortality rates that occurred in triploid populations following sea water transfer in the present study (described in Chapter 2) were in accordance with the latter studies. Evidence from diploids of several species indicates that stress, particularly in association with extended periods of anaerobic activity, can result in mortality (Graham *et al.*, 1982; Wood *et al.*, 1983; Ferguson and Tufts, 1992). Higher mortality in triploids could result from differences in stress response, but with the exception of a study on brook trout *Salvelinus fontinalis* (Biron and Benfey, 1994) this remains to be investigated.

There are differences in cellular morphology between triploids and diploids, but the physiological basis for the apparent different response during suboptimal husbandry practices and sea water transfer is unclear, despite previous comparisons of a range of physiological parameters in diploid and triploid fish (reviewed by Benfey, 1999). Triploids differ genetically to diploids in that they possess an extra set of chromosomes. As a result of the extra DNA, triploids are more heterozygous (Allendorf and Leary, 1984; Leary *et al.*, 1985), display an increased cell size with a consequent decrease in cell surface area to volume ratio, and a reduction of cell number at the tissue level (Swarup, 1959a, Small and Benfey, 1987).

The primary response of teleost fishes to a stressor is the secretion of catecholamines by the chromaffin cells and the secretion of cortisol by the interrenal cells into the

blood stream (reviewed by Barton and Iwama, 1997; Wendelaar Bonga, 1997; Sumpter, 1997). These hormones give rise to secondary changes including elevated plasma levels of glucose and lactate. Lactate is the product of anaerobic metabolism, generated under conditions of exercise or hypoxia, hence causes of increasing lactate under conditions of stress and exercise are difficult to separate. The measurement of plasma cortisol can be used to determine the primary stress response of fish to certain stressors (reviewed by Sumpter, 1997). Plasma lactate is a useful indicator of the secondary stress response as well as the extent of anaerobic metabolism (Wood and Perry, 1985; Barton and Iwama, 1991). Confinement, or the dropping of water levels, provides a standardised reference stressor that has been shown to induce an acute primary stress response in Atlantic salmon. Such a treatment is an excellent means of comparing the stress response of different fish groups and remains relevant to culture conditions (Einarsdottir and Nilssen, 1996).

The current study examined the primary stress response in four populations of Atlantic salmon; mixed sex diploids, mixed sex triploids, all-female diploids and all-female triploids; during the freshwater (FW) parr phase and 4 weeks post-transfer to sea water (SW smolt). Plasma cortisol and lactate levels were determined in fish prior to a confinement (= pre-stress), immediately following confinement (= stress), and during recovery from confinement. This allowed assessment of whether ploidy status, population sex ratio or the physiological challenge associated with SW transfer, affects the primary stress response of juvenile Atlantic salmon.

3.3 MATERIALS AND METHODS

3.3.1 Fish production and husbandry

All-female diploid, all-female triploid, mixed sex diploid and mixed sex triploid Atlantic salmon populations were produced and maintained in separate tanks under standardised husbandry conditions, during both the freshwater (FW) and seawater (SW) phases, as previously described in Chapter 2. Populations were unrelated and

each was produced by fertilising eggs pooled from 15 - 18 females with sperm pooled from 7 - 12 males. Population sex ratios were confirmed to be 100% female for all-female populations and equal sex ratios for the mixed sex populations (see results Chapter 2). The smolts were acclimated to seawater conditions for 4 weeks prior to experimentation. Fish remained unfed and undisturbed for 12 h prior to experimentation.

3.3.2 Experimental protocols

All experimental sampling protocols commenced at 08:00 - 09:00 h, in unfed fish. Mean fork length (cm FL) and total wet weight (g TWWt.) were recorded for each fish sampled and condition factor ($K = (TWWt. / FL^3) \times 100$) was calculated. The ploidy status of each fish sampled was assessed by determining the mean erythrocyte nucleus length (ENL) (Benfey *et al.*, 1984) using air dried blood smears stained with haematoxylin (method described in section 2.3.4).

Experiment 1: Cortisol levels of 'pre-stress' FW parr

Ten fish were randomly selected from each of the four experimental populations at the FW parr stage (13 month post-fertilisation) and a blood sample was collected by caudal venepuncture, within 10 min of initial tank disturbance, using heparinised syringes, after which fish were killed by cranial impact.

Experiment 2: Confinement of FW parr

Ten fish were randomly selected from each of the four experimental populations at the FW parr stage (14 month post-fertilisation) and a blood sample was collected within 10 min of initial tank disturbance as described above (= pre-stress fish). Ten more fish were selected from each population and were subjected to 1 h and 20 min of confinement in a mesh catch bag which compressed the fish against one another within the confines of the respective holding tank. The mesh bag was tied next to the inflow pipe of the respective holding tank, to ensure that the confined fish had access

to adequately oxygenated water. A blood sample was taken from each confined fish as for pre-stress fish.

Experiment 3: Prolonged confinement of SW smolt

Ten SW smolt (18 month post-fertilisation), from each of the all-female diploid, all-female triploid, mixed sex diploid and mixed sex triploid populations, were netted from their respective tanks and blood sampled by caudal venepuncture within 10 min of initial tank disturbance (= pre-stress fish). Another thirty smolt from each population were netted and confined in a 40 L bin of aerated sea water (initial stocking density 60 $kg.m^{-3}$). Ten fish were sampled after 1, 3 and 6 h of confinement.

Experiment 4: Stress recovery in SW smolt

Due to the low number of fish available, fish from the all-female and mixed sex populations were pooled according to ploidy status at a ratio of 4:3. A blood sample was taken by caudal venepuncture from seven pre-stress diploid and triploid SW smolt (18.5 month post-fertilisation) within 10 min of initial tank disturbance. In addition, 28 diploid and 28 triploid SW smolt were subjected to confinement in one of two 20 L bins of aerated seawater for 2 h (initial stocking density of 120 $kg.m^{-3}$). Of these fish, blood samples were taken from 7 diploids and 7 triploids following confinement, while the remainder were evenly distributed between three 2000 L holding tanks. Each tank was randomly allocated a 'recovery period' of 6, 24 or 48 h post-confinement, after which time fish were netted and blood samples taken within 10 min of tank disturbance.

3.3.3 Plasma cortisol and lactate analysis

Blood samples were transferred to 1.5 mL tubes on ice and then centrifuged at 12,000 *g* for 3 min. The plasma was aliquotted and stored at - 20° C prior to cortisol and lactate determination. Plasma cortisol was measured by radioimmunoassay using standard procedures (Pankhurst and Sharples, 1992). Extraction efficiency

(measured by recovery of ^{3}H-labelled steroid) ranged from 94 - 96% and assay values were corrected accordingly. Assay detection limit was 2.4 ng.ml^{-1} plasma. Different internal standards were used between FW and SW experiments and interassay variability (%CV), using a pooled internal standard was 23% (n = 3) and 18.3% (n = 3) respectively. Lactate was measured spectrophotometrically using an enzymatic kit (Sigma 826-B).

3.3.4 Statistical Analysis

Data were tested for normality and equal variances within treatments using the Shapiro-Whilk W test and Bartletts test, respectively. A square root transformation was used for data that did not conform to normality. Multivariate ANOVA was used to determine the effects of ploidy, population sex ratio and confinement stress or stress recovery period on respective features (JMP, version 3.1 for Windows). We acknowledge that the experimental design did not preclude possible tank effects.

3.4 RESULTS

3.4.1 Triploidy assessment, fish weight and length

The mean ENL values of triploid FW parr and SW smolt were significantly higher than those of diploid FW parr and SW smolt, respectively ($P < 0.05$, Table 1). In addition, all triploid individuals had mean ENL values in excess of the maximum mean ENL value observed in diploid fish in section 2.2.2 (maximum mean for population = 6.9 μm), which confirmed their ploidy status. Mean TWWt., FL and K values of fish sampled from each population, at the FW parr stage (experiments 1 and 2), did not differ significantly between populations ($P > 0.05$, Table 2). Diploid SW smolt, sampled for experiment 3, had significantly higher TWWt., shorter FL and higher K values than triploid SW smolt sampled in the same experiment ($P < 0.05$, Table 2). In experiment 4, diploid SW smolt had significantly higher K values

than triploid SW smolt; however, TWWt. and FL were not statistically different with ploidy status ($P < 0.05$, Table 2).

Table 1. Mean erythrocyte nucleus length (ENL) of sampled diploid and triploid Atlantic salmon.

Developmental stage	Ploidy	n (fish)	ENL (μm) Mean	S.D.	S.E.
FW parr	Diploid	10	6.200	0.366	0.116
	Triploid	35	7.723	0.308	0.052
SW smolt	Diploid	87	6.189	0.339	0.036
	Triploid	80	8.893	0.779	0.087

Table 2. Mean total wet weights (TWWt), fork length (FL) and condition factor (K = (TWWt. / FL^3) × 100) of Atlantic salmon sampled from 4 populations, all-female diploids, all-female triploids, mixed sex diploids and mixed sex triploids. Asterix (*) denotes significant difference between diploid and triploid populations of the same sex status.

	Experiment	Population Type	TWWt (g)	FL (cm)	K ($g.cm^{-3}$)
1	pre-stress FW parr	All-female diploids	41.58 ± 6.0	15.24 ± 0.5	1.17 ± 0.1
		All-female triploids	43.28 ± 3.7	15.42 ± 0.4	1.17 ± 0.02
		Mixed sex diploids	41.10 ± 3.3	15.31 ± 0.5	1.14 ± 0.05
		Mixed sex triploids	48.48 ± 7.0	15.79 ± 0.6	1.17 ± 0.03
2	pre-stress and confined FW parr	All-female diploids	45.21 ± 1.7	15.79 ± 0.1	1.14 ± 0.2
		All-female triploids	43.10 ± 1.6	15.63 ± 0.2	1.12 ± 0.01
		Mixed sex diploids	39.76 ± 1.2	15.38 ± 0.1	1.08 ± 0.01
		Mixed sex triploids	43.15 ± 1.9	15.70 ± 0.2	1.10 ± 0.01
3	pre-stress and confined SW smolt	All-female diploids	91.01 ± 1.9 *	20.63 ± 0.1 *	1.02 ± 0.01 *
		All-female triploids	78.68 ± 2.0	21.2 ± 0.1	0.92 ± 0.01
		Mixed sex diploids	87.30 ± 1.8	20.44 ± 0.1 *	1.01 ± 0.01 *
		Mixed sex triploids	86.66 ± 2.0	20.86 ± 0.1	0.94 ± 0.01
4	recovery from confinement in SW smolt	Diploids	86.52 ± 15.8	20.80 ± 1.2	0.94 ± 0.06 *
		Triploids	82.96 ± 14.1	21.00 ± 1.0	0.87 ± 0.04

3.4.2 Experiment 1: Cortisol levels of pre-stress FW parr

All mean plasma cortisol values for pre-stress parr were below 10 $ng.ml^{-1}$. There was no significant difference in the mean cortisol values between experimental population types ($P > 0.05$, Fig. 1).

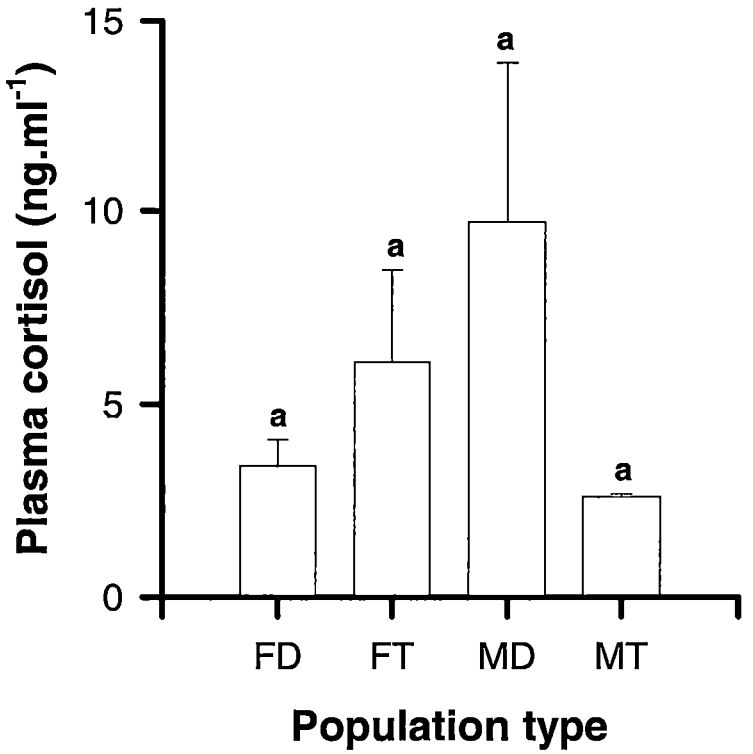

Figure 1. Mean (± SE) plasma cortisol values for different populations of Atlantic salmon FW parr. FD = all-female diploids, FT = all-female triploids, MD = mixed sex diploids, MT = mixed sex triploids (n = 10). Superscripts indicate values are not significantly different ($P < 0.05$).

3.4.3 Experiment 2: Confinement of FW parr

Mean plasma cortisol values were below 10 $ng.ml^{-1}$ in all pre-stress FW parr (Fig. 2a). An eight- to ten-fold elevation of plasma cortisol levels occurred following 1 h 20 min confinement, regardless of population type ($P < 0.05$, Fig. 2a). Mixed sex diploids had significantly higher plasma cortisol levels than all-female triploids.

Following confinement, plasma lactate levels were significantly different between population types and were higher in mixed sex triploid parr than in all-female

diploid parr ($P < 0.05$, Fig. 2b). Plasma lactate values for pre-stress FW parr were not obtained due to a technical error.

3.4.4 Experiment 3: Prolonged confinement of SW smolt

Plasma cortisol levels for each population of pre-stress SW smolt (mean ± SE: all-female diploid (FD) = 5.73 ± 1.75, all-female triploid (FT) = 19.34 ± 8.0, mixed sex diploid (MD) = 35.82 ± 10.64, mixed sex triploid (MT) = 14.46 ± 5.03 $ng.ml^{-1}$, Fig. 3) were significantly higher than respective values at the FW parr stage (mean ± SE: FD = 4.2 ± 0.37, FT = 5.09 ± 0.84, MD = 6.13 ± 0.52, MT = 6.89 ± 0.94 $ng.ml^{-1}$, $P < 0.05$).

Plasma cortisol levels were significantly elevated above those of pre-stress fish after 1 and 3 h of confinement in all-female diploid and mixed sex triploid populations ($P < 0.05$, Fig. 3). In the all-female triploid population, plasma cortisol levels were significantly higher than pre-stress levels following 3 h confinement ($P < 0.05$). In the mixed sex diploid population, plasma cortisol levels were significantly higher than pre-stress levels after 1 h confinement ($P < 0.05$), but not different from pre-stress levels after 3 h confinement ($P > 0.05$). Within each population, cortisol levels following 1 and 3 h confinement were not significantly different from each other ($P > 0.05$). After 6 h, all groups of fish had cortisol levels that were not different from those of pre-stress fish ($P > 0.05$).

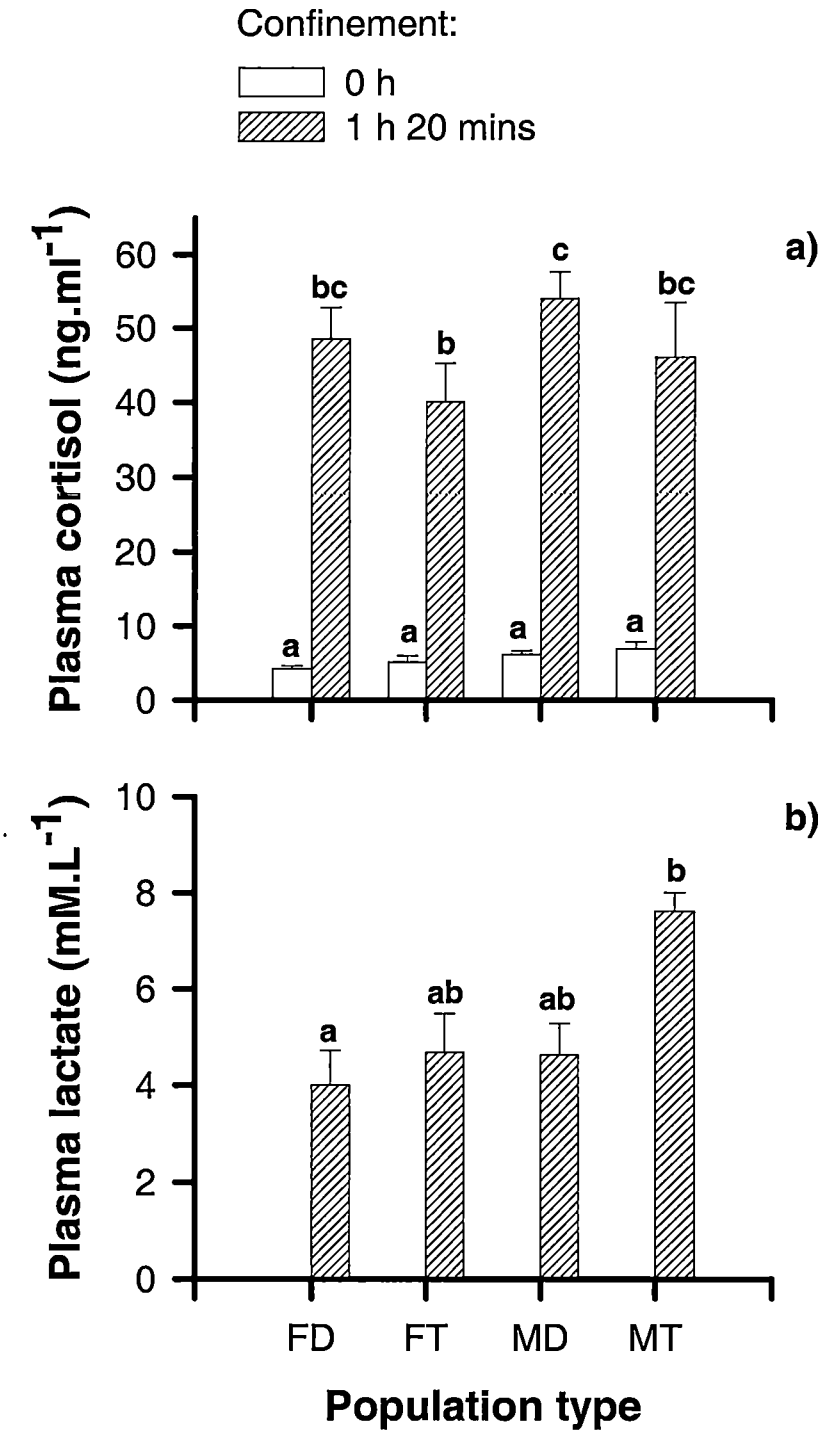

Figure 2. Mean (+ SE) plasma cortisol levels (a) and plasma lactate levels (b), before or after 1 h 20 minutes of confinement in four different populations of Atlantic salmon FW parr; FD = all-female diploids, FT = all-female triploids, MD = mixed sex diploids, MT = mixed sex triploids (n = 10). Different superscripts denote values that are significantly different ($P < 0.05$).

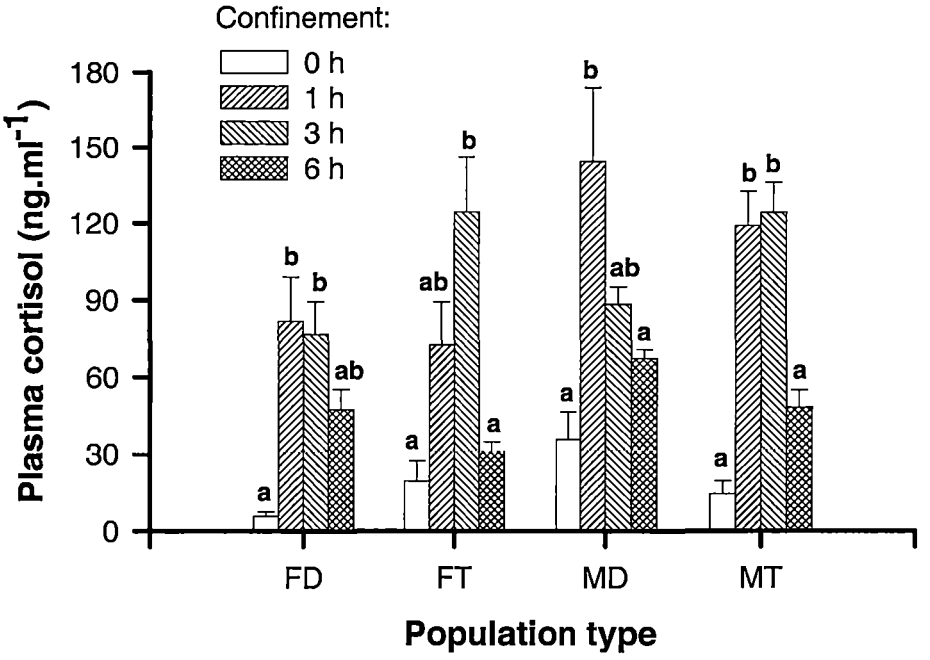

Figure 3. Change in mean (+ SE) plasma cortisol following 1, 3 or 6 h of confinement in four different populations of Atlantic salmon SW smolt; FD = all-female diploids, FT = all-female triploids, MD = mixed sex diploids, MT = mixed sex triploids (n = 10). 0 h = pre-stress fish. Different superscripts denote differences between confinement treatments within population types ($P < 0.05$).

Within sampling times, mean plasma cortisol levels were higher in mixed sex populations of SW smolt (data pooled for mixed sex diploid and triploid populations) compared to all-female populations (data pooled for all-female diploid and triploid populations, $P < 0.05$, Fig. 3). In case the effect of population sex status masked possible ploidy effects, data for each sex type were analysed separately. Within all-female populations there was no statistical difference in mean cortisol values between diploid and triploid female SW smolt following confinement ($P = 0.5286$, Fig. 4). In contrast, plasma cortisol levels of mixed sex populations were significantly different between diploid and triploid individuals following 3 and 6 h confinement ($P < 0.05$, Fig. 4). A general trend was evident between ploidy groups within both population sex ratio types. Triploid individuals had slightly lower

cortisol levels than diploids following 1 h confinement, higher levels following a 3 h confinement and lower levels than diploids following a 6 h confinement. Plasma cortisol levels peaked after 1 h confinement in diploid populations and after 3 h of confinement in triploid populations.

Plasma lactate levels of pre-stress SW smolt were less than $1mM.L^{-1}$ and were not different between populations ($P > 0.05$, Fig. 5). Lactate levels were elevated in fish following 1 and 3 h of confinement ($P < 0.05$), but were statistically similar to pre-stress levels in smolt subjected to 6 h confinement ($P > 0.05$). Within each population, plasma lactate levels of fish following 3 h confinement were not statistically different to those following 1 h confinement ($P > 0.05$). After 1 h of confinement, plasma lactate levels of all-female triploids were significantly higher than those of all-female diploids and mixed sex triploids ($P < 0.05$), while lactate levels in mixed sex diploids were not significantly different to any other population ($P > 0.05$). Consequently, variation in plasma lactate between populations following 1 h confinement could not be attributed to ploidy status. Following 3 and 6 h confinement, lactate levels did not differ between populations ($P > 0.05$).

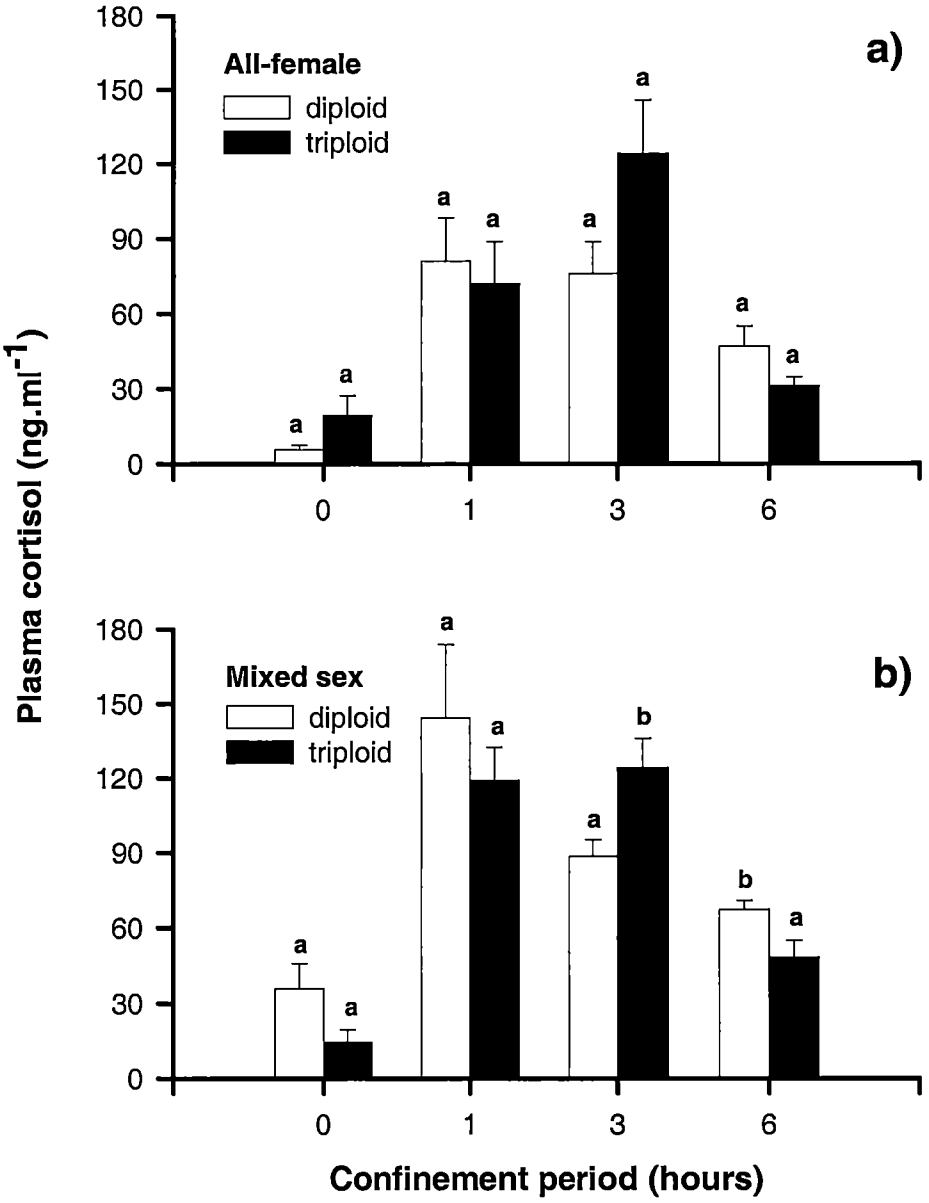

Figure 4. Change in mean (+ SE) plasma cortisol levels following 1, 3 or 6 h of confinement for diploid and triploid Atlantic salmon SW smolt from (a) all-female populations and (b) mixed sex populations (n = 10). 0 h = pre-stress fish. Different superscripts denote differences within confinement treatments ($P < 0.05$).

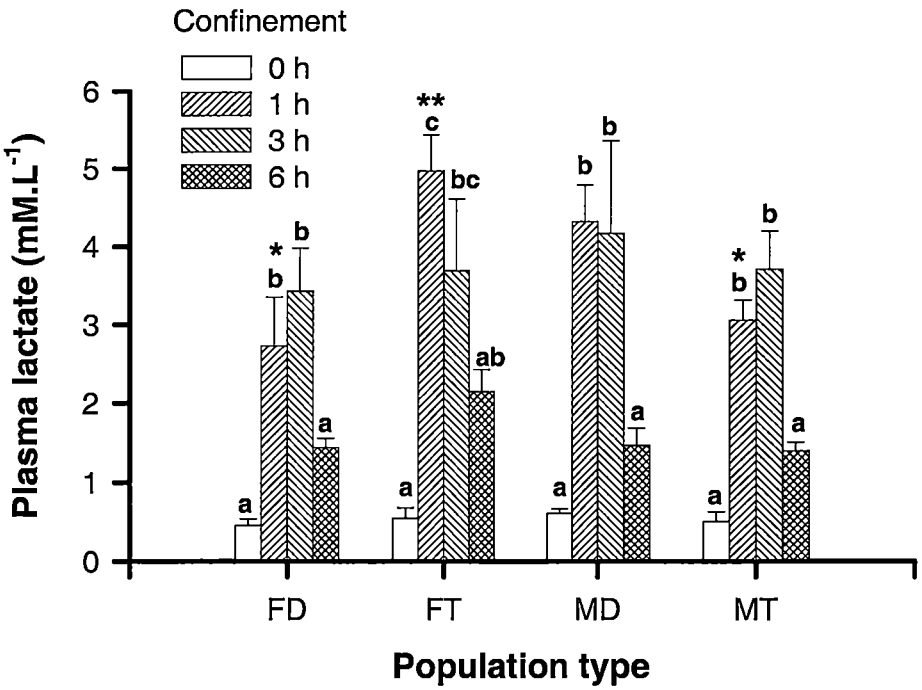

Figure 5. Change in mean (+ SE) plasma lactate levels following 1, 3 or 6 h of confinement in four different populations of Atlantic salmon SW smolt; FD = all-female diploids, FT = all-female triploids, MD = mixed sex diploids, MT = mixed sex triploids (n = 10). 0 h = pre-stress fish. * denotes significant difference to ** at 1 h confinement ($P < 0.05$). Different superscripts denote differences between confinement treatments within population types ($P < 0.05$).

3.4.5 Experiment 4: Recovery from confinement in SW smolt

Plasma cortisol concentrations of pre-stress mixed sex diploid and mixed sex triploid SW smolt did not differ significantly (< 20 $ng.ml^{-1}$, $P > 0.05$, Fig. 6a). After 2 h of confinement (0h recovery time), plasma cortisol was elevated five-fold ($P < 0.05$)., irrespective of ploidy status ($P > 0.05$). Recovery of cortisol to basal levels occurred within 6 h post-confinement in both diploid and triploid smolt ($P > 0.05$, Fig. 6a).

Plasma lactate concentrations in pre-stress fish were not statistically different between ploidy groups ($P > 0.05$, Fig. 6b). Following 2 h of confinement (0 h

recovery), plasma lactate levels were significantly elevated in triploid fish only ($P < 0.05$). At 6 h post-confinement, plasma lactate levels were significantly elevated in both diploid and triploid fish ($P < 0.05$), and at 24 h post-confinement plasma lactate levels were elevated in triploids relative to pre-stress fish ($P < 0.05$). By 48 h, lactate levels were not significantly different from those of pre-stress fish ($P > 0.05$). Within each treatment, there was no significant difference between ploidy groups ($P > 0.05$, Fig. 6b).

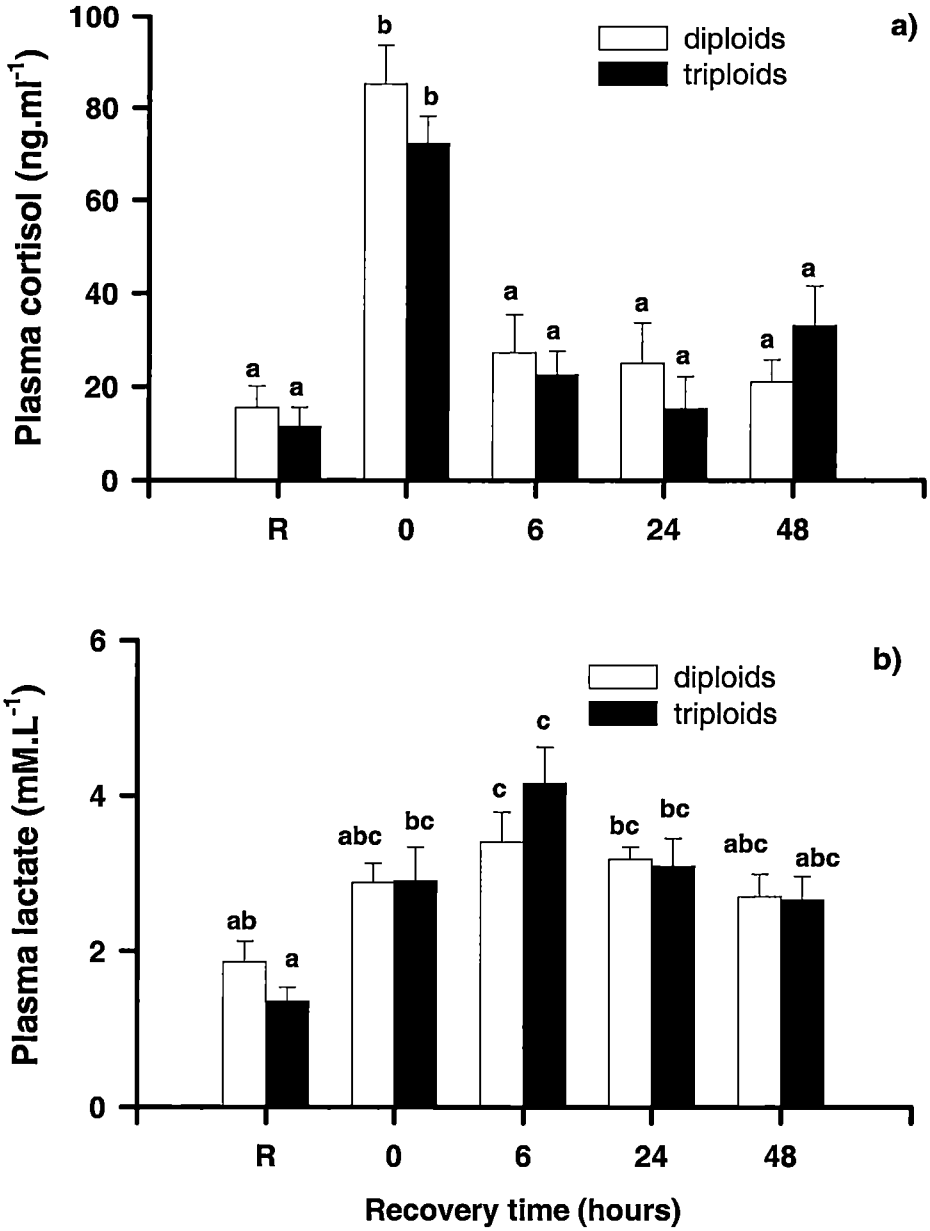

Figure 6. Mean (+SE) plasma cortisol (a) and mean (+SE) plasma lactate (b) levels of mixed sex diploid and mixed sex triploid Atlantic salmon SW smolt prior to confinement (R = pre-stress fish), after 2 h confinement (0 h recovery) and 6, 24 or 48 h post-confinement (n = 7). Different superscripts denote differences between treatments ($P < 0.05$).

3.5 DISCUSSION

Ploidy status had no consistent effect on plasma cortisol levels in Atlantic salmon FW parr in either pre-stress fish or those subjected to confinement. Similarly, Biron and Benfey (1994) found no significant difference in the plasma cortisol response of diploid and triploid juvenile FW brook trout *Salvelinus fontinalis,* following 5 minutes acute handling and confinement stress (Biron and Benfey, 1994). In the present study, there was a difference in the time course of change in plasma cortisol levels between mixed sex diploid and all-female triploid SW smolt, and mixed sex diploid and mixed sex triploid SW smolt suggesting that the stress response of triploid Atlantic salmon SW smolt may be slightly delayed compared to that of diploid smolt. Similarly, Biron (1993) found subtle differences in the cortisol response of triploid rainbow trout *Onchorynchus mykiss*, following acute handling (reviewed by Biron and Benfey, 1994). Previous authors have suggested that the decrease in cell surface area to volume ratio characteristic of tissues in triploid fish, may affect trans-membranous exchange rates and therefore response time to sensory input such that the ability of triploid fish to perceive and respond to environmental changes may be altered or delayed (reviewed by Benfey, 1999).

The mean levels of plasma cortisol in pre-stress Atlantic salmon SW smolt were higher than respective levels for pre-stress FW parr. Cortisol concentrations for pre-stress fish in the present study were similar to those previously recorded for Atlantic salmon in FW (< 10 $ng.ml^{-1}$: Thorpe *et al.*, 1987; Boesgaard *et al.*, 1993; Olsen *et al.*, 1995; Einarsdottir and Nilssen, 1996) and in SW smolt (1.4 - 32.3 $ng.ml^{-1}$: Waring *et al.*, 1992; Sverdrup *et al.*, 1994; Olsen *et al.*, 1995; Einarsdottir and Nilssen, 1996). The increase in baseline plasma cortisol levels of salmonids during smoltification, seaward migration and acclimation to SW, is well documented (Redding *et al.*, 1984b; Barton *et al.*, 1985; Bjornsson *et al.*, 1987; Thorpe *et al.*, 1987; Barton and Iwama, 1991; Bisbal and Specker, 1991; Fuentes *et al.*, 1996). Cortisol affects enzyme levels and activity of Na^+- K^+ - ATPase, thereby improving hypo-osmoregulatory capacity following entry to seawater (Bjornsson *et al.*, 1987;

review Fuentes *et al.*, 1996). The decrease in condition factor, particularly in triploid SW smolt following SW transfer, in this study, further indicates that SW transfer is a physiologically demanding and stressful process for smolt.

The magnitude of the stress response of SW smolt was greater than that of FW parr in the present study. Plasma cortisol levels in Atlantic salmon FW parr following 1 h 20 minutes of confinement (47 $ng.ml^{-1}$) agree with previously recorded levels for Atlantic salmon FW parr, immediately following exercise (28 $ng.ml^{-1}$, Boesgaard *et al.*, 1993), and Atlantic salmon FW post-smolt following 30 minutes of stress and exercise (50 $ng.ml^{-1}$, Thomas *et al.*, In press). In comparison, higher plasma cortisol levels of up to 139 $ng.ml^{-1}$ have been observed in Atlantic salmon FW parr after 30 minutes of acute stress (Olsen *et al.*, 1995). The magnitude of the stress response and the rapidity of recovery from stress can depend on the genetic strain, fish developmental stage, breeding history, water conditions, stressor type and intensity (Barton and Iwama, 1997). These factors may account for inter-study differences. The magnitude of plasma cortisol levels in Atlantic salmon SW smolt following 1 - 3 h confinement in the present study (140 $ng.ml^{-1}$), was similar to that recorded in Atlantic salmon within 20 minutes of dropping water levels (193 $ng.ml^{-1}$, Einarsdottir and Nilssen, 1996) or within 1 h following 9 minutes net confinement (165 $ng.ml^{-1}$, Waring *et al.*, 1992). In the current study, the increase in the magnitude of the stress response under SW conditions is most likely due to the increase in water salinity and the smoltification / hypo-osmoregulatory status of the fish sampled and not the increase in the body mass of salmon, since plasma cortisol levels in Atlantic salmon (Hemre and Krogdahl, 1996) and greenback flounder (*Rhombosolea taparina*, Barnett and Pankhurst, 1998) have been shown to be independent of size.

In addition, it is possible that plasma cortisol levels of confined FW parr were alleviated by an increased level of activity enforced by experimental conditions. In the FW experiment, fish were held with a water flow rate of approximately 20 $L.min^{-1}$ for 1 h 20 minutes. In the SW experiments, confinement occurred under still water conditions. It has been shown that moderate levels of exercise in Atlantic salmon (0.5 body $length.s^{-1}$) can induce a decrease in plasma cortisol to levels below

that of controls after 4 h duration (Boesgaard *et al.*, 1993). The physiology of the SW smolt may also have contributed to low levels of activity in SW experiments. The increase in cortisol levels associated with smoltification has been associated with an observed reduction in swimming activity at this stage, via symptomatic muscle fatigue induced by changes in muscle electrolyte concentrations (Thorpe *et al.*, 1987), thereby exacerbating the elevation of plasma cortisol levels with stress. The decrease in cortisol with continued moderate exercise in fish could be due to a cessation or decrease in cortisol secretion possibly in combination with an increase in metabolic clearance rate (MCR) (Boesgaard *et al.*, 1993). Chronic (> 12 h), but not acute stress may increase MCR in both FW and SW fish (Redding *et al.*, 1984). It has been shown that SW conditions facilitate a higher MCR of plasma cortisol compared to FW conditions, in coho salmon (*O. kisutch*, Redding *et al.*, 1984). In comparison, Nichols *et al.* (1985), found that the cortisol MCR was unaltered during acclimation to SW in brook trout and that cortisol secretion and plasma cortisol concentration increased. High levels of cortisol secretion in Atlantic salmon SW smolt, following acute stress, may counteract any possible increase in cortisol MCR under SW conditions.

Population sex ratio in SW smolt had a significant effect upon levels of plasma cortisol in pre-stress fish and post-confinement. A flexibacter infection was observed in experimental populations, as described in Chapter 2, and was concomitant with higher mortality in both mixed sex populations compared with the all-female populations. However, there were no data pertaining to the incidence of the flexibacter in live fish, or the pathogenicity of the bacteria, hence it is difficult to quantify the demographics of the infection between populations. It is not clear whether the presence of the flexibacter contributed to the primary stress response in experimental fish (Pickering and Pottinger, 1985; Thorpe *et al.*, 1987), or whether the elevated primary stress response was due to the effect of population sex ratio. It is possible that an increased susceptibility to the flexibacter infection may have represented a tertiary stress response to the elevated plasma cortisol levels. Increased susceptibility to infectious disease has been associated with high cortisol levels in salmonids (Pickering and Duston, 1983; Pickering and Pottinger, 1989; Pickering *et al.*, 1989; al., 1989; Maule *et al.*, 1989; Barton and Iwama, 1991; Schreck, 1996).

Other possible factors contributing to increased cortisol levels in mixed-sex populations include the interaction between male and female individuals. Growth of male and female individuals has been shown to differ significantly within the same population of brook trout (Johnstone *et al.*, 1979).

Within each population type, peak cortisol levels were maintained between 1 and 3 h confinement but were decreased after 6 h confinement under still water conditions, irrespective of ploidy status. The lower stocking density of fish subject to 6 h of confinement may have partially obviated the stress effects of the confinement treatment. In addition, the fish subject to this particular treatment may have partially recovered from the initial sampling disturbances. This is supported by the subsequent confinement recovery experiment, in which plasma cortisol levels of SW smolt subject to 2 h confinement returned to pre-stress levels within 6 h post-confinement, concomitant with a reduction in stocking density.

Ploidy status had no effect on recovery of plasma cortisol levels following confinement to pre-stress control levels in Atlantic salmon SW smolt. Recovery of plasma cortisol levels in Atlantic salmon SW smolt was similar to that in previous studies in which a decline of plasma cortisol was shown to occur from 2 to 8 h post-stress in Atlantic salmon (Waring *et al.*, 1992; Boesgaard *et al.*, 1993; Einarsdottir and Nilssen, 1996) and other salmonids (Barton *et al.*, 1980; Pickering *et al.*, 1982; Pickering and Pottinger, 1989). However, decline to pre-stress control levels can take 24 - 48 h. Plasma cortisol levels returned to pre-stress control levels: within 48 h in Atlantic salmon subject to capture and 9 minutes net confinement (Waring *et al.*, 1992); within 24 h in Atlantic salmon subject to water level reduction (Einarsdottir and Nilsen, 1996); after 24 h post-capture in wild rainbow trout (Pankhurst and Dedual, 1994); 48 h following catheterisation in rainbow trout (Brown *et al.*, 1986); after 24 h in chinook salmon (*O. tshawytscha*) subjected to 30 seconds handling stress (Barton and Schreck, 1987); and by 4 h in rainbow trout subjected to exercise (Nielson *et al.*, 1994). The magnitude and rapidity of recovery from stress can depend on fish species, strain, fish developmental stage, water conditions, stressor type and intensity (Barton and Iwama, 1997). In the present

study, the stress response to confinement was comparatively short lived, which may reflect the intensity of the stressor, and domestic strain of the fish.

Basal lactate levels did not differ with ploidy status in Atlantic salmon SW smolt nor was there any consistent indication that plasma lactate levels differed with ploidy status following confinement in both FW parr and SW smolt. Plasma lactate levels increase as a result of increased anaerobic activity. It has been suggested that triploid rainbow trout may have a decreased aerobic capacity compared to diploid counterparts (Virtanen *et al.*, 1990) based on a more rapid elevation of plasma lactate and depletion of liver glycogen. The results of this study do not support this view. The aerobic capacity of diploid and triploid Atlantic salmon appeared to be similar under conditions of confinement, which is supported by another investigation in which haematological responses to confinement stress were similar between diploid and triploid Atlantic salmon SW smolt (Chapter 4; Sadler *et al.*, 2000a). Other studies have shown that oxygen consumption rates, critical swimming speed and the time to exhaustion at a constant swimming speed, is similar for diploid and triploids, further indicating that aerobic capacity does not differ with ploidy status (reviewed by Benfey, 1999).

Basal plasma lactate levels for SW smolt (< 2 $mM.L^{-1}$) agreed with those from previous investigations in SW Atlantic salmon (Tang *et al.*, 1989), FW Atlantic salmon (Tufts *et al.*, 1991; Booth *et al.*, 1995; Brobbel *et al.*, 1996; Wilkie *et al.*, 1997; Thomas *et al.*, 1999) and other FW salmonids (brown trout *Salmo trutta*: Pickering *et al.*, 1982; rainbow trout: Pankhurst and Dedual, 1994). The magnitude of the peak plasma lactate level in FW parr following confinement (8 $mM.L^{-1}$) was similar to some studies of FW Atlantic salmon subject to stress and exercise (9 $mM.L^{-1}$: Booth *et al.*, 1995; 7 $mM.L^{-1}$: Brobbel *et al.*, 1996; 9 $mM.L^{-1}$: Thomas *et al.*, 1999), but was lower than others (10 $mM.L^{-1}$: Tang *et al.*, 1989; 26 $mM.L^{-1}$: Tufts *et al.*, 1991; 12 $mM.L^{-1}$: Wilkie *et al.*, 1997). Likewise, peak plasma lactate levels in SW smolt following confinement (5.5 $mM.L^{-1}$) were lower than that of previous studies of SW Atlantic salmon (16 $mM.L^{-1}$: Tang *et al.*, 1989) and FW Atlantic salmon (as above). These differences may reflect inter-study variation in

fish size, activity levels and extent of anaerobic metabolism. The occurrence of peak lactate levels between 1 and 6 h following initial confinement of SW smolt was consistent with the timecourse of increase described in previous studies on SW Atlantic salmon (4h: Tang *et al.*, 1989), FW Atlantic salmon (2h: Tufts *et al.*, 1991; Booth *et al.*, 1995; Brobbel *et al.*, 1996; 1h: Wilkie *et al.*, 1997) and other salmonids (brown trout, 2h: Pickering *et al.*, 1982; chinook salmon, 6h: Barton *et al.*, 1986; rainbow trout, 1h: Pankhurst and Dedual, 1994).

In the present study, the recovery of plasma lactate levels to baseline levels in SW smolt occurred within 24 h post-confinement. Blood lactate levels can recover within 2-12 h post-exercise or post-stress in FW Atlantic salmon (Tang *et al.*, 1989; Tufts *et al.*, 1991; Booth *et al.*, 1995; Brobbel *et al.*, 1996), by 4 h in brown trout (4h: Pickering *et al.*, 1982) and in less than 24 h in rainbow trout (Pankhurst and Dedual, 1994). Inter-study variation may reflect differences in fish species, developmental stage, size, activity levels or the extent of anaerobic metabolism.

Although data on behavioural responses to confinement stress are not presented in the current study, triploid fish were observed to be more docile upon disruption, particularly following confinement. This is in accordance with previous studies that indicate triploid salmonids are less aggressive than diploid counterparts (McGeachy *et al.*, 1995, reviewed by Benfey, 1999). Decreased aggression may be attributed to possible differences in androgen levels; however, it has also been suggested that triploid fish are less sensitive to changes in sound and light, and that decreased cell numbers in the brain and sensory systems could have a profound effect on their ability to recognise and respond to changes in their surroundings (reviewed by Benfey, 1991). In the current study, there were no significant differences in the primary stress response of triploid fish to support this suggestion. Differences in the behaviour of diploid and triploid fish, however, may indicate there are other sources of physiological variation, rather than the primary stress response, that contribute to a perceived differential response to stress in triploid fish. Stevenson (1991) indicated that triploid rainbow trout tend to be distributed lower in the water column than

diploid counterparts, particularly at higher water temperatures. The implication of this behaviour with regards to the physiology of triploid fish remains unclear.

3.5.1 Summary

In summary, the primary and secondary stress responses of triploid Atlantic salmon following confinement were not different to diploid fish. This supports the outcomes of a comparison study in which haematological responses to confinement stress were not different between diploid and triploid Atlantic salmon SW smolt, despite differences in cellular morphology with ploidy status (Chapter 4; Sadler *et al.*, 2000a). It is unlikely that the physiological stress response contributes to any difference in the performance of triploids during husbandry, or in response to management practices, which begs the question as to the basis of these reports. This issue will be addressed further in Chapters 4 and 6.

Chapter 4:

BLOOD RHEOLOGY, OXYGEN AFFINITY AND HAEMATOLOGICAL RESPONSES TO CONFINEMENT STRESS IN DIPLOID AND TRIPLOID ATLANTIC SALMON

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4. BLOOD RHEOLOGY, OXYGEN AFFINITY AND HAEMATOLOGICAL RESPONSES TO CONFINEMENT STRESS IN DIPLOID AND TRIPLOID ATLANTIC SALMON

4.1 ABSTRACT

It was hypothesised that the different cellular morphology of triploid Atlantic salmon contributes to a decreased aerobic capacity in triploids compared to diploids, particularly under stressful conditions. Stress responses and haematological parameters were measured for diploid and triploid Atlantic salmon smolt prior to and following 2.5 hrs confinement in seawater (SW). The following parameters were measured: plasma cortisol, plasma lactate, plasma glucose, haematocrit (Hct), red blood cell count (RBCC), mean cell volume (MCV), blood haemoglobin concentration (Hb), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), total protein and erythrocyte adenosine triphosphate (ATP). The magnitude of the stress response following confinement, as indicated by an increase in plasma cortisol, plasma lactate and plasma glucose levels, was similar between diploids and triploids. In one experiment, total blood Hb was lower in triploid fish than diploid fish, but in a subsequent experiment, total blood Hb was similar between ploidy groups. There was no difference in Hct, MCHC, [ATP], Hb:ATP or total plasma protein between diploid and triploid fish. Oxygen affinity (PO_{50} values) and percentage oxygen saturation of Hb were similar between diploid and triploid fish at various levels of physiological pH (6.76 - 7.99) at 15° C, which indicates that the oxygen carrying capacity of triploids may only be compromised by low blood Hb content, if it at all. Blood viscosity was dependant on Hct and shear rate, but did not differ between diploid and triploid fish and therefore it is unlikely to contribute to increased vascular resistance in triploid fish. Despite having larger, fewer erythrocytes, triploid SW smolt had similar oxygen carrying capacity and haematological stress responses to diploid smolt, under experimental conditions.

4.2 INTRODUCTION

It has been shown that triploid salmonids have higher mortality than diploids under conditions of high oxygen demand and / or low oxygen availability (Quillet *et al.*, 1987; Quillet and Gaignon, 1990; Aliah *et al.*, 1991; Johnstone *et al.*, 1991; Ojolick *et.al.*, 1995, reviewed by Benfey, 1999), which are conditions likely to occur during routine farm management of fish. These instances indicate that the respiratory mechanisms or aerobic capacity of triploid fish may be compromised in some way, particularly in view of the characteristic difference in red blood cell size and number with ploidy status (Benfey, 1996; Virtanen *et al.*, 1990). However, previous studies examining the haematological parameters of triploid and diploid salmonids offer conflicting results.

With the exception of Virtanen *et al.* (1990), investigators agree that haematocrit (hct) levels of diploid and triploid salmonids are similar despite differences in cellular morphology (review Benfey, 1996). Some authors report that diploids and triploids have equivalent total haemoglobin levels (Hb) (Stillwell and Benfey, 1994, 1996a), while others have shown that triploids have lower Hb levels than diploids (Benfey and Sutterlin, 1984a; Graham *et al.*, 1985; Small and Randall, 1989; Yamamoto and Iida, 1994a), in which case, oxygen carrying capacity may be compromised.

At the whole animal level, oxygen consumption rates of diploid and triploid rainbow trout *Oncorhynchus mykiss* are comparable under normoxic and hypoxic conditions, under different temperature conditions and during different developmental stages (Oliva-Teles and Kaushik, 1987b, 1990a, 1990b; Yamamoto and Iida, 1994b). This suggests that triploids are able to compensate for an observed decrease in red blood cell count (RBCC) and blood Hb content. Lowered oxygen carrying capacity may be compensated for by increased cardiac output, ventilation rate, ventilation volume and blood oxygen affinity.

While comparison of the cardiac output of diploid and triploid salmonids has yet to be made, data pertaining to ventilation rate, like haematological parameters, provide apparently conflicting results. For example, King and Lee (1984) observed an increased ventilation rate in triploid Atlantic salmon individuals compared to that of diploids, whereas, Benfey and Stillwell (1994, 1996a) showed ventilation rate was similar in diploid and triploid brook trout *Salvelinus fontinalis*. Clearly these parameters require further investigation.

Oxygen affinity may be affected by a number of intermediary metabolites (adenosine triphosphate (ATP), lactate, CO_2) and ultimately plasma pH levels (Milligan and Wood, 1987a) and as such may provide a compensation mechanism for a perceived lowered oxygen carrying capacity in triploids. A decline in plasma pH occurs following chronic exercise and stress in Atlantic salmon due to an accumulation of plasma CO_2, lactate, protons and other metabolites (Thomas, 1998). It has been suggested that a lowered oxygen carrying capacity may not be deleterious to pre-stress triploid Atlantic salmon; however, conditions that require exertion may challenge the ability to uptake oxygen (Graham *et al.*, 1985).

The extent to which respiratory function of triploid Atlantic salmon differs to diploids, particularly under stressful conditions is an area that requires further investigation if management / harvest protocols in aquaculture are to be optimised. In this chapter the haematological status of diploid and triploid Atlantic salmon smolt acclimated to seawater and held under resting maintenance conditions is examined. The same parameters are examined in fish subject to a standardised confinement stress treatment. In addition, blood carrying capacity and oxygen affinity is examined in diploid and triploid fish under a range of physiological pH levels and we test the hypothesis that differences in erythrocytic volume in diploid and triploid fish result in different blood viscosity.

4.3 METHODS AND MATERIALS

4.3.1 Fish production and husbandry

All-female diploid, all-female triploid, mixed sex diploid and mixed sex triploid smolt were produced and maintained, as described in Chapter 2. At 17 months post-fertilisation, the smolt were transferred to SW facilities where they were acclimated to sea water conditions for 4 weeks. On one occasion, the salmon were subjected to aberrant water temperatures as low as 1° C for one night due to a system fault. This occurred 17 days prior to blood sampling and did not interfere with the fishes acclimatisation, as indicated by baseline cortisol levels (Chapter 3).

4.3.2 Haematology of pre-stress and stressed SW smolt

Blood sampling and stress treatment

Smolt were sampled at approximately 4700° days. All fish were fasted and were left undisturbed for 24 h prior to manipulations (= pre-stress fish). Ten pre-stress fish from each population were randomly sampled from each tank using a scoop net. Approximately 500 µl of blood was sampled from each live fish by caudal venepuncture using heparinised syringes. Blood was sampled within 10 minutes of initial tank disturbance and transferred to eppendorph tubes on ice. A further ten fish were sampled from each population and were subject to 2.5 hours confinement in one of four 20 L containers of aerated sea water (= stressed fish), separated according to population type, prior to blood sampling. The following blood parameters were determined for each fish: mean erythrocyte nucleus length (ENL), plasma cortisol, plasma lactate, plasma glucose, red blood cell count (RBCC), haemoglobin content (Hb), haematocrit (Hct), mean cell haemoglobin concentration (MCHC), mean cell volume (MCV), Met-haemoglobin (MetHb), total plasma protein and adenosine triphosphate (ATP). The ploidy status of each fish sampled

was confirmed using mean ENL values (Benfey *et al.*, 1984), as described in Chapter 2.

Due to the time constraints of haematological sample processing, the haematological parameters of pre-stress and stressed fish from each population were determined in two separate experiments, conducted consecutively within one week of one another. Experiment 1, which examined the haematological parameters of pre-stress and stressed fish from the all-female diploid and all-female triploid populations, will be referred to as HP1F. Experiment 2, which examined the same parameters in fish from the mixed sex diploid and triploid populations, will be referred to as HP2M.

Plasma cortisol

Plasma cortisol was measured by radioimmunoassay (RIA) at the endocrinology laboratory, School of Aquaculture, University of Tasmania. Cortisol was extracted from 50 µl aliquots of plasma in 1 ml ethyl acetate then 50 µl aliquots of the subsequent extract were evaporated in assay tubes. Assay protocol followed Pankhurst and Sharples (1992). Extraction efficiency, determined as the recovery of ^{3}H-labelled steroid extracted with plasma, was 94% and assay values were corrected accordingly. Assay detection limit was 2.4 ng.ml^{-1} plasma and interassay variability (%CV), using a pooled internal standard was 5.4% (n = 3).

Plasma glucose and blood lactate

Plasma glucose levels were determined using an hexokinase enzymatic Glucose test kit (No. 15-u.v., Sigma Co., St. Louis, USA). An enzymatic lactate kit (No. 826-u.v., Sigma Co.) was used to quantify blood lactate levels with the following alterations. A 50 µl sample of whole blood was added to 100 µl of cold 0.6 M PCA. This mixture was allowed to stand on ice for 5 minutes and was then centrifuged at 15,000 *g*, at 4° C, for 3 minutes. The supernatant extract was stored at - 20° C prior to testing (= test extract). Two standard samples were made in the same way (50 µl std + 100 µl PCA). To each vial of NAD from the test kit, 2 ml Glycine buffer, 4 ml water and 100 µl LDH was added (= assay reagent). A 57 µl aliquot of test extract or

'standard' was added to 800 µl of assay reagent. The blank was 57 µl PCA in 800 µl reagent. After 45 minutes reaction time the absorbance of test samples and standards were measured at 340 nm against the blank.

Red blood cell count, Haemoglobin content and Haematocrit

The red blood cell count (RBCC) per unit volume of blood was determined in an improved Nuebauer counting chamber. Haemoglobin concentration [Hb] was estimated using the cyanmethaemoglobin method (Dacie and Lewis, 1975) with the added step of centrifugation to remove cell debris. Haematocrit (Hct) was determined by centrifuging ≈ 75 µl of whole blood in microhaematocrit tubes at 15,000 *g* for 3 minutes. Hct, mean corpuscular haemoglobin concentration (MCHC), mean cell volume (MCV) and mean cell haemoglobin, were calculated from the following formulae:

Hct = packed cell volume / total volume × 0.01

MCHC = [Hb] / (Hct / 100)

MCV = Hct / RBCC

MCH = [Hb] / RBCC

Methaemoglobin

Methaemoglobin content (MetHb) was estimated using the multiple wavelength method (Benesch *et al.,* 1973). This technique is based on the extinction coefficients of oxy-, deoxy- and methaemoglobins of mammalian blood. It has been tested as a reliable analytical method for measuring methaemoglobin in tropical marine fish species (Wells *et al.*,1997). A 20 µl sample of haemolysate was added to 1 ml of 100 mM HEPES-HCl buffer (pH 7.3) in a cuvette and mixed by inversion. The absorbances at 560 nm, 576 nm, and 630 nm were read against a buffer blank on a Novaspec II spectrophotometer. The percentage of Methaemoglobin was calculated from the relationship:

$M = [\ C_{metHb} / (C_{oxyHb} + C_{deoxyHb} + C_{metHb})]$

where the molar concentrations were calculated as follows:

$C_{oxyHb} = (\ 1.013A_{576} - 0.3269A_{630} - 0.7353A_{560}\ ) \times 10^{-4}$

$C_{deoxyHb} = (1.373A_{560} - 0.747A_{576} - 0.737A_{630}) \times 10^{-4}$

$C_{metHb} = (2.985A_{630} + 0.194A_{576} - 0.4023A_{560}) \times 10^{-4}$

Total plasma protein

Plasma Protein levels were determined spectrophometrically by the biuret method (Dawson *et al.,* 1969) using a certified bovine serum albumin / globulin standard (10 $g.dL^{-1}$, No. 541-1, Sigma Co.).

Total nucleoside triphosphate

A 100 µl sample of whole blood was combined with 100 µl of cold 12% trichloroacetic acid (TCA). The mixture was left on ice for five minutes then spun at 15,000 *g* for three minutes. The supernatant extract was stored at - 20° C for 3 days prior to analysis. The whole blood levels of nucleoside triphosphate (NTP) were quantified spectrophotmetrically using an enzymatic NTP test kit (No.366-u.v., Sigma Co.). The method does not distinguish ATP from other nucleoside triphosphates, but ATP is essentially the only NTP in salmonid erythrocytes (Wells and Weber, 1990).

NADH solution was prepared by adding 1 ml of PGA buffer and 1.5 ml of water to a 0.3 mg vial of NADH. A 100 µl aliquot of extract was added to 1 ml of NADH solution. The initial absorbance at 340 nm was recorded and 16 µl of GAPD/PGK enzyme was added. The absorbance was read again at 340 nm after 10 minutes. Hb: ATP was calculated using the formula:

Haemoglobin ATP ($\mu mol.g\ Hb^{-1}$) = [ATP] / [Hb]

4.3.3 Blood oxygen transport

Blood oxygen transport of all-female diploid and all-female triploid smolt was examined using pooled blood from 5 pre-stress fish from each population. Blood was sampled by caudal venepuncture with a heparinised syringe.

Oxygen equilibrium curves were measured using a series of 50 $mmol.l^{-1}$ Hepes buffers at constant (125 $mmol.l^{-1}$) chloride concentration (Weber, 1992), and decreasing pH (6.76, 7.11, 7.39, 7.7, 7.99), in a modified tonometric system (Wells and Weber, 1989). The partial pressure of oxygen in the tonometer was calculated using the formula:

PO_2 (Torr) = (PB - (H × PH_20) / 100) × (0.2095 / (V- v)) × ((Tb / Ta) × A)

where: PB = atmospheric pressure in Torr, H = relative humidity percent, PH_20 = saturated vapour pressure at room temperature in Torr, 0.2095 = fraction of O_2 in air at the time of the experiment, V = volume of tonometer in ml, v = volume of contents in ml, A = volume of air added in ml, Tb = temperature of water bath and Ta = air temperature.

Oxygen saturation was determined for each pH level at 15° C with a 5nm bandpass Novaspec II spectrophotometer for at least six points of equilibration. The percentage saturation of haemoglobin at a known partial pressure was calculated using the spectral absorbances of deoxy-, oxy-, and partially oxygenated Hb samples at both 560 nm and 578 nm and the following formula:

% Saturation at Anm = ((A deoxy - A partially oxy) / (A deoxy - A oxy)) × 100

The affinity coefficient, P_{50}, and cooperativity coefficient, n_{50}, were determined by interpolation and slope of hill plots according to Weber and Wells (1989). The effect of pH on oxygen transport at various oxygen partial pressures was evaluated from the Bohr factor, $\phi = \Delta \log P_{50} / \Delta$ pH. The root effect was estimated from the change in saturation (P_{100}) with decreasing pH using the method of Pelster and Weber (1990).

4.3.4 Blood viscosity

In vivo

Blood was sampled from 5 all-female diploid fish and 5 all-female triploid fish by caudal venesection using clean syringes and was transferred to pathology tubes containing K_2 EDTA anticoagulant on ice. The hct values of each blood sample was determined. Corresponding *in vivo* blood viscosity was determined using 500µl of whole blood in a cone plate viscometer with a cone angle of 8° (LVTD CP/11, Brookfield Engineering Laboratories, USA.), at a shear rate of 12 rpm. Shear rate is the gradient of velocity between adjacent fluid planes during flow ((cm.s) / cm = s^{-1}) (Fletcher and Haedrich, 1986). The sample cup was regulated at a temperature range of 15.0 ± 0.2 °C using a circulating water bath. Calibration of the viscometer was checked with Brookfield standards and found to be within specification.

In vitro

Blood samples used for the *in vivo* experiment above were pooled for each ploidy group, centrifuged at 3000 *g* and the plasma was removed. Erythrocytes were resuspended in Cortland's solution (Wolf, 1963; Appendix C), a physiological saline, to preclude erythrocyte aggregation at low shear rates which arises from the bridging effects of large plasma protein molecules such as fibrinogen and globulins (Fletcher and Haedrich, 1987). A range of haematocrits were reconstituted from each pooled sample and the viscosity of each hct was measured at the following shear rates: 1.5 rpm, 3 rpm, 6 rpm, 12 rpm, 30 rpm, 60 rpm. Blood viscosity measurements could not be obtained for high haematocrit samples at high shear rates. The effect of hct and shear rate on *in vitro* blood viscosity was compared between diploids and triploids. The *in vitro* blood viscosity experiment was repeated using blood of fish sampled from the same populations (5 all-female diploids + 5 all-female triploids). For descriptive purposes, the *in vitro* blood viscosity experiments will be referred to as BV1 and BV2, respectively.

4.3.5 Statistical Analysis

Data from blood haematology experiments were tested for normality and homogeneity of variances within treatments using Shapiro-Wilk and Bartletts tests, respectively, prior to analysis. A 2-way ANOVA analysis ($\propto = 0.05$) was used to determine the effects of ploidy (p) and confinement stress (s) on respective parameters. It is understood that the experimental design did not preclude possible tank effects; however, precluding the fluctuation of parameters due to photoperiod, feeding status and disturbance was of greater concern. A Welch ANOVA was also used for data with unequal variances (JMP 3.1 Software). A students t-test ($\propto = 0.05$) was used to compare *in vivo* blood viscosity and corresponding hct values between diploids and triploids (Excel Software). A 3-way ANOVA was used to compare the effects of ploidy (p), hct (h) and shear rate (sr) on viscosity (v). Haematocrits were grouped within each ploidy treatment for the purposes of analysis. A distinction was made between low hct (< 35 %) and high hct (> 35 %) values based on the overall mean of *in vivo* hct values.

4.4 RESULTS

4.4.1 Triploidy assessment, fish weight and length

The mean ENL value of triploid SW smolt (data pooled for mixed sex and all-female triploids) was significantly greater than that of diploid SW smolt (data pooled for mixed sex and all-female diploids, $P < 0.05$, Table 1). Each triploid fish sampled had a mean ENL value that was greater than the maximum mean ENL value observed in diploid fish (6.9 μm), which confirmed their ploidy status.

Table 1. Mean erythrocyte nucleus length (ENL) for sampled diploid and triploid Atlantic salmon SW smolt.

Ploidy	n (fish)	ENL (μm) mean	S.D.	S.E.
Diploid	87	6.189	0.339	0.036
Triploid	80	8.893	0.779	0.087

All-female diploids had a significantly greater mean total wet weight, lower mean fork length and greater mean condition factor than the all-female triploids ($P < 0.05$, Table 2). Mixed sex diploids had a significantly lower fork length and greater condition factor than mixed sex triploids ($P < 0.05$), but the two groups did not differ in total wet weight ($P > 0.05$, Table 2).

Table 2. Mean total wet weight, fork length (FL) and condition factor ($K = TWWt./FL^3) \times 100$) for SW smolt sampled from four populations of Atlantic salmon, all-female diploids, all-female triploids, mixed sex diploids and mixed sex triploids. Asterix (*) denotes significant difference between diploid and triploid populations of the same sex status ($P < 0.05$).

Population	TWWt. (g)	FL (cm)	K ($g.cm^{-3}) \times 100$
all-female diploids	91.0 ± 1.9 *	20.6 ± 0.1 *	1.02 ± 0.01 *
all-female triploids	78.7 ± 2.0	21.2 ± 0.1	0.92 ± 0.01
mixed sex diploids	87.3 ± 1.8	20.4 ± 0.1 *	1.01 ± 0.01 *
mixed sex triploids	86.7 ± 2.0	20.9 ± 0.1	0.94 ± 0.01

4.4.2 Haematology of pre-stress and stressed SW smolt

The mean values of the haematological parameters for diploid and triploid SW smolt, sampled in experiments HP1F and HP2M, both prior to- and following 2.5 hours of confinement are given in Table 3. In both HP1F and HP2M, plasma cortisol levels were significantly elevated in fish subjected to confinement compared to pre-stress fish ($P < 0.05$, Table 3). In each experiment, the mean plasma cortisol levels of SW smolt, both prior to- and following 2.5 hours of confinement, did not differ statistically with ploidy status ($P > 0.05$, Table 3). These results indicate that it is unlikely there was any tank effect within treatments.

In both experiments (HP1F and HP2M), plasma glucose and blood lactate levels were significantly elevated in fish subject to confinement, compared to pre-stress fish ($P < 0.05$, Table 3). Plasma glucose levels did not differ between ploidy groups, in each experiment ($P > 0.05$, Table 3). In HP1F, blood lactate levels did not differ between all-female diploid and all-female triploid fish ($P > 0.05$, Table 3); however, in HP2M, mixed sex triploids had lower blood lactate levels than mixed sex diploids ($P < 0.05$, Table 3).

Triploid fish had a lower RBCC and larger MCV than diploid fish in both experiments HP1F and HP2M ($P < 0.05$, Table 3). Changes in the RBCC and MCV values following confinement were not statistically significant in experiment HP1F ($P > 0.05$, Table 3), but mixed sex diploid and triploid fish displayed an increase in RBCC following confinement in experiment HP2M ($P < 0.05$, Table 3). Hct values did not differ between diploids and triploids in each experiment ($P > 0.05$, Table 3). There was a significant increase in Hct following confinement in HP2M ($P < 0.05$, Table 3), but there were no significant changes in Hct with confinement in HP1F ($P > 0.05$, Table 3).

The total blood Hb content of all-female triploid blood was significantly lower than that of all female diploids (HP1F, $P < 0.05$, Table 3); however, the same effect of ploidy status was not seen between the mixed sex populations (HP2M, $P > 0.05$,

Table 3). There was no change in blood Hb levels with confinement during HP1F, however, during HP2M, there was a significant increase in blood Hb levels following confinement ($P < 0.05$), which was associated with an increase in Hct in the same fish. In both experiments, triploid fish had significantly higher MCH than diploid fish ($P < 0.05$) which was concurrent with the increase in MCV; however, the MCHC of triploid fish remained similar to that of diploids ($P > 0.05$, Table 3).

Mean MetHb values of 31.78 ± 1.88 % and 34.96 ± 1.86 % were observed in pre-stress all-female diploids and all-female triploids, respectively, and these values decreased significantly following confinement ($P < 0.05$, Table 3). MetHb values obtained during HP1F were questionable, due to a delay in the processing of the blood samples. The prompt processing of fresh blood samples in experiment HP2M, provided indication that MetHb levels of less than 10% can occur in pre-stress diploids and triploids; however, technical problems precluded the acquisition of sufficient sample numbers and the results were inconclusive. A trial of the cyanide derivative technique for determining MetHb (Wells *et al.*, 1997) was undertaken, but this technique also proved problematic and unsatisfactory.

In both experiments HP1F and HP2M, the amount of ATP associated with the red blood cells was similar between diploid and triploid fish ($P > 0.05$, Table 3). Although the amount of ATP associated with each red blood cell was significantly higher in triploids compared to diploids ($P < 0.05$, Table 3), values were proportional to MCV. The amount of ATP per red blood cell decreased significantly following confinement in experiment HP2M ($P < 0.05$, Table 3). No ATP values were attained for pre-stress fish in experiment HP1F due to a technical error.

No significant differences were detected in the total plasma protein concentrations between all-female diploid and all-female triploid fish, in experiment HP1F ($P > 0.05$), and there were no changes in total protein concentrations following confinement ($P > 0.05$, Table 3). By contrast, in experiment HP2M, total plasma protein levels were higher in mixed sex triploid fish compared to mixed sex diploid fish ($P < 0.05$), and protein levels were lower in fish subject to confinement ($P < 0.05$, Table 3).

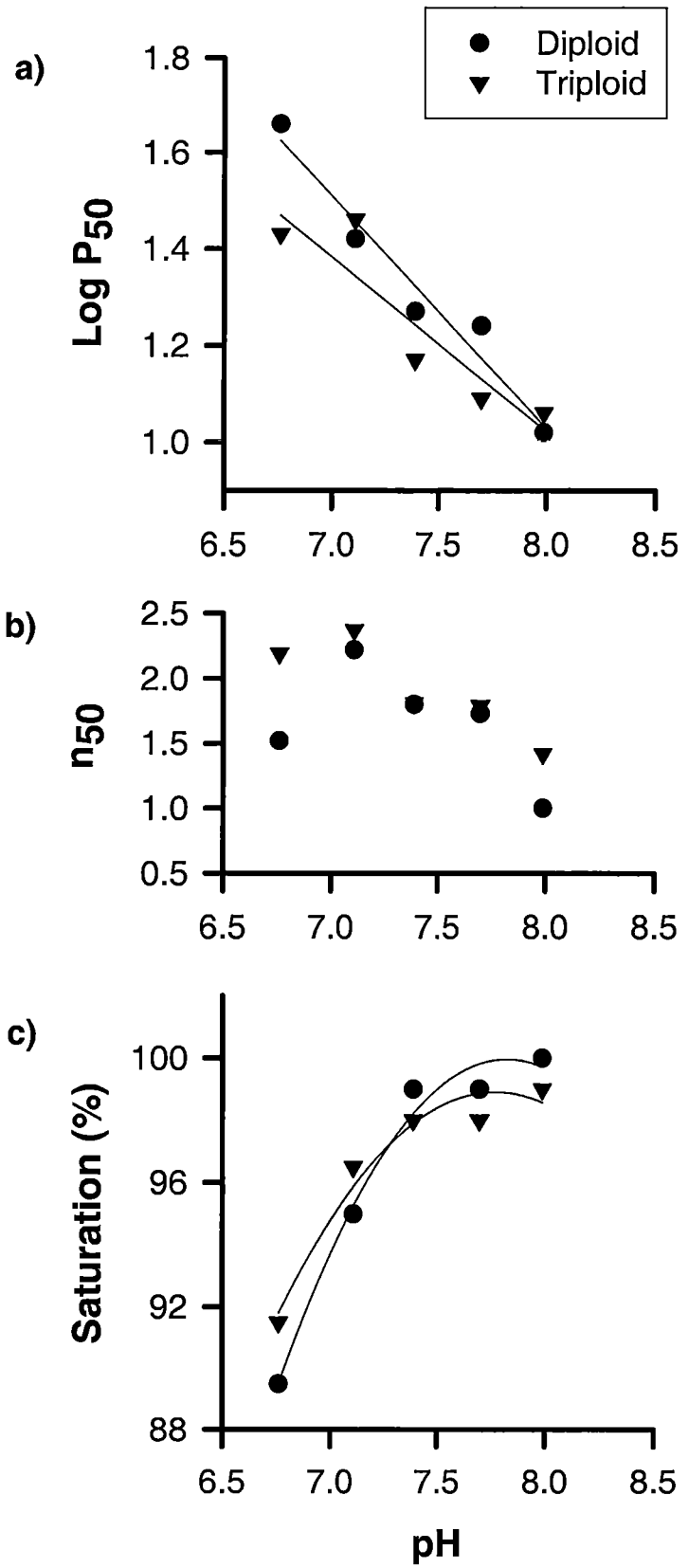
Table 3. Haematological parameter means (± SE values) for SW smolt from different population types (all-female diploids, all-female triploids, mixed sex diploids, mixed sex triploids), prior to- (= 'pre-stress') and following 2.5 hours confinement stress (= 'stressed') determined over the course of two experiments HP1F and HP2M. Light gray shaded values indicate significant difference with ploidy status. Dark gray shaded values indicate significant difference with stress status ($P < 0.05$).

Population	Treatment	Hct %	RBCC $\times 10^5.\mu L$	MCV fL	MCH pg	MCH C $g.L^{-1}$	Hb $g.dL^{-1}$	MetHb %	Total Protein $g.L^{-1}$	ATP $\mu mol.dL^{-1}$	Hb-ATP $g.\mu mol^{-1}$	ATP-rbc $\times 10^{-9}$ $\mu mol.L^{-1}$	ATP-hct $\mu mol.dL^{-1}$	Glucose $mg.L^{-1}$	Cortisol $ng.ml^{-1}$	Lactate $mmol.L^{-1}$
Experiment HP1F																
all-female diploid	pre-stress	32.44 ± 1.1	0.938 ± 0.048	35.3 ± 2.1	90.9 ± 0.74	259.9 ± 57.8	83.66 ± 5.47	31.78 ± 1.88	23.73 ± 3.08					22.24 ± 1.3	24.6 ± 6.07	0.89 ± 0.13
	stressed	35.70 ± 2.3	0.966 ± 0.040	36.9 ± 2.2	82.7 ± 0.33	227.5 ± 29.7	79.73 ± 3.21	28.39 ± 1.56	18.32 ± 2.30	177.7 ± 2.96	22.52 ± 0.81	1.86 ± 0.08	513.7± 32.96	29.67 ± 2.08	75.46 ± 11.62	3.67 ± 0.79
all-female triploid	pre-stress	31.16 ± 3.4	0.702 ±0. 044	43.5 ± 3.4	102.3 ± 0.79	201.8 ± 49.4	68.79 ± 2.33	34.96 ± 1.86	19.27 ± 2.04					25.27 ± 1.88	32.58 ± 5.03	0.79 ± 0.14
	stressed	31.19 ± 2.7	0.616 ± 0.050	50.2 ± 1.69	120.3 ± 1.01	214.3 ± 15.2	69.13 ± 3.91	27.50 ± 0.76	23.96 ± 1.87	158.40 ± 11.21	22.71 ± 1.18	2.66 ± 0.18	537.3± 44.84	28.39 ± 3.23	74.3 ± 15.67	3.73 ± 1.08
Experiment HP2M																
mixed sex diploid	pre-stress	30.75 ± 2.73	0.758 ± 0.074	41.5 ± 3.0	82.8 ± 0.56	200.49 ± 8.5	60.38 ± 3.81		18.9 ± 4.2	103.48 ± 7.56	17.16 ± 0.59	1.43 ± 0.12	343.4± 16.58	26.91 ± 1.82	31.32 ± 7.89	1.14 ± 0.12
	stressed	41.18 ± 1.78	0.886 ± 0.047	47.4 ± 2.8	91.8 ± 0.61	193.0 ± 3.9	79.62 ± 4.1		9.68 ± 1.72	105.71 ± 8.09	13.34 ± 0.82	1.21 ± 0.094	255.7± 13.11	33.82 ± 2.0	76.18 ± 12.22	2.55 ± 0.24
mixed sex triploid	pre-stress	31.93 ± 1.84	0.382 ±0. 026	84.3 ± 4.8	152.9 ± 0.52	194± 10.65	61.97 ± 5.12		40.03 ± 3.33	96.33 ± 8.12	14.92 ± 1.90	2.44 ± 0.16	302.3± 14.15	28.92 ± 1.86	29.34 ± 6.20	0.75 ± 0.09
	stressed	35.37 ± 1.39	0.515 ±0. 025	69.0 ± 1.7	154.2 ± 0.92	222.6 ± 9.3	77.76 ± 2.69		14.58 ± 1.84	106.32 ± 4.46	13.79 ± 0.98	2.08 ± 0.092	294.7± 22.14	36.74 ± 2.02	104.23 ± 9.84	1.33 ± 0.24

4.4.3 Blood oxygen transport in diploid and triploid SW smolt

There was little difference between diploids and triploids in the partial pressure of oxygen required to attain 50% saturation (PO_{50}) at any given pH (Fig. 1a). Comparing the lines of regression in Fig.1a, it appears that triploids may require slightly lower partial pressures of oxygen than diploids to achieve 50% blood saturation at any given pH level. In addition, there appeared to be no difference in the percentage blood oxygen saturation of diploid and triploid Atlantic salmon with increasing *in vitro* pH levels at 15° C (Fig. 1c).

Figure 1. Oxygen partial pressure at 50% blood oxygen saturation (a), sigmoidal coefficient (b) and blood oxygen saturation levels (c) at various physiological pH levels in all-female diploid and all-female triploid Atlantic salmon SW smolt. Symbols are values obtained from blood pooled from 5 fish of each ploidy type.


a)
Diploid
Triploid
Log P_{50}
1.8
1.6
1.4
1.2
1.0
6.5
7.0
7.5
8.0
8.5
b)
n_{50}
2.5
2.0
1.5
1.0
0.5
6.5
7.0
7.5
8.0
8.5
c)
Saturation (%)
100
96
92
88
6.5
7.0
7.5
8.0
8.5
pH

4.4.4 Blood viscosity of diploid and triploid SW smolt

In vivo

Triploid smolt had a slightly higher mean *in vivo* blood viscosity than their diploid counterparts (Fig. 2a), which was coincident with a slightly higher hct (Fig. 2b); however, these differences were not statistically significant.

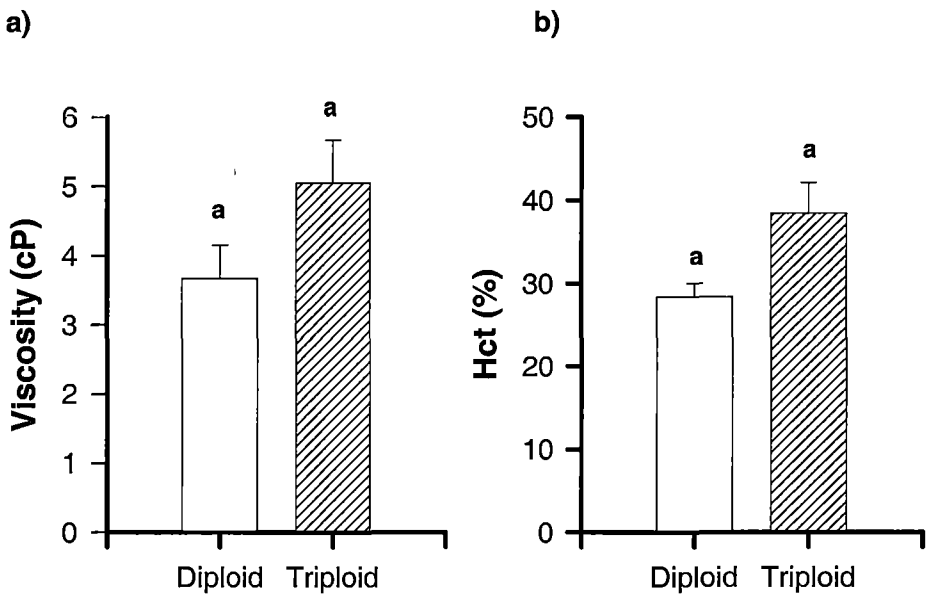


Figure 2. Mean *in vivo* blood viscosity at shear rate = 12 rpm (a) and haematocrit (b) of all-female diploid and all-female triploid SW smolt (n = 5). Superscripts indicate there was no significant difference between ploidy groups.

In vitro

In both experiments BV1 and BV2, the viscosity of red blood cells in suspension varied with Hct ($P < 0.05$) and shear rate ($P < 0.05$) but not with ploidy status ($P > 0.05$). Blood viscosity increased with an increase in haematocrit (Fig. 3 and 4) and decreased with an increase in shear rate (Fig 5 and 6). Significant differences were detected in *in vitro* blood viscosity, between diploid and triploid SW smolt at high hct levels only. Triploids had significantly lower blood viscosity than diploids at hct = 68 % in experiment BV1 ($P < 0.05$, Fig. 5), but had higher blood viscosity values at hct = 29 % and Hct = 54 % in experiment BV2 ($P < 0.05$, Fig. 6). The inconsistent results between ploidy groups at high hct values, and between experiments, suggests that the biological significance of the observed differences is most likely nominal.

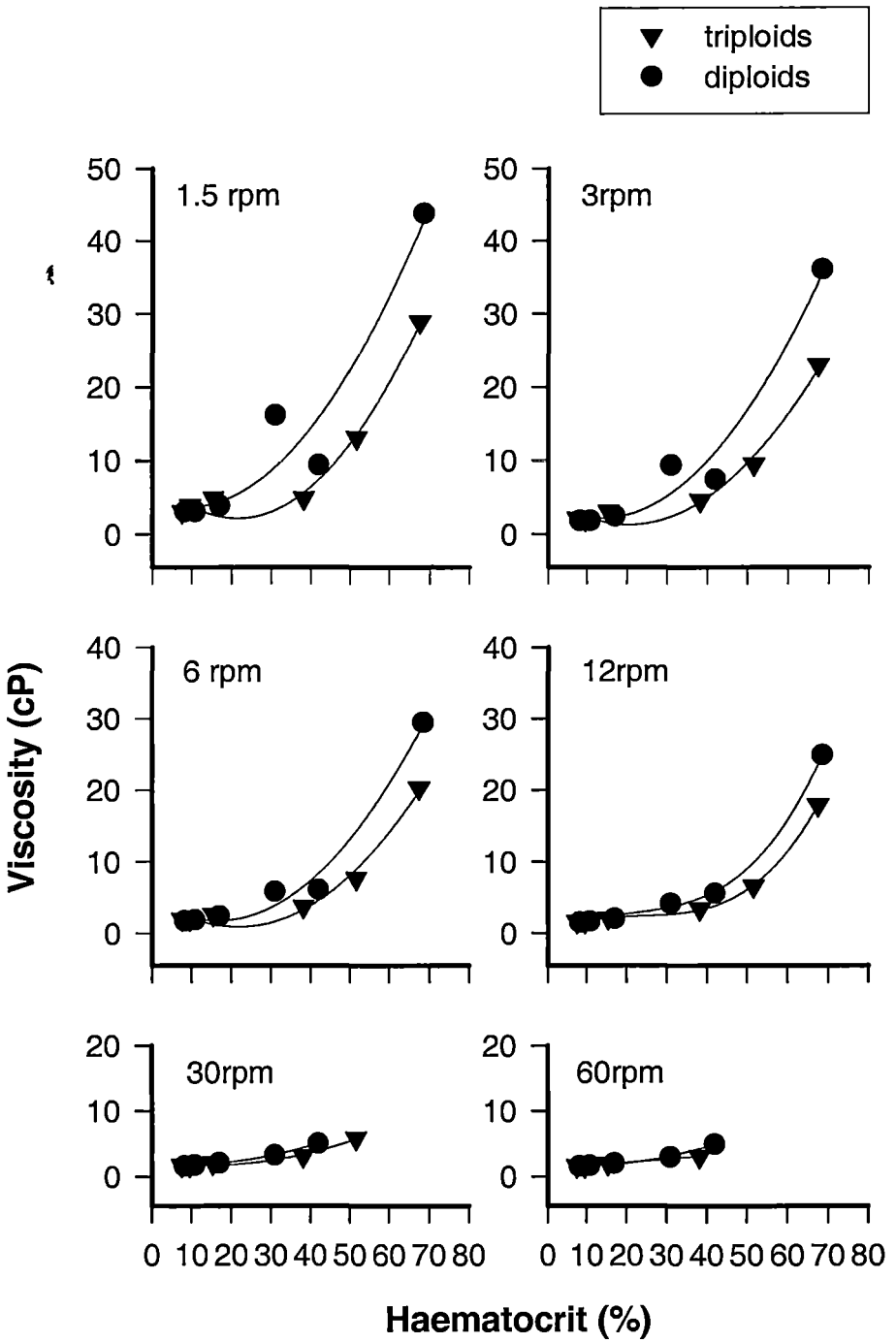


Figure 3: Change in viscosity of red blood cell suspensions, sampled from all-female diploid and all-female triploid Atlantic salmon SW smolt, with haematocrit value, at various shear rates (red blood cell suspensions pooled from 5 fish). Experiment BV1.

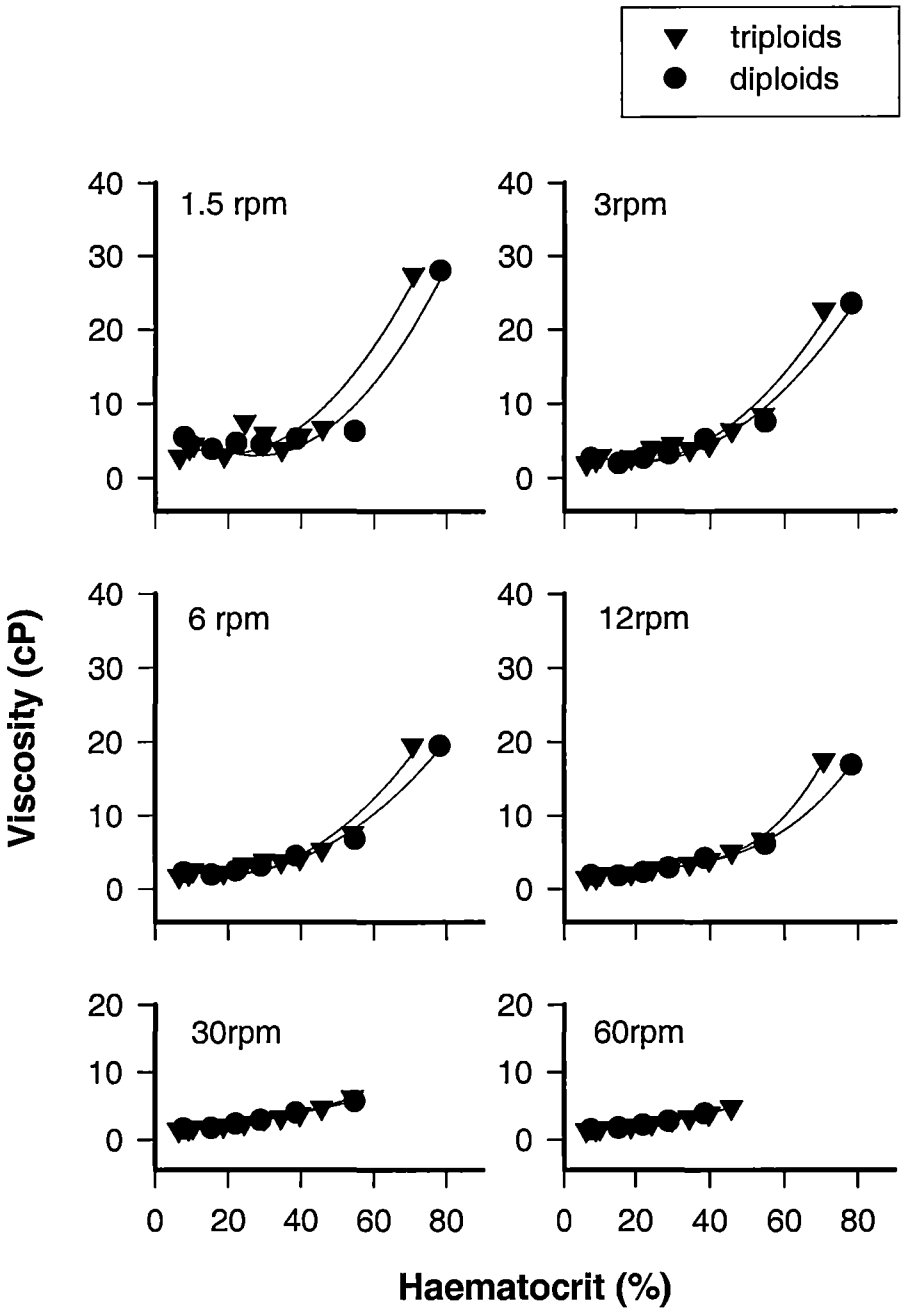


Figure 4: Change in viscosity of red blood cell suspensions, sampled from all-female diploid and all-female triploid Atlantic salmon SW smolt, with haematocrit (hct) value at various shear rates (red blood cell suspensions pooled from 5 fish). Experiment BV2.

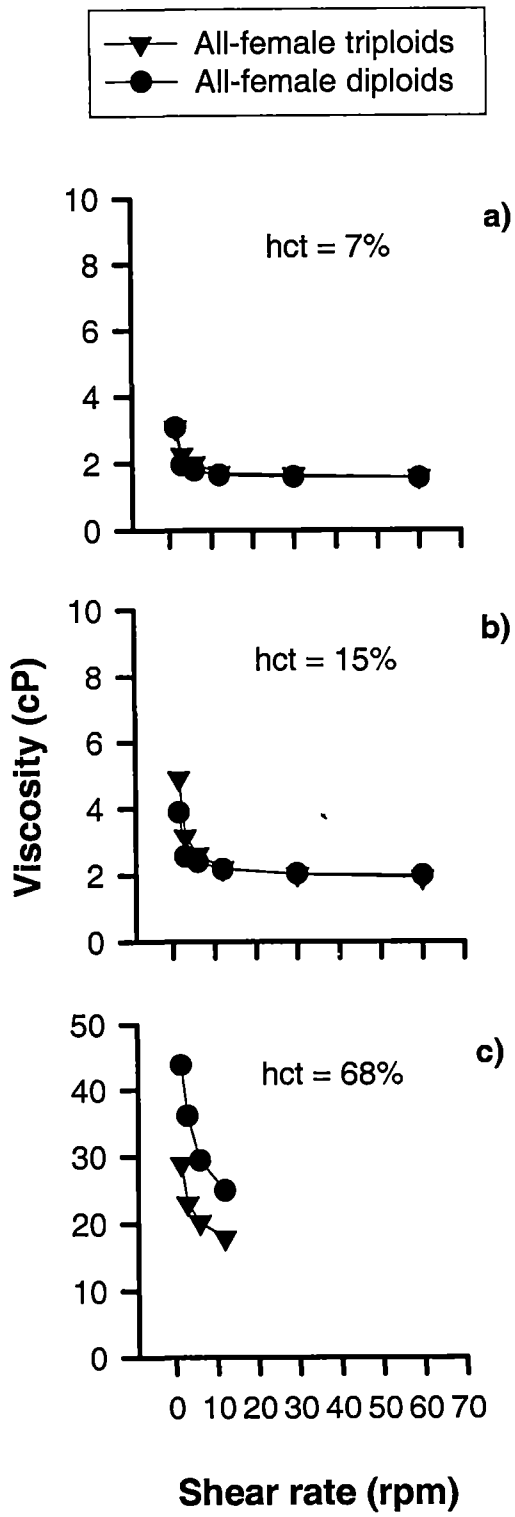


Figure 5. Change in *in vitro* viscosity of red blood cell suspensions, sampled from all-female diploid and all-female triploid SW smolt, with shear rate, at haematocrit (hct) levels; a) 7%, b) 15% and c) 68% (red blood cells for each ploidy group pooled from 5 fish). Experiment BV1.

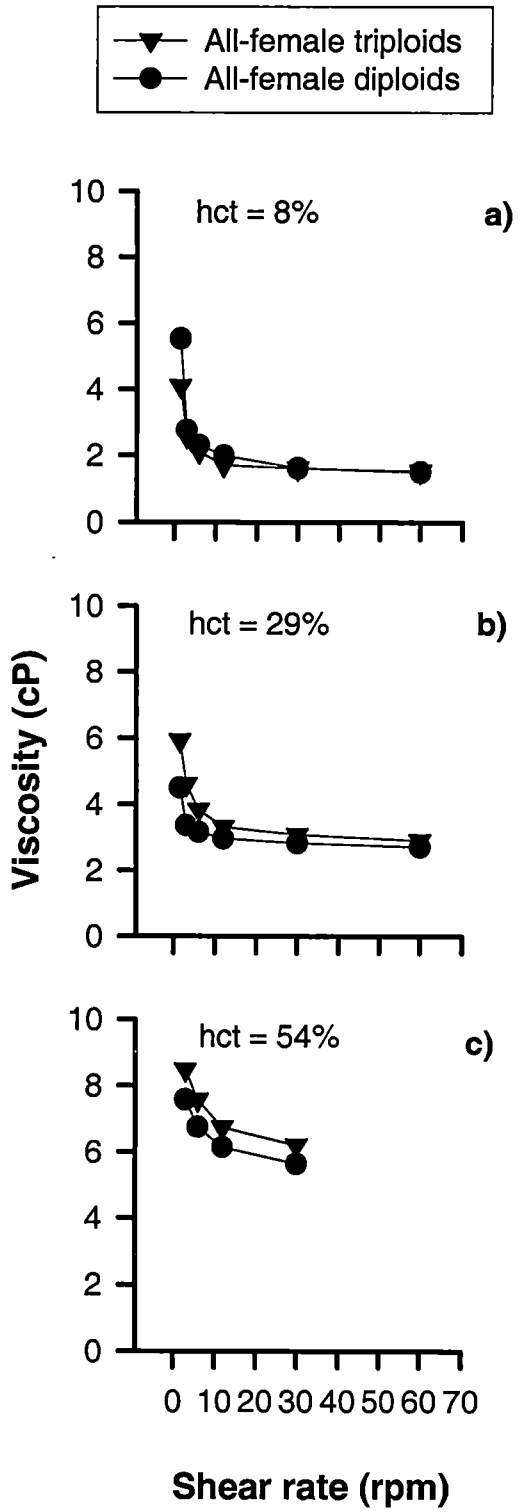


Figure 6. Change in *in vitro* viscosity of red blood cell suspensions, sampled from all-female diploid and all-female triploid SW smolt, with shear rate, at haematocrit (hct) levels a) 8%, b) 29% and c) 54%. (red blood cells for each ploidy group pooled from 5 fish). Experiment BV2.

4.5 DISCUSSION

4.5.1 **Haematology**

Diploid and triploid SW smolt subject to confinement stress showed elevated levels of plasma cortisol that are characteristic of the primary stress response in salmonids (Pickering, 1992; Mazur and Iwama, 1993; Pankhurst and Van Der Kraak, 1997) and the magnitude of the elevation did not differ with ploidy status. The difference in fish size between all-female diploid and all-female triploid groups was not a confounding factor, since plasma cortisol levels in Atlantic salmon (Hemre and Krogdahl, 1996) and greenback flounder (*Rhombosolea taparina*, Barnett and Pankhurst, 1998) have been shown to be independent of size. The results of the current study support those of Chapter 3 (Sadler *et al.*, 2000b), which examined the plasma cortisol response of diploid and triploid Atlantic salmon to confinement, and are in agreement with those of a previous study which examined the plasma cortisol response of diploid and triploid brook trout to acute handling stress (Biron and Benfey, 1994).

Plasma glucose and plasma lactate levels were elevated in diploid and triploid Atlantic salmon SW smolt following confinement, which is characteristic of the secondary stress responses in healthy salmonids (Barton, 1997; Morgan and Iwama, 1997). The persistence of altered states of glucose, lactate, and protein, and recovery rates following stress are well defined indicators of the robustness of the stress response (Barton, 1997; Morgan and Iwama, 1997). This response is an important indicator of available energy reserves, and healthy rainbow trout may show elevated plasma lactate for 24 hours post-stress (Pankhurst and Dedual, 1994). Plasma glucose levels did not vary with ploidy status which supports the findings of Biron and Benfey (1994), who examined plasma glucose levels in triploid brook trout following acute handling stress. In the present study, lower plasma lactate levels in mixed sex triploid smolt compared to mixed sex diploid smolt suggested that triploid fish were not subject to the same degree of anaerobic metabolism as diploid

fish of a similar size, under the same conditions. By contrast, plasma lactate levels of all-female diploid smolt and all-female triploid smolt were similar following confinement, which indicated similar levels of anaerobic metabolism between ploidy groups under the same conditions. Although plasma lactate levels have been shown to be dependant on fish size (Somero and Childress, 1980, 1990; Ferguson *et al.*, 1993), the magnitude of the difference in mean wet weight between all-female diploid and all-female triploid fish (≈10.0 g) is sufficiently small and does not warrant consideration. Furthermore, the results of the present study are supported by the results of Chapter 3 (Sadler *et al.*, 2000b), in which the magnitude of the plasma lactate response in Atlantic salmon SW smolt following different periods of confinement, and the recovery of plasma lactate to basal levels post-confinement, did not differ with ploidy status. The results of both experiments HP1F and HP2M are contrary to a previous study in which it was suggested that triploid salmonids have a lowered aerobic capacity compared to diploids (Virtanen *et al.*, 1990).

Disturbances to plasma protein; however, occur only under nutritional stress or extreme physical disturbance (Wood *et al.*, 1983; Wells *et al*, 1986). In the current study all-female diploid and triploid fish did not display a significant shift in protein following confinement, whereas, in the second experiment, mixed sex triploid fish and stressed mixed sex diploid displayed an inconsistent fluctuation of plasma protein levels. The high plasma protein levels of pre-stress mixed sex triploid fish may reflect ill-health in fish (Huggett *et al.*, 1992) from this population prior to experimentation.

As expected, this study confirms previous findings that triploid salmonids have larger erythrocytes and lower cell counts than diploids, with the result that the Hct of diploid and triploid individuals were not different (Benfey and Sutterlin, 1984a; Graham *et al.*,1985; Small and Randall, 1989; Biron and Benfey, 1994; Yamamoto and Iida, 1994a, 1994b). Decreased Hct values were observed in triploid rainbow trout by Virtanen *et al* (1990); however, their results appear to be anomalous and cannot be explained. The similarity of hct values between diploids and triploids,

despite differences in cellular morphology, highlights the internal regulation of oxygen carrying capacity by the maintenance of an optimal Hct (Rand *et al.*, 1964).

Despite evidence of primary and secondary stress responses to confinement in diploid and triploid SW smolt, Hct did not change in fish from all-female populations in response to stress (experiment HP1F), whereas Hct increased in fish from mixed sex populations under the same conditions (experiment HP2M). Although another study found no change in Hct following acute handling of triploid brook trout (Biron and Benfey, 1994), previous haematological studies of teleosts have shown that stressors such as exercise, anaesthesia and hypoxia induce an increase in RBCC and Hct (Houston *et al.*, 1971; Primmet *et al.*, 1986; Lowe-jinde and Nimni, 1983; Milligan and Wood, 1987a, 1987b; Korcock *et al.*, 1988; Tetens and Lykkeboe, 1981; Soivio *et al.*, 1980; Virtanen *et al.*, 1990). Both increase in RBCC and erythrocytic swelling contribute to the increase in Hct under stressful conditions (Milligan and Wood, 1987a, 1987b; Tetens and Lykkeboe, 1981; Virtanen *et al.* 1990; Wells and Weber,1991). Wells and Weber (1990) showed that adrenergic contraction of the spleen at the onset of a stress response causes the release of fresh immature red blood cells into the circulation system. This reaction usually occurs very rapidly and may not have been detected in the time sequence of experiment HP1F. The relative contributions of the initial and rapid adrenergic flush, and the slower, more persistent cortisol response are complex and depend on the nature and duration of the stress imposed. The responses are qualitatively and quantitatively different in confined fish and fish swimming freely (Lowe and Wells, 1996). We would therefore anticipate that adrenergic responses are fully manifested in pre-stress all-female fish acutely sampled, and that cortisol effects will persist beyond the restoration of baseline catecholamine levels (Gamperl *et al.*, 1994). Further, complex interactions between the two endocrine responses occur at the level of the erythrocyte which appear to regulate oxygen transport in response to either acute or chronic stress (Perry and Reid, 1993). Accordingly, it is most likely the corticosteroid, rather than the adrenergic, stress response which has been evaluated in our study and is relevant to fish husbandry protocols.

The slight increase in MCV observed in both diploid and triploid SW smolt in experiment HP1F and in diploid SW smolt in experiment HP2M, following confinement was not statistically significant, but indicates adrenergic swelling of the erythrocytes under stressful conditions. Erythrocytic swelling under such conditions involves the osmotic influx of water and ionic exchanges at the erythrocytic membrane (Nikinmaa and Huestis, 1984). It has been suggested that the resultant increase in MCV of rbc's facilitates an increase in oxygen affinity by alleviating the allosteric interaction of Hb and ATP, decreasing Hb and NTP concentrations, and increasing intracellular pH (review Wells and Weber, 1991), although it has been noted that an observed increase in MCV of rbc's following stress may not necessarily be integral to normal physiological mechanisms (Wells and Weber, 1991). The relatively high MCV of pre-stress mixed sex triploids compared to the MCV for all other triploids appeared to be anomalous and the reason for this is unclear.

The differences in total Hb content between diploids and triploids were not consistent between experiments. The results of experiment HP1F implied that the oxygen carrying capacity of triploids is compromised compared to that of diploids; however, according to the results of experiment HP2M, triploids had a similar oxygen carrying capacity to diploids. The results of the former experiment (HP1F) are in agreement with previous studies on salmonids (Benfey and Sutterlin, 1984a; Graham *et al.*,1985; Small and Randall, 1989; Yamamoto and Iida, 1994a). The results of the latter experiment (HP2M) highlight the ability of fish to regulate total blood haemoglobin levels with Hct. If it exists, the lower blood Hb content of triploid fish is most likely compensated for by various regulatory mechanisms including increased blood flow and ventilation rates. Assuming that the *in vivo* blood pH levels are similar for diploid and triploid Atlantic salmon, it is unlikely that Hb oxygen affinity would compensate for a decrease in total Hb in triploids since it is shown in this study that oxygen affinity, as indicated by P_{50} values, is similar between diploids and triploids at various physiological pH levels which are likely to occur under stressful conditions. Graham *et al.* (1985) found similar P_{50} values and *in vivo* blood pH levels for pre-stress diploid and triploid Atlantic salmon.

In the present study the increase in MCH in triploid fish was such that MCHC was similar between diploid and triploid fish in both experiments. Generally, there was greater variation in MCHC values for triploid individuals. Given that immature erythrocytes, which have lower MCHC than mature cells (Milligan and Wood, 1987a), may have been released into circulation prior to sampling, it may be that the Hb content of immature erythrocytes is proportionately lower in triploids than in diploids, or that the number of immature erythrocytes is higher in triploids than diploids. This may account for the wide variation in MCHC values obtained for triploid Atlantic salmon compared to diploids and / or it may account for the lower blood Hb levels in all-female triploids. Triploid Atlantic salmon also displayed slightly lower Hct values than their diploid counterparts. Although the subtle differences in MCHC and Hct were not statistically significant, the combination of these two parameters may have contributed to a significantly lower blood Hb in all-female triploids. This demonstrates the influence of subtle differences in cellular parameters on larger scale physiological parameters.

It is unlikely that MetHb, the oxidised non-functional form of haemoglobin, contributes to a reduction of oxygen carrying capacity in triploid salmon. Previously recorded methaemoglobin levels for healthy teleosts range from 1 - 8% (Wells *et al.*, 1997; Powell and Perry, 1997). The MetHb levels observed in this study were comparatively high due to a delay in processing the samples. Oxidation of Hb occurs rapidly, but assuming a linear rate of oxidation we may postulate that there is no difference in the percentage content of MetHb between diploids and triploids.

The observations of ATP levels support our findings regarding oxygen affinity. ATP levels are known to affect the oxygen affinity of Hb by two modes of action. Firstly by allosteric interaction and secondly by the Haldane effect, that is by contributing to a proximal increase in protons at the cellular membrane (Wells and Weber, 1983). The amount of ATP associated with individual rbc's of diploid and triploid Atlantic salmon individuals appeared to be proportional to the size of the blood cell resulting in the comparative levels of ATP per unit volume of blood and comparative Hb:ATP

ratios. This facilitates in part the similarity in oxygen affinity observed between diploids and triploids under different pH conditions as indicated by P_{50} values.

In addition, there was a 25% decrease in ATP levels associated with the red bood cells of mixed sex diploid smolt following 2.5 hours of confinement, whereas, mixed sex triploids displayed only a 5% decrease in ATP levels ($p = 0.0000$). Due to individual variation, no significant differences were detected in ATP levels between diploids and triploids ($p = 0.6975$). In both diploid and triploid fish there was a decrease in the Hb: ATP with confinement stress ($p = 0.0000$). Previous work has indicated that ATP levels decrease following exercise, anaesthesia, during chronic or acute hypoxia (Bushnell *et al.,* 1984; Lane *et al.*, 1981; Milligan and Wood, 1987a; Weber and Wells 1983, 1989, 1991). The reduction in ATP levels is usually greater than explained by an increase in MCV alone and may be caused by metabolic degradation of ATP, or the inhibition of ATP synthesis. In rainbow trout, ATP levels decrease under hypoxic conditions over a period of 7 days (Soivio *et al.*,1980). The observed decrease in ATP with stress would facilitate an increase in oxygen affinity (Wells and Weber, 1983, 1989, 1991), via the concurrent decrease in ATP:Hb and increase in erythrocytic proximal acidity. A decrease in ATP:Hb particularly occurs in immature erythrocytes that are released by splenic contraction under such conditions (Milligan and Wood, 1987a; Lane *et al.*, 1981). In experiment HP2M, the decrease in ATP levels of triploids appeared to be delayed, which may have repercussions on the regulation of Hb oxygen affinity. However, P_{50} values and % Hb saturation values indicate that oxygen affinity of diploid and triploid Atlantic salmon is similar.

4.5.2 Blood oxygen transport

Atlantic salmon diploid and triploid smolt display a similar dependance of % blood oxygen saturation (P_{100}) on plasma pH (Root effect). This implies that at any given pH, a direct comparison of the blood oxygen carrying capacity between diploid and triploid SW smolt can be made by examination of the total Hb determinations. These results are quite different to those of Graham *et al.* (1985) who showed that although

the oxygen affinity (P_{50} values) and *in vivo* blood pH values were similar between landlocked diploid and triploid Atlantic salmon individuals, the Hb-oxygen loading ratio of triploids was only 77% of diploid values.

This *in vitro* experiment showed that the dependance of the oxygen affinity coefficient, P_{50}, and cooperativity coefficient, n_{50}, on plasma pH (Bohr effect) was similar between diploid and triploid Atlantic salmon. This suggests that oxygen uptake is similar between diploids and triploids, despite differences in cellular morphology and possible differences in total Hb content. This may be explained by the similarity in MCHC, Hb: ATP and erythrocytic depth dimensions between ploidy status. Triploid erythrocytes are greater in length and width than those of diploids but not in depth (Benfey, 1996), hence it is likely that oxygen diffusion within erythrocytes is similar between ploidy groups. In both diploid and triploid fish, a higher partial pressure of oxygen is required to achieve 50% blood oxygen saturation at lower plasma pH levels, thereby regulating oxygen unloading at tissues in response to carbon dioxide and lactate loading. Under stressful conditions such as exercise, anaesthesia, acute or chronic hypoxia and hypercapnia, blood oxygen transport of triploid salmon would be regulated by the Bohr effect and the allosteric action of phosphates, in a similar fashion to that of diploid salmonids (Soivio *et al.*, 1980; Bushnell *et al.*, 1984; Milligan and Wood, 1987a; Weber and Wells, 1989).

If the aerobic capacity of triploids is compromised at all, it is most likely due to lowered Hb content and/or differential blood flow dynamics. A decrease in the relative gill surface area of triploid SW smolt compared to diploid SW smolt (Chapter 6), might be expected to result in compensation of haematological and oxygen transport characteristics. In addition, a high incidence of deformities observed in the triploid populations (Chapter 6), such as gill filament deformity (GFD), short opercula and jaw deformity may impact on the branchial apparatus.

4.5.3 Blood viscosity

Blood viscosity contributes to vascular resistance and hence is a determinant of the cardiac output required to generate adequate peripheral blood circulation and oxygen delivery to metabolic tissues (Graham and Fletcher, 1983). Triploid smolt had a slightly higher mean *in vivo* blood viscosity than their diploid counterparts (Fig. 3) which can be attributed to a correspondingly higher mean hct. This was confirmed by the *in vitro* experiments, which showed blood viscosity was dependant on shear rate and hct regardless of ploidy status. The non-newtonian nature of blood is such that an increase in blood viscosity results from an increase in Hct and a decrease in shear rate (Graham and Fletcher, 1985; Fletcher and Haedrich, 1987; Wells and Baldwin, 1990).

The effect of erythrocyte size and shape on blood flow dynamics is controversial (Wells and Baldwin, 1990; Baldwin and Wells, 1990). Fish display interspecies diversity in erythrocyte dimensions which is reflected in their rheological behaviour such that smaller erythrocytes tend to show less shear-dependence on viscosity (Wells and Forster, 1989; Baldwin and Wells, 1990; Wells and Baldwin, 1990). However, erythrocyte size is not constant in salmonids, and adrenergically mediated increases in MCV are thought to lower erythrocyte viscosity as well as modulate haemoglobin-oxygen affinity (Wells and Weber, 1991; Wells *et al.*, 1991). Wells *et al.* (1990) found blood viscosity of notothenids increased with a decrease in MCHC on an intraspecific basis. It was suggested that this occurs as a result of decreased erythrocytic deformability, or it is due to increased intercellular contact. Further, erythrocytic deformability in rainbow trout increases with adrenergic swelling during exercise and hypoxia (Hughes and Kikuchi, 1984). Confinement stress was shown to reduce blood viscosity in a marine teleost (Pankhurst *et al.*, 1992). We examined whether the large erythrocytes of triploid blood might have an affect on blood flow properties. This study indicates that a variation in erythrocyte morphology, as seen between diploid and triploid salmon, does not contribute to significant changes in blood viscosity with ploidy status.

The four main factors effecting blood viscosity at any given temperature and shear rate are cell concentration, cell deformity, plasma viscosity and cell aggregation. The plasma viscosity, which is a function of plasma protein concentration, is very low in salmonids (rainbow trout) compared to less active species (Fletcher and Haedrich, 1987). Cellular aggregation occurs under conditions of low flow rates, due to the bridging effects of large plasma protein molecules such as fibrinogen and globulins (Fletcher and Haedrich, 1987). Both plasma viscosity and cellular aggregation were factors eliminated in *in vitro* experiments by the reconstitution of plasma with isotonic saline solution. Viscosity of blood at high hcts (>50%) has been shown to be chiefly a function of deformability of the rbc's (Graham and Fletcher, 1985). The similarity of blood viscosity between triploid and diploid Atlantic salmon at any given hct and shear rate implies the deformability of respective erythrocytes was similar and that the increase in MCV in triploids had the equivalent effect of an increase in RBCC in diploids. Erythrocytic deformability is dependant on the red cell membrane flexibility and the viscosity of the internal fluid as determined by the quantity and structure of Hb and the presence or absence of a nucleus (review Graham and Fletcher, 1985). The cell surface to volume ratio of the erythrocyte and the tensile property of the membrane structure, determine membrane flexibility (Fletcher and Haedrich, 1987). The increase in nucleus size and cell volume to surface area ratio in triploids (Benfey, 1996) does not contribute to any difference in cell flexibility. This supports the previous suggestion that under normal conditions erythrocytic volume is not an important determinant of blood viscosity due to the high deformability of fish erythrocytes (Wells and Forster, 1989). Fletcher and Haedrich (1987) also noted that species differences in red cell size do not account for differences in blood viscosity.

These data highlight the internal regulation of blood viscosity via hct and explains the factors which limit RBCC, particularly in triploids. Triploids may be able to compensate for lower Hb content by an increase in cardiac output rather than increase in hct, since triploid hct did not vary from diploid hct following stress under these experimental conditions. Under different conditions, such as strenuous exercise, the increase in oxygen demand may require a differential increase in hct

and/or cardiac ouput in triploids. It is unlikely that the blood viscosity of triploids under such conditions would contribute to increased vascular resistance in capillary beds or in branchial lamellae and, consequently, be detrimental to aerobic efficiency. Particularly since blood viscosity tends to be maintained under conditions of high blood pressure (high shear rates) despite an increase in hct (Fig. 4). It is known that salmonids, eg. rainbow trout, as an active temperate species, have a low, narrow, viscosity range that is less dependant on shear rate and temperature than more sedentary species, such as winter flounder (*Pseudopleurnectes americanus*) and the short horn sculpin (*Myoxocephalus scorpius*) (Fletcher and Haedrich, 1986). Rainbow trout also have similar blood rheology to Arctic char *Salvelinus alpinus*, which highlights the interesting point that blood viscosity may be more a reflection of taxonomic relationships than environmental adaptations (Fletcher and Haedrich, 1986). Salmonids most likely experience relatively small changes in the viscosity component of vascular resistance (Fletcher and Haedrich, 1986). Assuming that blood vessel diameters do not change with ploidy status (review Benfey, 1996), the blood viscosity of triploids would not contribute to any possible aberrations in the blood flow dynamics of these fish. Clearly cardiac output and blood flow rates of triploids require investigation in order to fully address the possibility that triploid salmon display a decreased aerobic capacity and respiratory efficiency.

4.5.4 Summary and Conclusions

Under the experimental conditions described here, there appeared to be no significant differences in the blood oxygen carrying capacity, oxygen affinity and blood viscosity of diploid and triploid Atlantic salmon, despite a difference in erythrocyte morphology. Despite having fewer and enlarged erythrocytes, triploid Atlantic salmon displayed only slightly reduced, if not similar blood oxygen carrying capacity and similar haematological responses to a confinement stress compared to diploids. The stress response, as indicated by plasma cortisol, glucose, and lactate concentrations, was equivalent for both groups. Further, haemoglobin-oxygen affinity, Bohr and Root effects were similar for both diploids and triploids. The larger triploid erythrocytes, showed similar shear dependence on blood viscosity, and

thus oxygen transport is unlikely to be compromised. The similarity of the haematological response to stress in diploid and triploid fish suggests that the higher mortality reported for triploids reared under farm conditions is not generated by their failure to show respiratory homeostasis in the face of stress. The greater heterozygosity displayed by triploid salmonids (Allendorf and Leary, 1984) may result in different responses to environmental perturbations, although there is little evidence for this (Oliva-teles and Kaushik, 1987b, 1990a, 1990b; Yamamoto and Iida, 1994a; McCarthy *et al.*, 1996; Stillwell and Benfey, 1996a, 1996b, 1997). Metabolic gas diffusion at the gills, cardiac output and blood flow rates are potential areas of physiological variation between diploids and triploids which have yet to be examined, particularly under conditions that demand physical exertion.

Chapter 5:

SKELETAL DEVELOPMENT OF DIPLOID AND TRIPLOID ATLANTIC SALMON

5. SKELETAL DEVELOPMENT OF DIPLOID AND TRIPLOID ATLANTIC SALMON

5.1 ABSTRACT

This study used Taylor's staining and clearing technique to examine the ontogeny, morphology and temporal onset of ossification of the lower jaw, the jaw suspensorium, branchial apparatus (including the opercula), cranium and fin skeletons of Atlantic salmon from different population types (all-female diploid, mixed sex diploid, all-female triploid and mixed sex triploid), up until 2350° days post-fertilisation. Skeletal morphology and the onset of ossification was similar between normal diploid and triploid fish, irrespective of sex. This is the most definitive study to date describing skeletal ontogeny and morphology in Atlantic salmon and it is the first to use a temperature adjusted development scale. Atlantic salmon have a longer period of ontogeny and display more advanced skeletal development at the time of hatching and first feeding compared to other teleosts. Skeletal deformities that were not otherwise detected at the gross level are described.

5.2 INTRODUCTION

Fish bone may be broadly classified as cellular or acellular according to the presence or absence of osteocytes, respectively (Kolliker, 1859; Moss, 1961a; De Beer, 1971). In both cellular and acellular bone, the organic bone matrix is composed of collagen fibre which interacts with sulfated muco-polysaccharides of the ground substance and is calcified with a hydroxyapatite salt (Moss and Posner, 1961, reviewed by Moss, 1961b). Salmonids, including Atlantic salmon, are among some of the lower teleosts that have cellular bone (Kolliker, 1859; Moss, 1961b), although large areas of apparently acellular bone, distinguished by an extremely low frequency of osteocytes, have been observed in the skeleton of some salmonids (Moss, 1961b). Bone types may be arbitrarily classified according to their structural form (lamellar or woven/ fibrous), as well as the presence and morphology of vessels, tubules and uncalcified collagen fibril bundles (reviewed by Moss, 1961b, 1963). In addition, fish with acellular and/or cellular bone possess a wide spectrum of uncalcified tissues including hyaline cartilage, epiphyseal cartilage and chondroid tissue (Moss, 1965). Chondroid tissue is intermediate to cartilage and chordoid (notochord) tissue in structure and may become osteogenic (Moss, 1965).

The processes of chondrogenesis and osteogenesis involve a number of sequential events which are initiated during embryogenesis by epithelial-mesenchymal cellular interactions and culminate in the condensation and differentiation of specialist precursor cells, followed by the transformation of precursor cells into osteogenic blast cells and matrix deposition. Osteoprogenitor or osteogenic precursor cells within the mesenchyme give rise to chondroblasts, osteoblasts and fibroblasts of bone (Palmer, 1993). Presumably genetic determinants are responsible for the development of cell types from condensed mesenchyme of embryonic limb buds and certain physiological factors, such as oxygen tension, prolonged hyperparathyroidism and growth factors, can influence the direction of differentiation (Palmer, 1993). In bone, but not cartilage, there is terminal differentiation of osteogenic cells into osteocytes followed by matrix mineralisation

(reviewed by Hall and Miyake, 1995). Osteocytes may be formed during osteogenesis by direct metaplasia of fibrocytes, enclosure of osteoblasts within osteoid, or via an intermediate stage of chondrocytes (Moss, 1965).

The different types of osteogenesis known to occur in fish, include chondroidal osteogenesis, membranous osteogenesis, direct fibroblastic metaplasia and transformation of secondary cartilage (Moss, 1961a, 1963, 1965; De Beer, 1971; Simmons, 1971). Membranous ossification is the most common and involves the deposition and mineralisation of a plate of extracellular matrix between membranous layers of the dermis (= periosteum) by osteoblasts. The osteoblasts may become buried in the deposited matrix and subsequently transform into osteocytes to form cellular bone. Alternatively, the osteoblasts may either recede from the site of appositional growth, or if they are buried within the matrix and transform into osteocytes, they quickly become pycnotic and their lucanae are filled with mineral matrix to form acellular bone. Chondroidal osteogenesis is unique to teleosts and involves direct transformation of a framework of chondroidal tissue to bone. This occurs either by transformation of chondroid cells into osteoblasts resulting in the formation of cellular bone, or by nuclear pycnosis of the chondroid cells and subsequent mineralisation of cell spaces resulting in acellular bone (Moss, 1965; Simmons, 1971).

There is a paucity of information regarding the mechanisms of calcium deposition and bone mineralisation in teleosts. In vertebrates, intracellular calcium takes the form of amorphous calcium phosphate and mitochondrial granules (Martin and Matthews, 1970; Mathews, 1970). The intracellular accumulation of amorphous calcium phosphate and cellular regulation or deposition of calcium within the extracellular matrix precedes the nucleation of crystaline hydroxyapatite and mineralisation (Bonucci, 1967; Anderson, 1969; Mathews, 1970). Cytoplasmic processes and extracellular deposition of calcium globules (Bonucci, 1967) or vessicles filled with amorphous calcium are thought to be involved in initial mineralisation (Bonucci, 1969; Martin and Matthews, 1970; Mathews, 1970). The tissues thought to be primarily involved in hormonal control of calcium regulation in

fish include the pituitary gland, which secretes pro-lactin, and the corpuscles of stannius (CS) (Copp, 1969; Lopez *et al.*, 1976; Pang and Pang, 1986). The thyroid and ovaries are of secondary importance (Pang and Pang, 1986), although it is difficult to separate the growth promoting and morphogenetic roles of the thyroid gland since the thyroid may act synergistically with the pituitary growth hormone (Simmons, 1971). Parathyroid glands, which secrete parathyroid hormone in most vertebrates, are absent in fish (Wendelaar Bonga and Flik, 1991). The possible role of the calcitonin secreting ultimobranchial body in calcium regulation and osteogenic activity of teleosts remains controversial (Copp, 1969; Lopez *et al.*, 1976; Pang and Pang, 1986, Wendelaar Bonga and Flik, 1991).

Cellular parameters that are fundamental morphological determinants during skeletal development include the number of cells in cellular aggregations, the time of initiation of cell condensation, the mitotically active cell fraction, the rate and orientation of cell division and the rate of cell death (reviewed by Hall and Miyake,1995; Kimmel *et al.*, 1998). Further, extracellular matrix and cell surface molecules are important determinants because cell shape is maintained by cytoskeletal - cell surface - extracellular matrix interactions (Hall and Miyake, 1995). Molecular factors, such as growth factors, cell adhesion molecules, proteoglycans, cell surface proteoglycan receptors and homeobox genes influence skeletal patterning (reviewed by Hall and Miyake, 1995). The homeobox is a highly conserved string of nucleotides (180 bases long) that is part of the coding sequence of genes expressed during embryonic development (homeiotic genes) and which are believed to be involved in the regulation of embryogenesis (Joly *et al.*, 1992). The influence of genotype on skeletal morphology has been clearly demonstrated in zebrafish, with craniofacial and pharyngeal defects arising from genetic mutation (reviewed by Schilling and Kimmel, 1997; Kimmel *et al.*, 1998). An example of genotypic influence on normal skeletal morphology, environmental parameters notwithstanding, may be the consistent morphological differences observed between wild and domestic strains of red sea bream *Pagrus major* and plaice *Pleuronectes platessa* (reviewed by Matsuoka, 1987).

Previous studies of skeletal development of salmonids appear to focus on a limited number of skeletal structures for a limited period of development (De Beer, 1927; Saunderson, 1935; Verraes, 1974a, 1974b; reviewed by Jollie, 1984). More comprehensive studies of skeletal development of non-salmonid species, including those of red sea bream *Pagrus major* by Matsuoka (1985, 1987), add to our knowledge of skeletal ontogeny throughout teleost development. The sequence of osteological development involves a number of qualitative and quantitative changes starting with cell condensation and the initial appearance of cartilage structures. This is followed by the development of the full complement of cartilage structures, the onset of ossification and finally the full development of ossified structures. As described previously, not all structures initially appear as cartilage, but appear as osseous structures from the outset, in which case bone matrix is deposited prior to ossification. In fish, development of structures of the mouth, opercula and gills precede the sequencial development of the head, fins and trunk (reviewed by Matsuoka, 1985, 1987). In salmonids and other teleost species, the first skeletal structures of skeleton to appear are the cartilaginous elements of the gills (ceratobranchials and hyoid arch) and lower jaw (Meckel's cartilage), as well as the osseous structures of the upper jaw (maxilla), the lower jaw (dentary), the pectoral fin girdle (cliethrum), and the opercula bones, which are all formed by membranous ossification (De Beer, 1927; Saunderson, 1935; Jollie, 1984; Matsuoka, 1985, 1987). These osseous structures appear prior to first feeding, in preparation for their respective supportive roles in feeding, respiration and locomotion, whereas the remainder of the skeleton generally becomes ossified later (Jollie, 1984; Matsuoka, 1985, 1987).

As discussed previously, triploid fish have larger but fewer cells in their tissues, including cartilage (Swarup, 1959a; Lou and Purdom, 1984), with a reduction of cell surface to volume ratio compared to diploid fish (Benfey, 1999). On the basis of differences in cell morphology with ploidy status, one might expect skeletal development to differ between diploid and triploid fish since the processes which determine skeletal morphology and mineralisation, including cell condensation, cell accumulation and differentiation, intra-cellular calcium accumulation and

extracellular calcium deposition, may differ with cell morphology. In addition, triploids are more heterozygous than diploids (Allendorf and Leary, 1984), and this may result in greater variation of skeletal phenotypic expression. The comparison of skeletal development between diploid and triploid fish is completely novel and offers the unique opportunity to examine the possible effects of intra-specific differences in cell size and heterozygosity on normal skeletal development.

The aim of this study was to examine the ontogeny of normal skeletal morphology and the temporal onset of ossification of each bone in the lower jaw, the jaw suspensorium, the cranium, the branchial apparatus and the fin skeleton (pectoral, dorsal, anal, caudal, pelvic girdle) of Atlantic salmon. This study examined whether there are differences in normal skeletal development of Atlantic salmon with ploidy or sex status and provides a foundation for assessment of abnormal skeletal development (Chapter 6). Different types of skeletal deformity that were detected in stained and cleared (Taylor and Van Dyke, 1985) embryos and juveniles, but were not otherwise detected at the gross level, are described.

5.3 METHODS AND MATERIALS

5.3.1 Fish production and husbandry

All-female diploid, all-female triploid, mixed sex diploid and mixed sex triploid populations were produced using commercial techniques and maintained in separate tanks under standardised husbandry conditions, during both the freshwater (FW) and seawater (SW) phases, as previously described in Chapter 2. Population sex ratios were determined to be 100% female for the all-female and 1 male: 1 female for the mixed sex populations (see results Chapter 2). Population ploidy status was found to be 96% and 100% triploid in all-female and mixed sex triploid populations, respectively (see results chapter 2).

5.3.2 Skeletal morphology during development

Fish (n = 10 - 20) were randomly sampled from each population at various stages of development as determined by accumulated temperature units (ATU = ° days) (Table 1). Fish were anaesthetised in 25 ppm Benzocaine and total wet weight (TWWt.) and fork length (FL) were recorded. Fish were then fixed in 10% neutral buffered formalin, and were subject to a staining and clearing technique (Taylor and Van Dyke, 1985, Appendix D) to differentiate between cartilage and bone structures *in situ*. The morphology of bones within a) the cranium, b) the branchial apparatus and hyoid arch, c) the lower jaw, the jaw suspensorium and upper jaw, d) the fin skeleton and e) the vertebral column, was recorded at each stage of development using a camera lucida. Bone nomenclature followed that of Jollie (1984) and Matsuoka (1985). A descriptive qualitative approach to skeletal ontogeny was taken. Fish from each population were examined to determine at which stage each cartilage and bone structure first appeared, first became fully delineated in shape and first became ossified. Ontological characteristics observed in individual fish were attributed to population characteristics if it occurred in more than 50% of the individual fish observed at each developmental stage. The process by which each bone developed was noted to be either by membranous secretion or by chondral ossification. Membranous secretion was distinguished in cleared and stained fish by the initial appearance of transparent red-staining osseous material at the site of bone development. Chondral ossification was characterised by the initial appearance of dense blue-staining cell matrix which subsequently developed into cartilage/chondroid and then became ossified, either at the periphery (epichondral) or within (endochondral) the cartilage structure, as indicated by the appearance of red-staining osseous material.

Table 1. Developmental stages and total number of fish sampled (n) for the determination of skeletal ontogeny of four Atlantic salmon populations; all-female triploids (FT), mixed sex triploids (MT), mixed sex diploids (MD) and all-female diploids (FD). Accumulated temperature units (ATU) represents development post-fertilisation.

Date	Development Stage	Weeks (post-fertilisation)	ATU (° days)	FT (n)	MT (n)	M D (n)	FD (n)
May 1996	Fertilisation		0				
		5	280	10	10	10	10
		6	340	10	10	10	10
		7	390	10	10	10	10
July 1996	Hatching						
	ALEVINS	8	470	10	10	10	10
		9	528	10	10	10	10
		10	583	10	10	10	10
August 1996		11	642	10	10	10	10
		12	706	20	20	20	20
	Swim up & First Feeding FRY	13	772	10	10	10	10
		14	843	10	10	10	10
September 1996		15	913	20	20	20	20
October 1996		19	1510	10	10	10	10
January 1997	PARR	31	2350	20	20	20	20

5.4 RESULTS

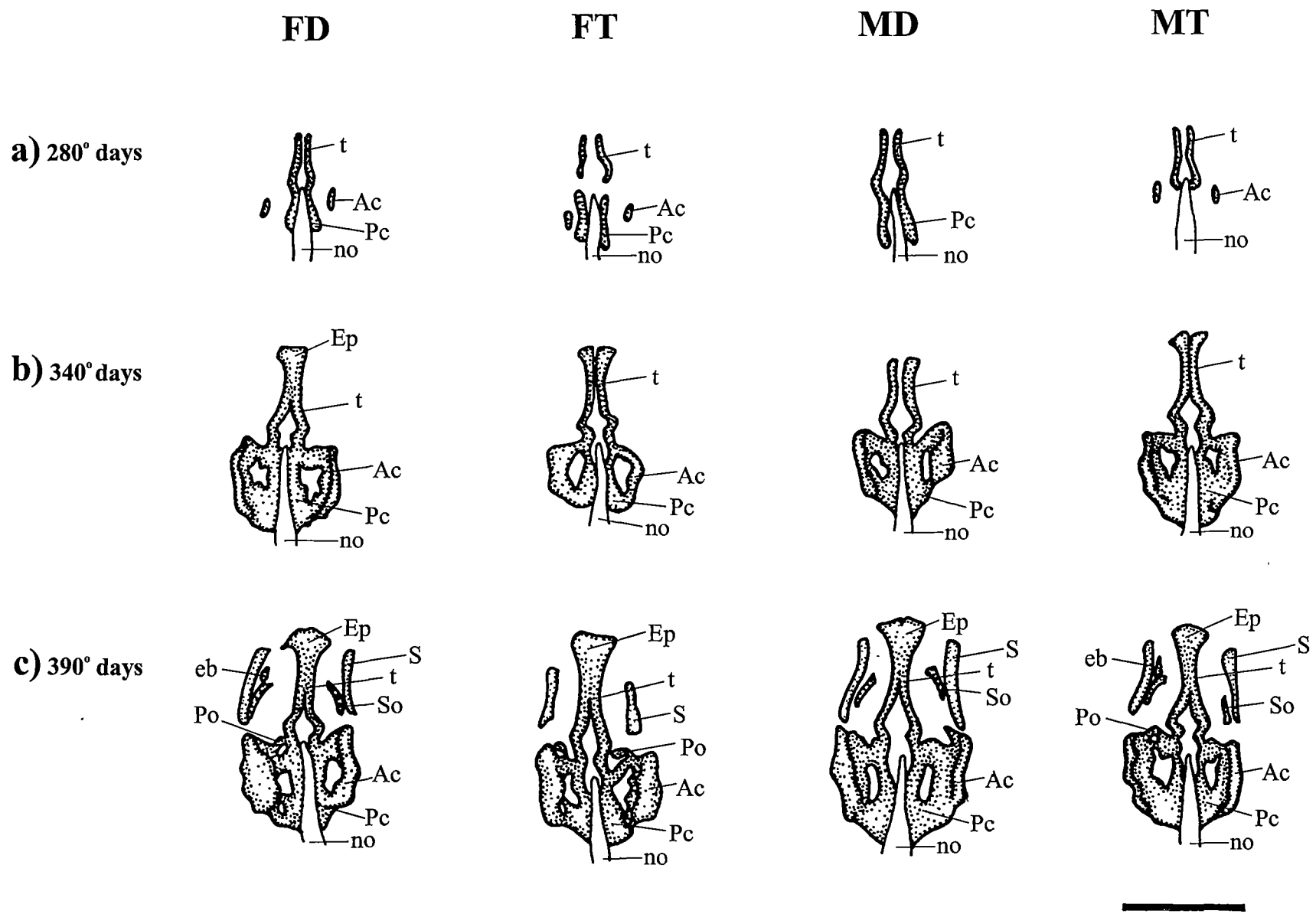
5.4.1 Ontogeny of cranial skeleton

Five weeks post-fertilisation (280° days, Fig. 1a), cellular condensation was observed in cleared and stained preparations at the anterior tip of the notochord of Atlantic salmon embryos. The cellular condensation was associated with presumptive sites of cartilage development, including the region of the auditory capsule, the parachordals and the rostral cartilage at the anterior tip of the trabeculae. At this stage fish from each population had started to develop trabecula cartilages and, with the exception of the mixed sex triploid fish, the parachordal cartilages.

Six weeks post-fertilisation (340° days, Fig. 1b), the cartilaginous base and lateral walls of the auditory capsule had started to form lateral to the parachordals in fish from the all female diploid, mixed sex diploid and mixed sex triploid populations. In addition, the trabeculae were fused posteriorly to the parachordals and, in the all-female diploid fish, the anterior tips of the adjacent trabecula cartilages had fused to form the ethmoid plate.

Seven weeks post-fertilisation (390° days, Fig. 1c), the ethmoid plate was present in fish from all populations and dispersed cells had accumulated at the dorsal surface of the ethmoid plate at the future site of the ethmoid cartilage. At this stage the sclerotic cartilage was formed in all fish, regardless of population type. In addition, the supraorbital cartilage was observed in fish from all populations, with the exception of the all-female triploids. The cartilaginous ectethmoid bar had started to develop in all-female diploid and mixed sex triploid fish, but was not observed in the all-female triploid and mixed sex diploids. The cartilage precursor of the prootic was evident in all fish with the exception of the mixed sex diploids. The lateral walls of the auditory capsule had extended to the dorsal surface of the cranium. The cartilaginous precursor for the intercalar was evident, but only in the all-female populations.

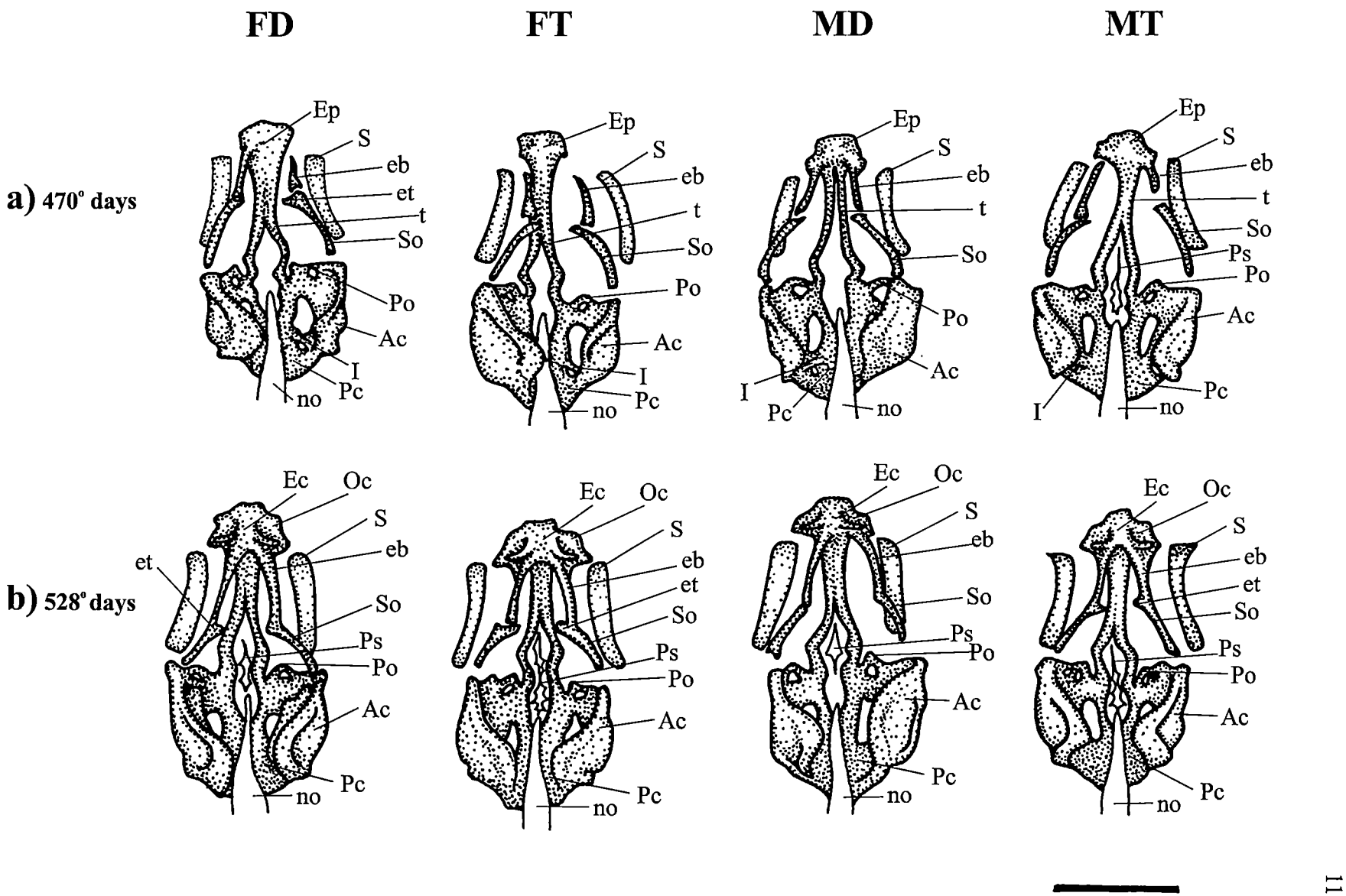
Figure 1. Camera lucida diagrams of the dorsal view of the cranial skeleton in stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at a) 5 weeks (280° days), b) 6 weeks (340° days) and c) 7 weeks (390° days) post-fertilisation. Stippled areas denote cartilage and, with the exception of the notochord, unfilled areas denote bone. Abbreviations: Ac = auditory capsule, eb = ectethmoid bar, Ep = ethmoid plate, I = intercalar, no = notocord, Pc = parachordal, Po = prootic, S = sclerotic, So = supraorbital cartilage, t = trabecula. Scale bar = 3 mm.


FD
FT
MD
MT
a) 280° days
b) 340° days
c) 390° days
t
Ac
Pc
no
Ep
S
eb
Po
So

One week post-hatching (470° days, Fig. 2a), the auditory capsule was well delineated and the dermis surrounding the lateral wall of the capsule stained darkly blue with Alcian Blue indicating deposition of proteoglycans in the mesenchyme tissues of this region. With the exception of the all-female triploid fish, the ethmoid plate had broadened and was laterally fused to the ectethmoid bar in every fish. A lateral process, the epiphysial tectum, had started to develop at the anterior tip of the supraorbital cartilage and was oriented towards the medial line of bilateral symmetry. The cartilaginous precursor of the prootic and the intercalar bones were now evident in all fish. The parasphenoid bone, formed by membranous secretion, was first apparent in mixed sex triploids.

Two weeks post-hatching (528° days, Fig. 2b), the olfactory capsules were delineated in the ethmoid cartilage of all fish. The supraorbital and epiphyseal tectum were fused anteriorly to the ethmoid bar to form a continuous framework surrounding the dorsal and rostral edges of the sclerotic. The parasphenoid had started to form in all fish, regardless of population type. In the all-female triploids and all-female diploids, the dermal tissue at the dorsal surface of the cranium, particularly at the junction of the epiphyseal tectum and ectethmoid bar, was stained blue with Alcian Blue indicating the accumulation of proteoglycans in the mesodermal tissue.

Figure 2. Camera lucida diagrams of the dorsal view of the cranial skeleton in stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at a) 1 week (470° days) and b) 2 weeks (340° days) post-hatching. Stippled areas denote cartilage and, with the exception of the notochord, unfilled areas denote bone. Abbreviations: Ac = auditory capsule, eb = ectethmoid bar, Ec = ethmoid cartilage, Ep = ethmoid plate, et = epiphysial tectum, I = intercalar, no = notocord, Oc = olfactory capsule, Pc = parachordal, Po = prootic, Ps = parasphenoid, S = sclerotic, So = supraorbital cartilage, t = trabecula. Scale bar = 3 mm.


FD
FT
MD
MT
a) 470° days
b) 528° days
Ep
S
eb
et
t
So
Ps
Po
Ac
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Pc
no
Ec
Oc

At 5 weeks post-hatching (706° days, Fig. 3), the frontal and supratemporal bones had started to form by membranous ossification at the dorsal surface of the cranium in fish from all populations. In addition, the parasphenoid was well delineated, extending to the ethmoid cartilage, and the basioccipital, exoccipital and prootic bones had started to ossify. The supraorbital and auditory capsule sections of the lateral line system were well delineated and had started to ossify within the dermis. With the exception of the all-female diploids, the basisphenoid was first apparent in fish of this age. In the all-female triploids, the parietal, epiotic, pterotic, sphenotic, pterosphenoid and nasal dermal bones had started to form, but these bones were not observed in fish from other populations at this stage.

By 8 weeks post-hatching (913° days, Fig. 4), the frontal and supratemporal bones covered approximately 75 - 80% of the dorsal surface of the cranium in all fish. At this stage various dermal bones were formed by membranous secretion, including the parietal, epiotic, pterotic, sphenotic, pterosphenoid, nasal, ethmoid, lateral ethmoid, lacrymal and infraorbital bones in all fish regardless of population type. The nasal lateral line and infraorbital lateral line had started to ossify. The prootic and basisphenoid bones were ossified and well delineated by this stage and the ethmoid cartilage had extended posteriorly to approximately one third the total length of the dorsal surface of the cranium.

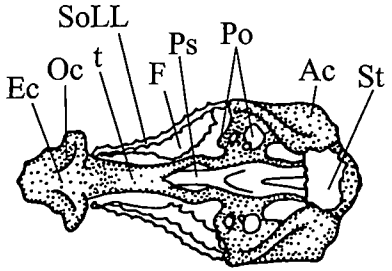
By 12 weeks post-hatching (1510° days, Fig. 5), all existing dermal bones had increased in area, covering 80 - 90% of the dorsal surface of the chondrocranium. The prootic bone had also increased in area, extending posteriorly to meet the exoccipital bone. The anterior orbitosphenoid bone had started to form at the posterior edge of the ethmoid cartilage in fish from each population, with the exception of the all-female triploids.

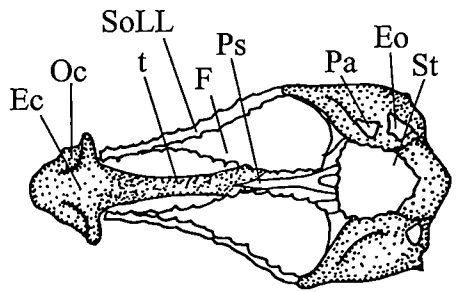
Figure 3. Camera lucida diagrams of a) the dorsal, b) lateral and c) ventral view of the cranial skeleton in stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at 5 weeks post-hatching (706° days). Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: Ac = auditory capsule, AcLL = auditory capsule lateral line, Bo = basioccipital, Bs = basisphenoid, eb = ectethmoid bar, Ec = ethmoid cartilage, Eo = epiotic, et = epiphysial tectum, Ex = exoccipital, F = frontal, I = intercalar, Io = infraorbital bones, Oc = olfactory capsule, Pa = parietal, Ps = parasphenoid, Po = prootic, Pts = pterosphenoid, Pto = pterotic, SoLL = supraorbital lateral line Sp = sphenotic, So = supraorbital, SoLL = supraorbital lateral line, St = supratemporal, t = trabecula. Sclerotic not shown. Scale bar = 3 mm.

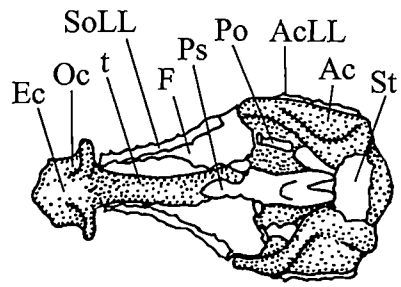
706° days

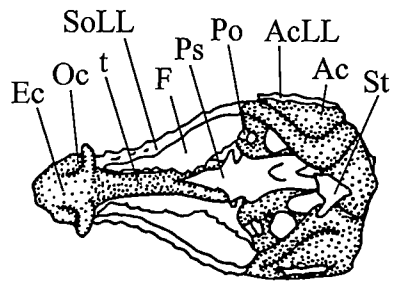
FD FT MD MT

a)









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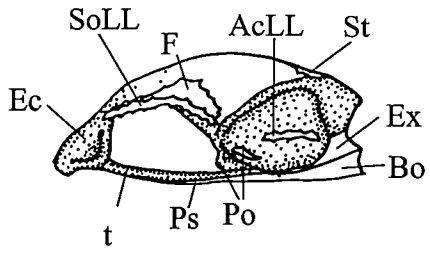
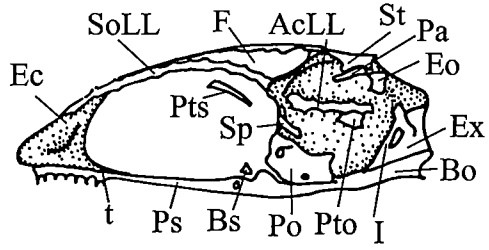
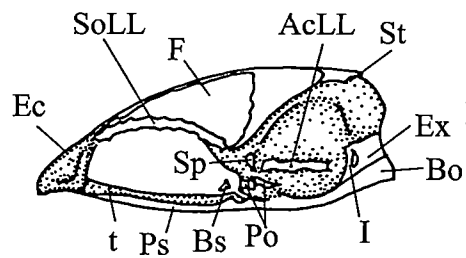
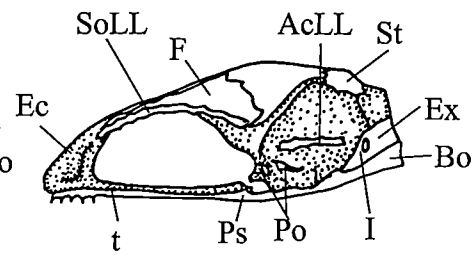
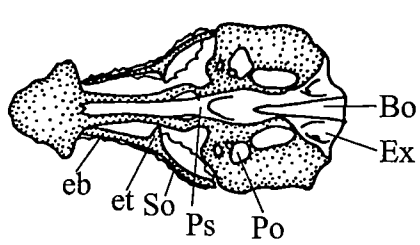


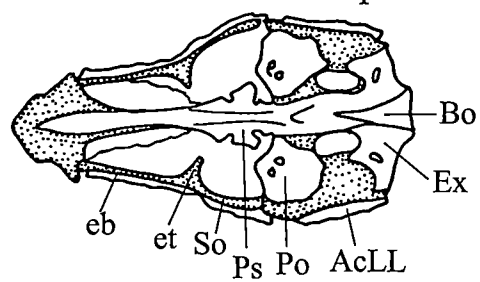


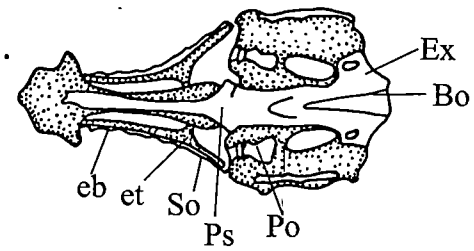




c)







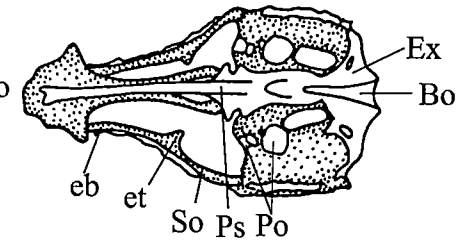
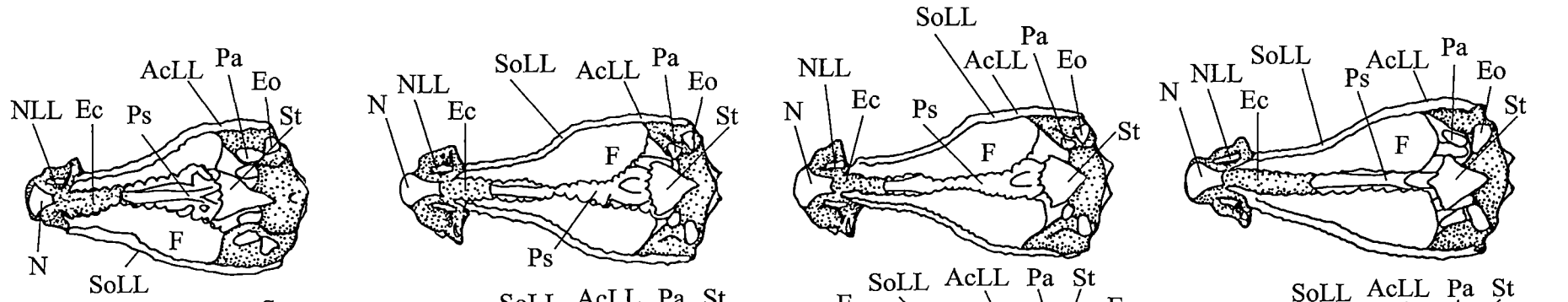


Figure 4. Camera lucida diagrams of a) the dorsal, b) lateral and c) ventral view of the cranial skeleton in stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at 8 weeks post-hatching (913° days). Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: AcLL = auditory capsule lateral line, Bo = basioccipital, Bs = basisphenoid, E = ethmoid, Ec = ethmoid cartilage, Eo = epiotic, et = epiphysial tectum, Ex = exoccipital, F = frontal, I = intercalar, Io = infraorbital bones, La = lacrymal, Le = lateral ethmoid, N = nasal, NLL = nasal lateral line, Pa = parietal, Po = prootic, Ps = parasphenoid, Pto = pterotic, Pts = pterosphenoid, SoLL = supraorbital lateral line, Sp = sphenotic, St = supratemporal. Sclerotic not shown. Scale bar = 3 mm.

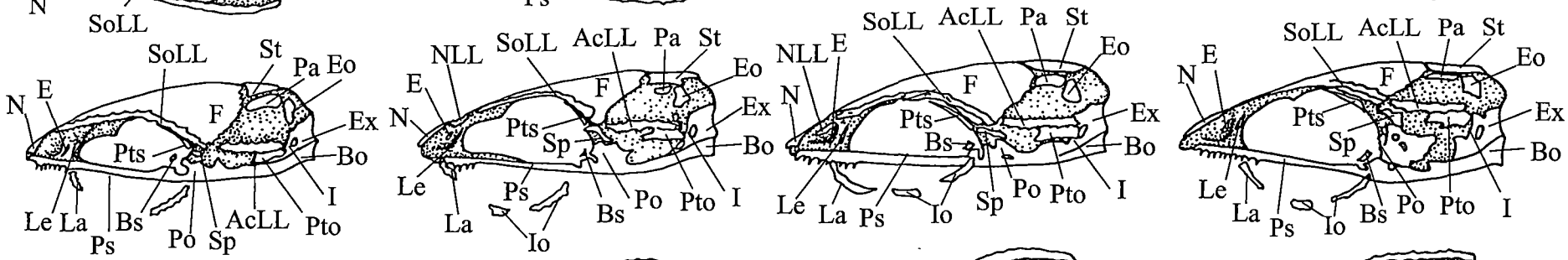
913° days

FD FT MD MT

a)



b)



c)

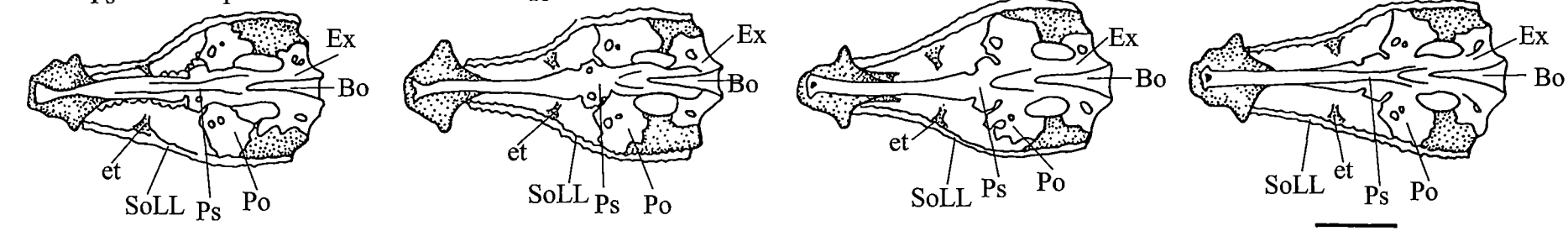
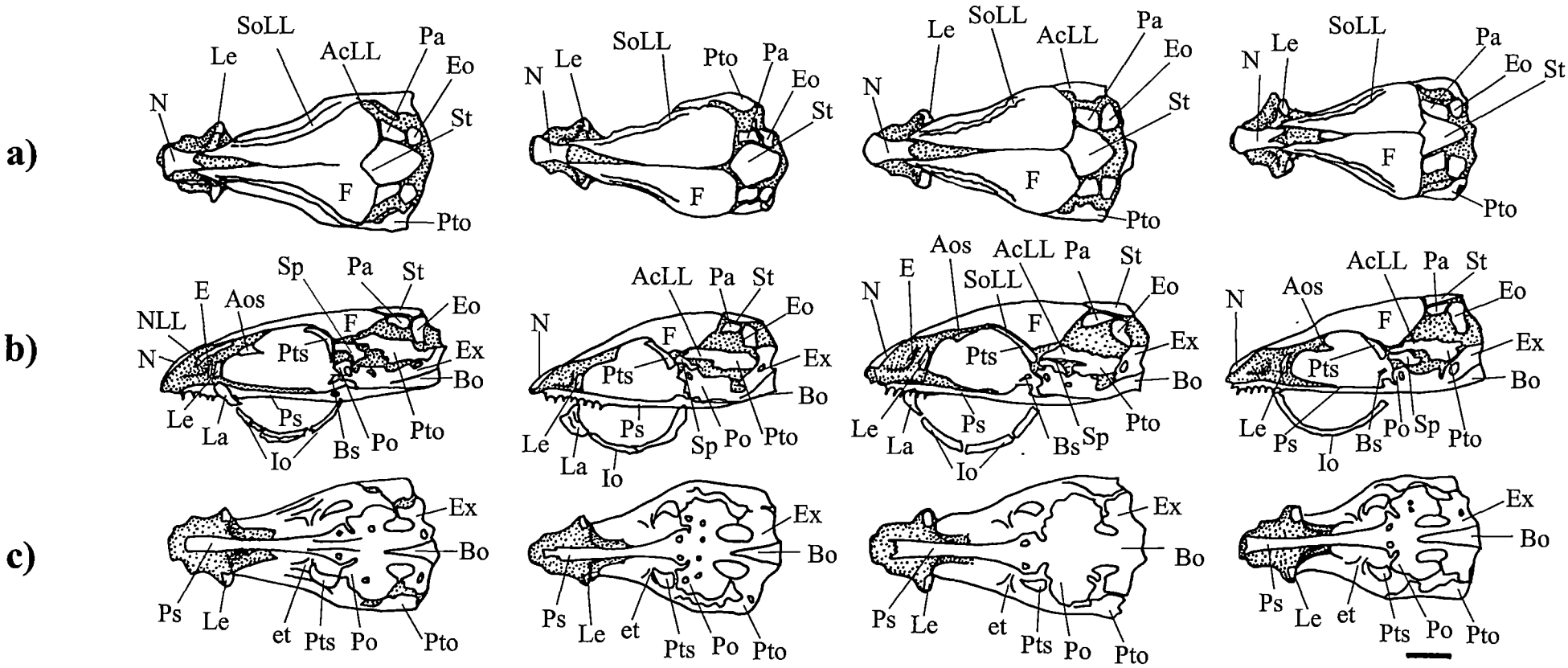


Figure 5. Camera lucida diagrams of a) the dorsal, b) lateral and c) ventral view of the cranial skeleton in stained and cleared (Taylor and Van Dyke, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at 12 weeks post-hatching (1510° days). Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: AcLL = auditory capsule lateral line, Aos = anterior orbitosphenoid, Bo = basioccipital, Bs = basisphenoid, E = ethmoid, Eo = epiotic, et = epiphysial tectum, Ex = exoccipital, F = frontal, Io = infraorbital bones, Le = lateral ethmoid, N = nasal, NLL = nasal lateral line, Pa = parietal, Po = prootic, Ps = parasphenoid, Pto = pterotic, Pts = pterosphenoid, S = sclerotic, SoLL = supraorbital lateral line, So = supraorbital, Sp = sphenotic, St = supratemporal. Scale bar = 3 mm.

1510° days


FD
FT
MD
MT
a)
b)
c)
N
Le
SoLL
AcLL
Pa
Eo
St
F
Pto
E
Aos
NLL
Sp
Pts
Ex
Bo
La
Ps
Io
Bs
Po
Sp
et

There were a few differences in the temporal differentiation of the cranial skeleton between fish of different ploidy and sex status (Fig. 6). The time at which the epiotic, pterotic, and sphenotic first appeared in all-female fish (706° days) preceded that in fish from the mixed sex populations (913° days). Furthermore, the parietal and pterosphenoid appeared in all-female triploid fish (706° days), before they appeared in the all-female diploid fish and fish from the mixed sex populations (913° days). Differences in skeletal ontogeny did not occur in conjunction with differences in weight or length at the same stage of development as the total wet weight and fork length of fish at 706° days was similar between populations (see section 2.4.4, Chapter 2).

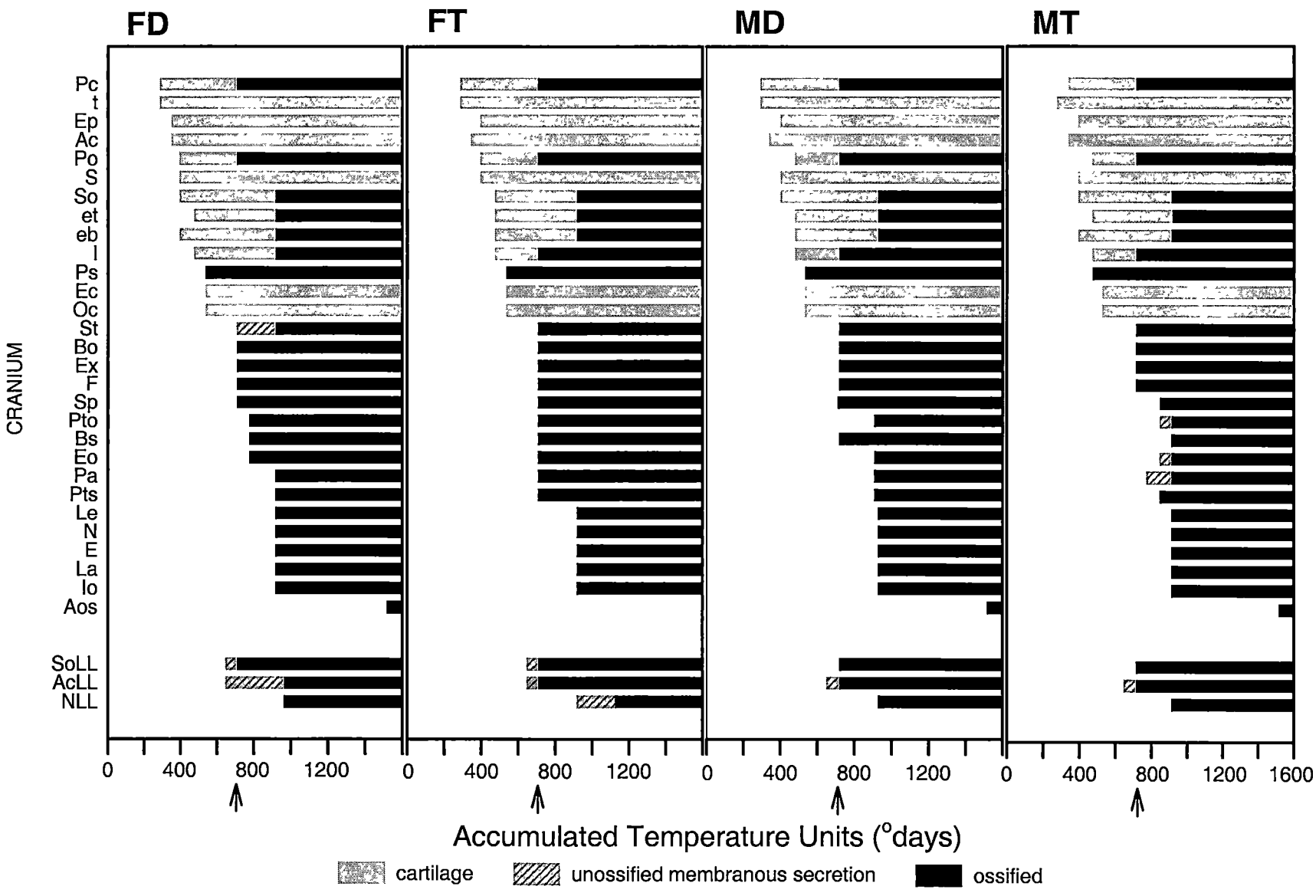
Figure 6. Ontogeny of each cartilage and bone element of the cranial skeleton of all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon populations, as defined by presence in more than 50% of fish examined (sample size as per Table 1). Stage of development is represented by accumulated temperature units post-fertilisation (ATU = ° days). Plain grey = initial appearance of cartilage, striped grey = initial appearance of structure formed by membranous secretion, black = ossification. Arrow indicates time of first feeding.

Abbreviations:

Ac	auditory capsule
Aos	anterior orbitosphenoid
Bo	basioccipital
Bs	basisphenoid
E	ethmoid
eb	ectethmoid bar
Ec	ethmoid cartilage
Eo	epiotic
Ep	ethmoid plate
et	epiphysial tectum
Ex	exoccipital
F	frontal
I	intercalar
Io	infraorbital bones/postorbital
La	lachrymal
Le	lateral ethmoid
N	nasal
no	notocord
Oc	olfactory capsule
Pa	parietal
Pc	parachordal
Po	prootic
Ps	parasphenoid
Pts	pterosphenoid
Pto	pterotic
S	sclerotic
So	supraorbital ca
Sp	sphenotic
St	supratemporal
t	trabecula

LATERAL LINE

AcLL	auditory capsule
NLL	nasal lateral line
SoLL	supraorbital lateral line


FD
FT
MD
MT
CRANIUM
Pc
t
Ep
Ac
Po
S
So
et
eb
I
Ps
Ec
Oc
St
Bo
Ex
F
Sp
Pto
Bs
Eo
Pa
Pts
Le
N
E
La
Io
Aos
SoLL
AcLL
NLL
0
400
800
1200
1600
Accumulated Temperature Units (°days)
cartilage
unossified membranous secretion
ossified

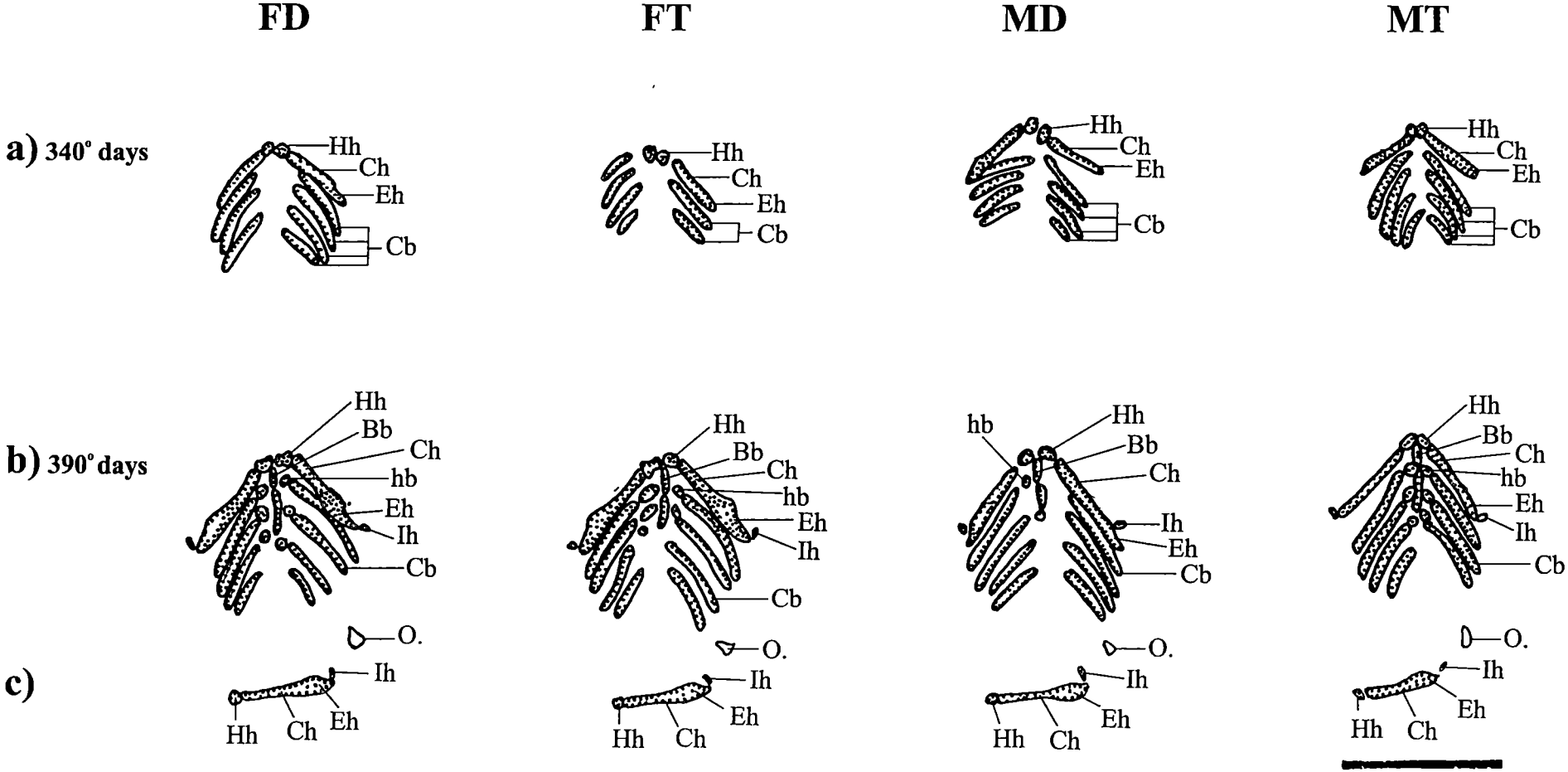
5.4.2 Ontogeny of the branchial apparatus, hyoid arch and opercula

The branchial elements initially appeared after the trabecula bone of the cephalic skeleton was present at **5 weeks post-fertilisation (280° days).** Only the all-female diploid fish had developed the ceratohyal, epihyal and hypohyal of the hyoid arch complex, or the ceratobranchial cartilages, at this time. Initially, the epihyal appeared at the posterior tip of the ceratohyal, differentiation of the two structures only becoming evident following ossification. The ceratobranchial elements developed sequentially towards the posterior end of the branchial chamber.

The bones associated with the hyoid complex and ceratobranchial elements did not appear in fish from the other populations until **6 weeks post-fertilisation (340° days, Fig. 7a),** concomitant with the appearance of the jaw suspensorium. With the exception of the all-female triploids, the full complement of ceratobranchials was evident in all fish, at this stage.

Seven weeks post-fertilisation (390° days, Fig. 7b), the basibranchial and hypobranchial cartilages started to develop from the anterior end of the branchial chamber in all populations. At this stage the interhyal cartilage of the hyoid arch was present. In addition, the opercula bone had started to develop at the posterior dorsal edge of the hyomandibular cartilage by membranous secretion within the epidermis (390° days, Fig. 7c).

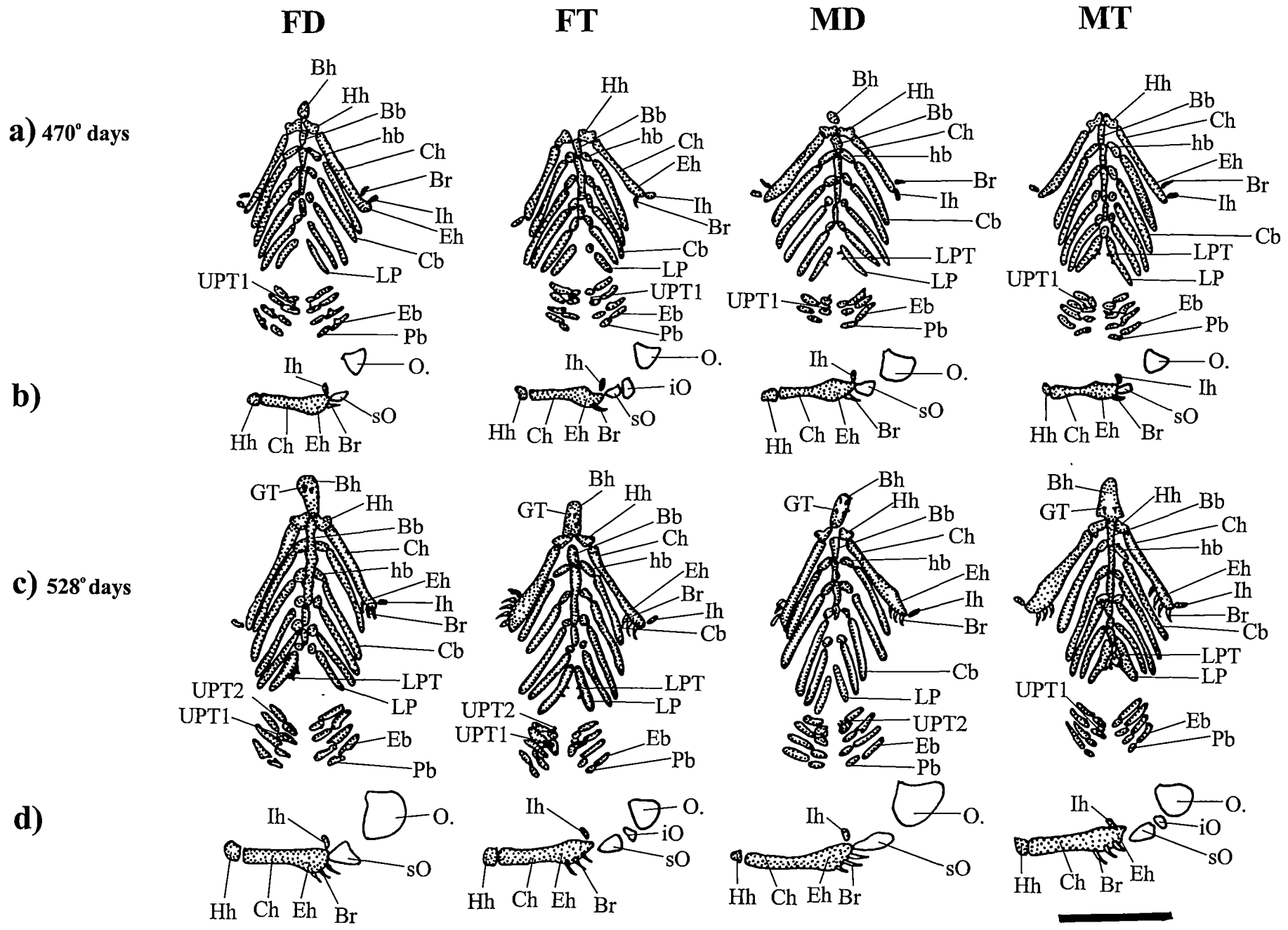
Figure 7. Camera lucida diagrams of a) the dorsal view of the branchial apparatus and hyoid arch at 6 weeks post-fertilisation (340° days), b) the dorsal view of the branchial apparatus and hyoid arch at 7 weeks post-fertilisation (390° days), and c) the lateral view of the hyoid arch and opercula at 7 weeks post fertilisation (390° days), dissected from stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon. Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: Bb = basibranchial, Cb = ceratobranchial, Ch = ceratohyal, Eh = epihyal, hb = hypobranchial, Hh = hypohyal, Ih = interhyal, O = opercula bone. Scale bar = 3 mm.


FD
FT
MD
MT
a) 340° days
b) 390° days
c)
Hh
Ch
Eh
Cb
Bb
hb
Ih
O.

The ceratobranchials and hyoid arch were fully delineated by **1 week post-hatching (470° days, Fig. 8a, b)** and the first branchiostegal ray was observed at the posterior tip of the epihyal. At this stage the epibranchial and pharyngobranchial cartilages were present in all fish, but the full complement of these bones was not present in mixed sex diploids (Fig. 8a). The epibranchials and pharyngobranchials developed sequentially starting at the posterior end of the branchial chamber. In fish from all populations, the lower pharyngeal had developed posterior to the 4 ceratobranchials and, in mixed sex populations had developed lower pharyngeal teeth on the 3rd pharyngobranchial cartilage (Upper pharyngeal teeth = UPT1) (Fig. 8a). The basihyal cartilage was present in mixed sex and all-female diploid fish at 470° days (Fig. 8a). In all fish the subopercula bone had started to form at the posterior tip of the epihyal and, in all-female triploid fish, the interopercula had also started to form at this stage (Fig. 8b).

The cartilage supports of primary gill filaments were present in fish **2 weeks post-hatching (528° days,** not shown in the diagram). Teeth were present on the basihyal, lower pharyngeal and 3rd and 4th pharyngobranchials in fish from all populations (Fig. 8c). The full complement of hypobranchial cartilages was present in fish from all populations and the full complement of basibranchial cartilages was present in mixed sex triploids, at this stage. The opercula and subopercula bones had increased in surface area and the mixed sex triploids had started to develop the interopercula bone (Fig. 8d). In addition, the dermal layers of the opercula were supported by three to four branchiostegal rays which extended from the posterior tip of the epihyal (Fig. 8d).

Figure 8. Camera lucida diagrams of a) the dorsal view of the branchial apparatus and hyoid arch at 1 week post-hatching (470° days), b) the lateral view of the hyoid arch and opercula at 1 week post-hatching (470° days), c) the dorsal view of the branchial apparatus and hyoid arch at 2 weeks post-hatching (528° days) and d) the lateral view of the hyoid arch and opercula at 2 weeks post-hatching (528° days), dissected from stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon. Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: Bb = basibranchial, Bh = basihyal, Br = branchiostegal rays, Cb = ceratobranchial, Ch = ceratohyal, Eb = epibranchial, Eh = epihyal, Gr = gill rakers, GT = glossohyal teeth, hb = hypobranchials, Hh = hypohyal, Ih = interhyal, iO = interopercula, LP = lower pharyngeal, LPT = lower pharangeal teeth, O = opercula bone, Pb = pharyngobranchial, pO = preopercula (see jaw Fig. 14 - 16), sO = subopercula, UPT1 = upper pharangeal teeth, UPT2 = upper pharyngeal teeth. Primary gill filaments not shown. Scale bar = 3 mm.


FD
FT
MD
MT
a) 470° days
b)
c) 528° days
d)
Bh
Hh
Bb
hb
Ch
Eh
Br
Ih
Cb
LP
LPT
UPT1
UPT2
Eb
Pb
O.
iO
sO
GT
Hh Ch Eh Br

Ossification of the ceratohyal, epihyal, and lower section of the hypohyal cartilage had started in fish from all populations by **5 weeks post-hatching (706° days, Fig. 9b)**. In addition, the glossohyal and urohyal bones had started to form by membranous secretion. The isolated basibranchial cartilage and the medial gular bone were evident in all fish at this stage (Fig. 9a); the latter formed by membranous secretion. The ceratobranchials had developed gill rakers and the full complement of branchiostegal rays was present, regardless of ploidy or sex status (Fig. 9a). At this stage a dental plate was present between the junction of the fourth epibranchial and pharyngobranchial cartilages in all fish and, with the exception of the mixed sex triploids, the dental plate of the glossohyal was well delineated (Fig. 9a). The upper section of the hypohyal, the ceratobranchials, the lower pharyngeal, and the first two basibranchial and hypobranchial cartilages had started to ossify in the all-female triploids and the mixed sex diploids by 706° days (Fig. 9). In the all-female triploids, the epibranchials had also started to ossify (Fig. 9a). The preopercula had started to ossify in fish from all populations, as shown in the diagrams of the jaw suspensorium (Figures 14 - 16). The preopercula was not shown in the present branchial figures to avoid obscuring other structures. At this stage the interopercula and up to 10 branchiostegal rays were present in fish from all populations adjoining the preopercula, subopercula and opercula bones to form a continuous sheet of dermal armour (Fig. 9).

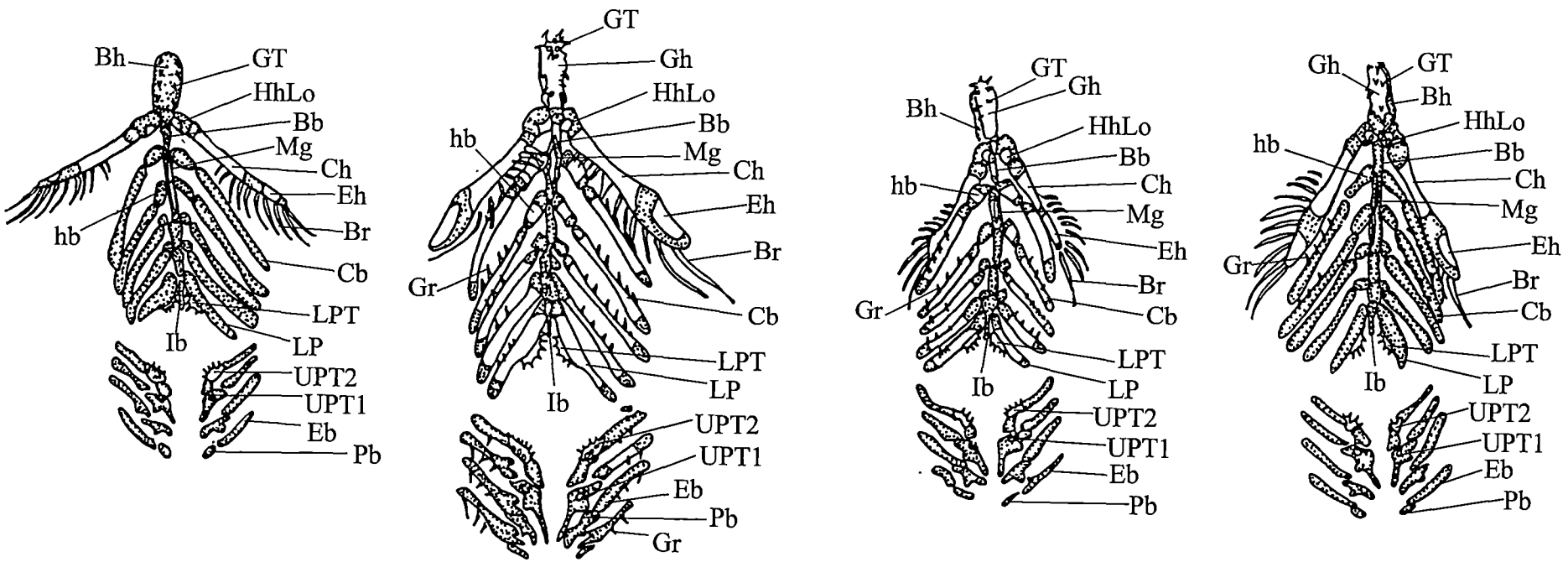
At 8 weeks post-hatching (913° days, Fig. 10), the interhyal cartilage as well as the first two basibranchial and hypobranchial cartilages were ossified in fish from all populations. At this stage the epibranchial cartilages and both the upper and lower surfaces of the hypohyal were ossified in fish from all populations. The third pharyngobranchial was ossified in the mixed sex diploid and mixed sex triploid fish. The preopercula, subopercula, interopercula and opercula bones were well delineated and had increased in area.

Figure 9. Camera lucida diagrams of a) the dorsal view of the branchial apparatus and hyoid arch, and b) the lateral view of the hyoid arch and opercula dissected from stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at 5 weeks post-hatching (706° days). Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: Bb = basibranchial, Bh = basihyal, Br = branchiostegal rays, Cb = ceratobranchial, Ch = ceratohyal, Eb = epibranchial, Eh = epihyal, Gh = glossohyal, Gr = gill rakers, GT = glossohyal teeth, hb = hypobranchials, Hh = hypohyal, HhLo = hypohyal lower, Ib = isolated basibranchial, Ih = interhyal, iO = interopercula, LP = lower pharyngeal, LPT = lower pharangeal teeth, Mg = medial gular, O = opercula bone, Pb = pharyngobranchial, Pf = primary filaments, pO = preopercula (see jaw Fig. 14 - 16), sO = subopercula, Uh = urohyal, UPT1 = upper pharangeal teeth, UPT2 = upper pharyngeal teeth. Primary gill filaments not shown. Scale bar = 3 mm.

706° days

FD FT MD MT

a)



b)

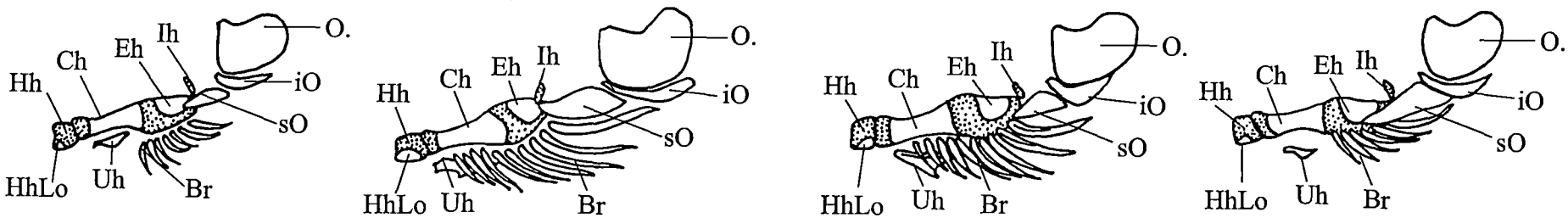
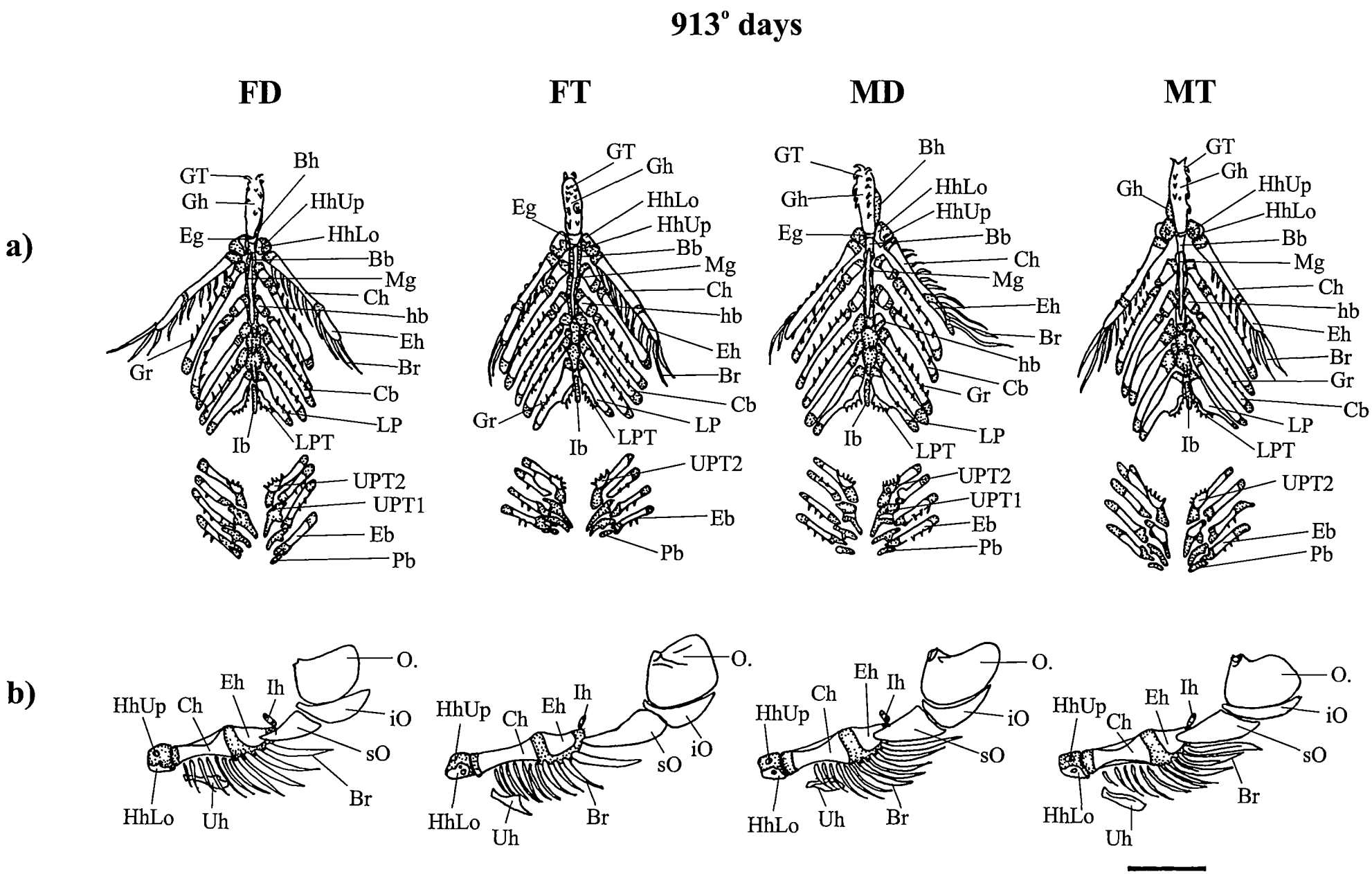


Figure 10. Camera lucida diagrams of a) the dorsal view of the branchial apparatus and hyoid arch, and b) the lateral view of the hyoid arch and opercula dissected from stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at 8 weeks post-hatching (913° days). Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: Bb = basibranchial, Bh = basihyal, Br = branchiostegal rays, Cb = ceratobranchial, Ch = ceratohyal, Eb = epibranchial, Eg = entoglossus, Eh = epihyal, Gh = glossohyal, Gr = gill rakers, GT = glossohyal teeth, hb = hypobranchials, HhLo = hypohyal lower, HhUp = hypohyal upper, Ib = isolated basibranchial, Ih = interhyal, iO = interopercula, LP = lower pharyngeal, LPT = lower pharangeal teeth, Mg = medial gular, O = opercula bone, Pb = pharyngobranchial, pO = preopercula (see jaw Fig. 14 - 16), sO = subopercula, Uh = urohyal, UPT1 = upper pharangeal teeth, UPT2 = upper pharyngeal teeth. Primary gill filaments not shown. Scale bar = 3 mm.


913° days
FD
FT
MD
MT
a)
b)
GT
Gh
Bh
HhUp
HhLo
Eg
Bb
Mg
Ch
hb
Eh
Br
Gr
Cb
LP
Ib
LPT
UPT2
UPT1
Eb
Pb
O.
iO
sO
Ih
Uh

By 12 weeks post-hatching (1510° days, Fig. 11), the first three basibranchials, first three hypobranchials and the pharyngobranchials were ossified in fish from all populations, irrespective of ploidy or sex status. All bones had doubled in size since 913° days; a period of 4 weeks.

There was little difference in the timing of the development of the branchial apparatus and hyoid arch of fish of different ploidy or sex status (Fig. 12) with the exception that the ceratobranchials, basibranchials, hypobranchials and epibranchials of the all-female triploid fish, and the ceratobranchials of the mixed sex diploid fish started to ossify by 706° days, prior to those of fish from other populations (913° days).

Figure 11. Camera lucida diagrams of a) the dorsal view of the branchial apparatus and hyoid arch, and b) the lateral view of the hyoid arch and opercula dissected from stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at 12 weeks post-hatching (1510° days). Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: Bb = basibranchial, Bh = basihyal, Br = branchiostegal rays, Cb = ceratobranchial, Ch = ceratohyal, Eb = epibranchial, Eg = entoglossus, Eh = epihyal, Gh = glossohyal, Gr = gill rakers, GT = glossohyal teeth, hb = hypobranchials, Hh = hypohyal, HhLo = hypohyal lower, HhUp = hypohyal upper, Ib = isolated basibranchial, Ih = interhyal, iO = interopercula, LP = lower pharyngeal, LPT = lower pharangeal teeth, Mg = medial gular, O = opercula bone, Pb = pharyngobranchial, pO = preopercula (see jaw Fig. 14 - 16), sO = subopercula, Uh = urohyal, UPT1 = upper pharangeal teeth, UPT2 = upper pharyngeal teeth. Primary gill filaments and branchiostegal rays not shown. Scale bar = 3 mm.

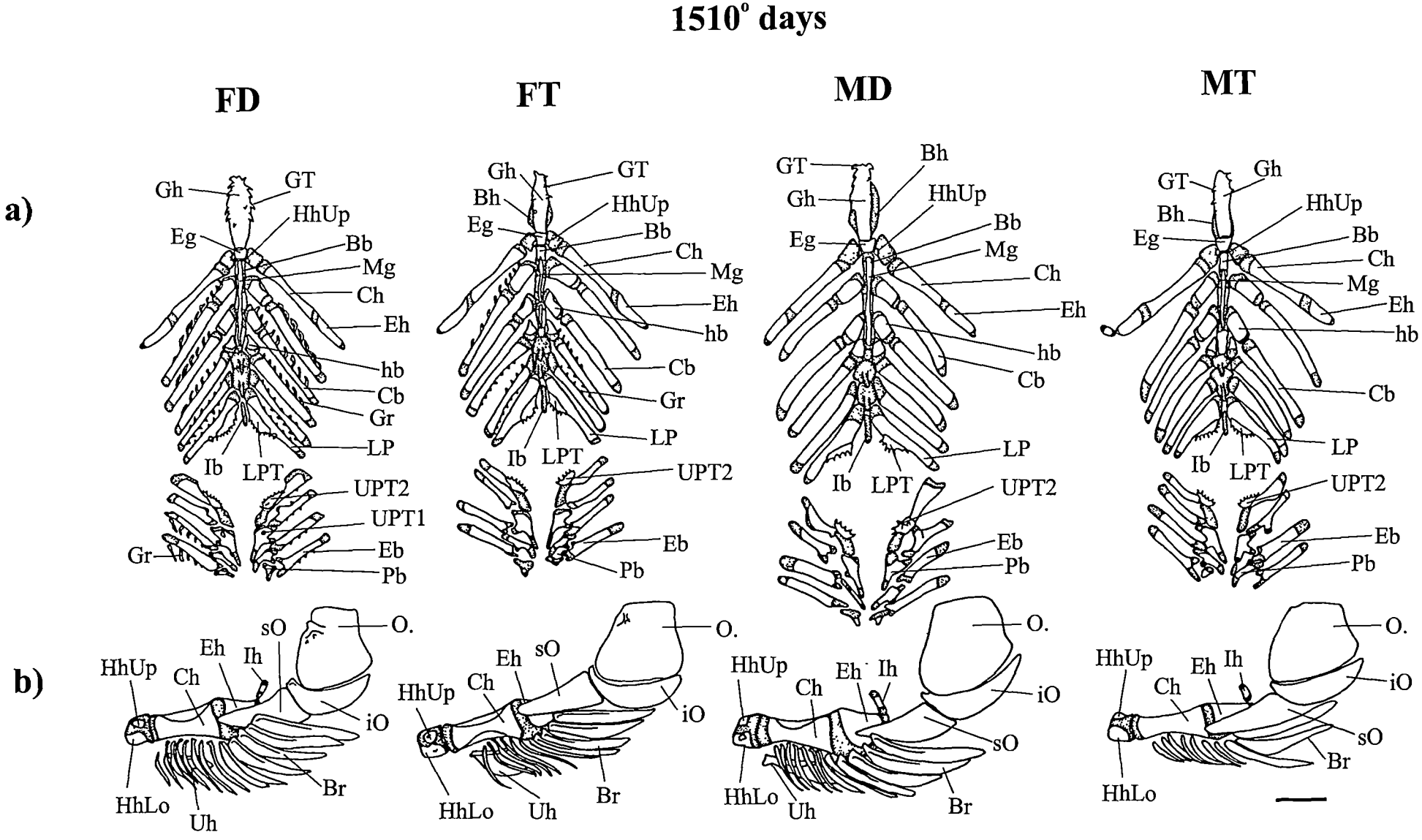
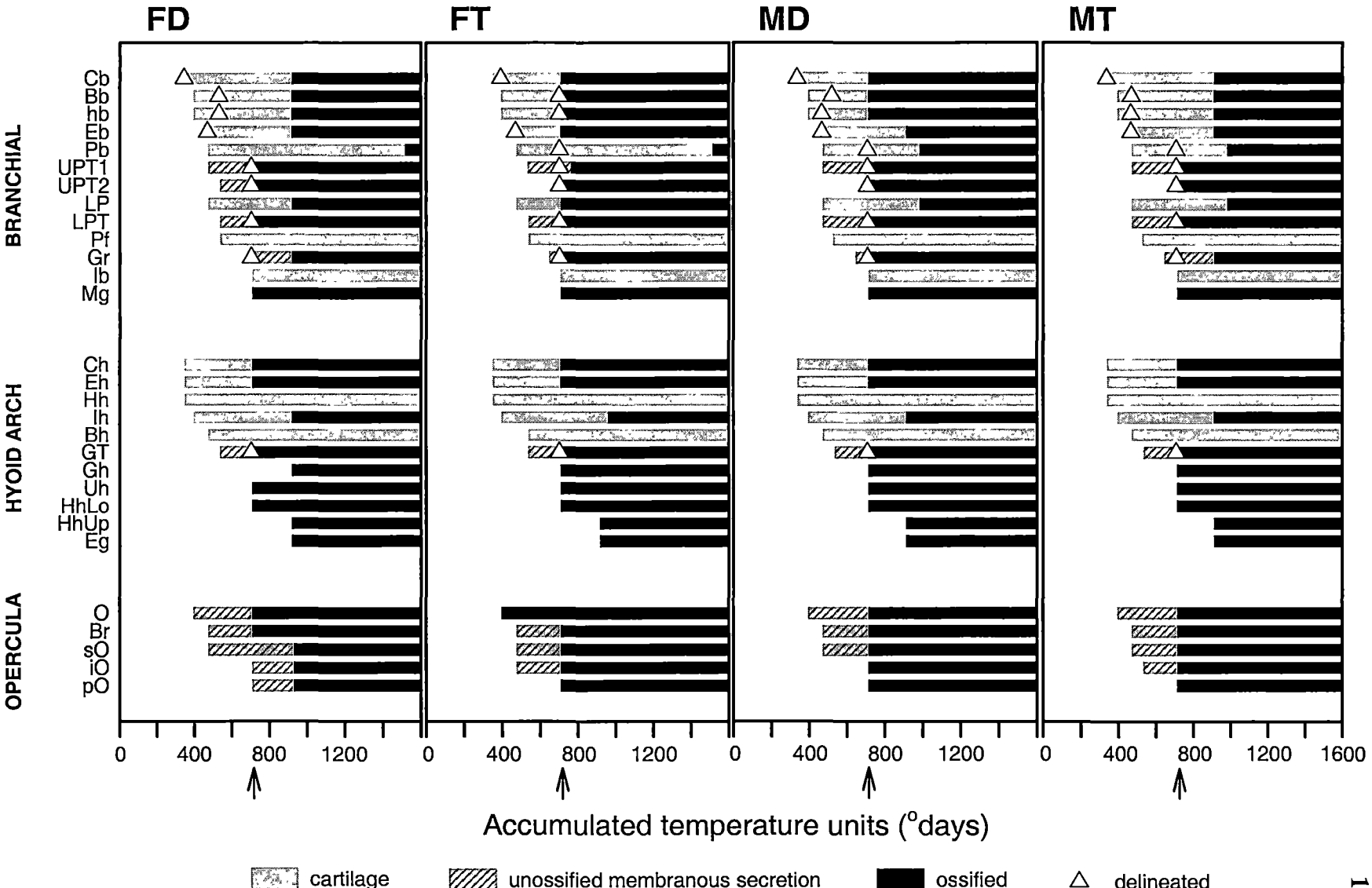

1510° days
FD
FT
MD
MT
a)
b)
Gh
GT
HhUp
Eg
Bb
Mg
Ch
Eh
hb
Cb
Gr
LP
Ib
LPT
UPT2
UPT1
Eb
Pb
Bh
sO
O.
iO
HhLo
Uh
Br

Figure 12. Ontogeny of each cartilage and bone element of the branchial apparatus, hyoid arch and opercula of all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon populations as defined by presence in more than 50% of the fish examined (sample size as per Table 1). Stage of development is represented by accumulated temperature units post-fertilisation (ATU = ° days). Plain grey = initial appearance of cartilage, striped grey = initial appearance of structure formed by membranous secretion, black = ossification, triangle = structure delineated in shape and/or number. Arrow indicates time of first feeding.

Abbreviations:

	BRANCHIAL APPARATUS		HYOID ARCH		OPERCULA
Bb	basibranchials	Bh	basihyal	O	opercula bone
Cb	ceratobranchial	Br	branchiostegal rays	iO	interopercula
Eb	epibranchial	Ch	ceratohyal	sO	subopercula
Gr	gill rakers	Eg	enotglossus	pO	preopercula (see jaw Fig. 14 - 16)
hb	hypobranchials	Eh	epihyal		
Ib	isolated basibranchial	GT	gloss teeth		
LPT	lower pharangeal teeth	Gh	glossohyal		
LP	lower pharyngeal	Hh	hypohyals		
Mg	medial gular	HhLo	hypohyal lower		
Pb	pharyngobranchial	HhUp	hypohyal upper		
Pf	primary filaments	Ih	interhyal		
UPT1	upper pharangeal teeth	Uh	urohyal		
UPT2	upper pharyngeal teeth				


FD
FT
MD
MT
BRANCHIAL
HYOID ARCH
OPERCULA
Cb
Bb
hb
Eb
Pb
UPT1
UPT2
LP
LPT
Pf
Gr
Ib
Mg
Ch
Eh
Hh
Ih
Bh
GT
Gh
Uh
HhLo
HhUp
Eg
O
Br
sO
iO
pO
0
400
800
1200
1600
Accumulated temperature units (°days)
cartilage
unossified membranous secretion
ossified
delineated

5.4.3 Ontogeny of the lower jaw, jaw suspensorium and upper jaw

The Meckel's cartilage of the lower jaw was formed in fish from all populations by **340° days (Fig. 13a)**, following the development of the trabecula of the cranial skeleton. In all but the all-female diploid population, the maxilla and the dentary bones had started to form by membranous secretion at this stage (Fig. 13a). In addition, the hyomandibular and metapterygoid-quadrate cartilages were present in all populations (Fig. 13a). The maxilla and dentary bones were observed in all-female diploid fish at **390° days** (Fig. 13b). **By 470° days** teeth had developed on the maxilla and the dentary bones of all fish, regardless of ploidy or sex status (Fig. 13c). In addition, the metapterygoid-quadrate cartilage had developed into a plate-like structure with an anterior process (Fig. 13c). The premaxilla was present in all fish by **528° days** (Fig. 13d).

By five weeks post-hatching (**706° days, Fig. 14**), the hyomandibular cartilage had differentiated into the symplectic and hyomandibular bones in fish from all populations. The hyomandibular had developed an anterior process by membranous secretion which extended to the metapterygoid-quadrate plate. The metapterygoid and quadrate bones were now ossified. A process extended posteriorly from the quadrate to support the symplectic. The anterior process of the metapterygoid-quadrate plate had differentiated into the pterygoid, consisting of the endopterygoid and the ectopterygoid bones. The palantine cartilage and dermal plate with teeth appeared as an anterior extension of the pterygoid. The dentary bone had fused with the lower jaw lateral line. In addition, the angular bone had formed by membranous secretion at the lateral surface of the Meckel's cartilage. A retroarticular bone was fused to the posterior tip of the angular bone in all fish at this stage.

The supramaxilla was evident in fish from all populations by **913° days (Fig. 15).** During the 4 weeks preceding **1510° days**, all bones of the jaw and jaw suspensorium had doubled in size (Fig. 16). The angular bone had increased in dorso-ventral depth over this time (Fig. 16).

Figure 13. Camera lucida diagrams of the lateral view of the lower jaw, upper jaw and jaw suspensorium dissected from stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at a) 6 weeks post-fertilisation (wpf) (340° days), b) 7 wpf (390° days), c) 1 week post-hatching (wph) (470° days) and d) 2 wph (528° days). Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: D = dentary, DT = dentary teeth, Hm = hyomandibular, M = maxilla, mc = Meckel's cartilage, Mt = maxilla teeth, mq = metapterygoid-quadrate plate, pm = premaxilla and teeth. Scale bar = 3 mm.

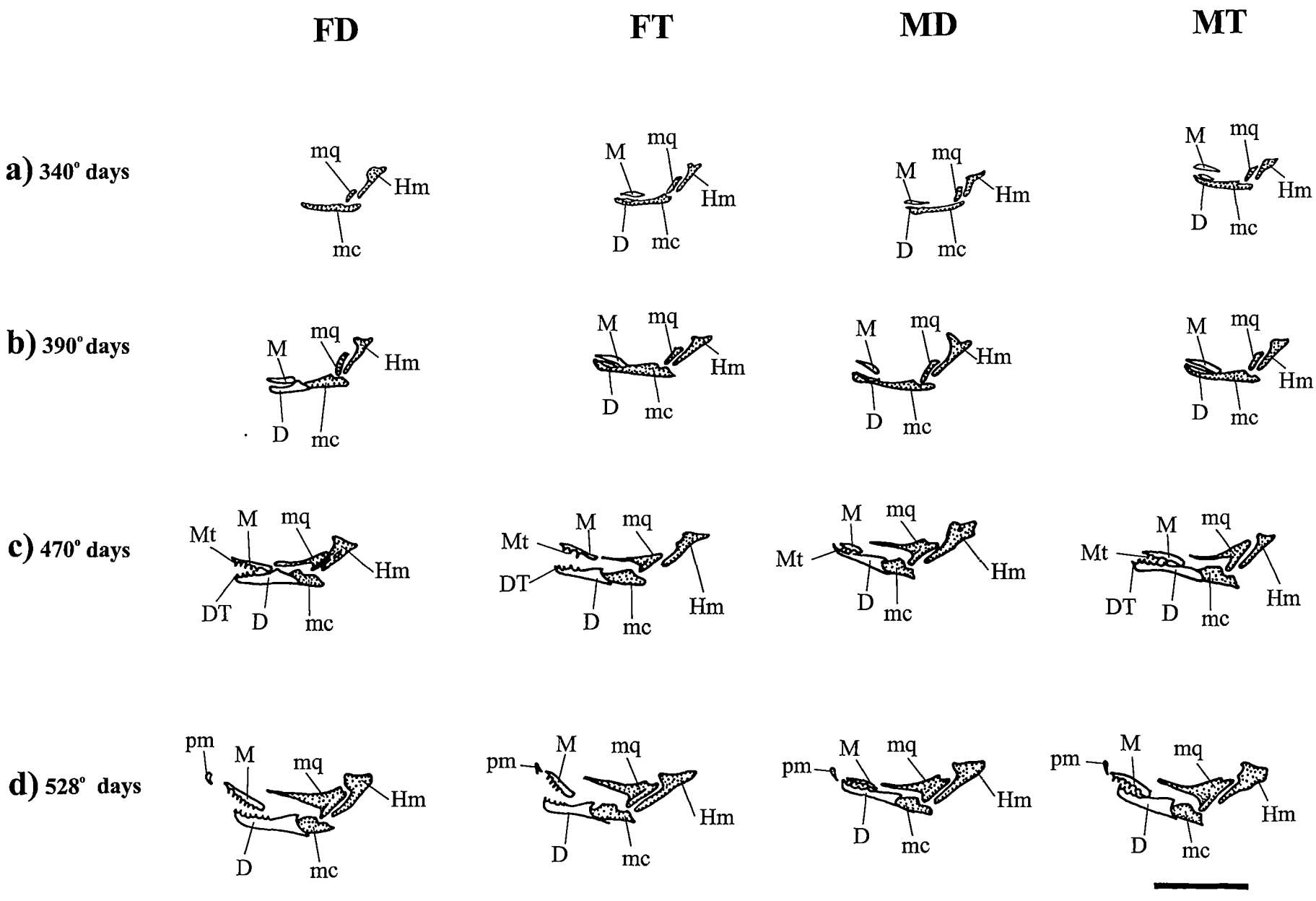
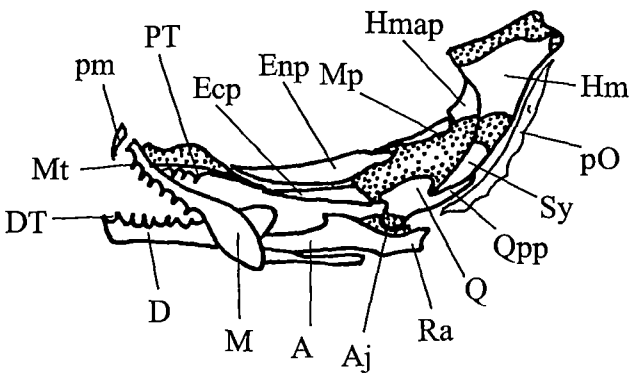

FD
FT
MD
MT
a) 340° days
b) 390° days
c) 470° days
d) 528° days
pm
M
mq
Hm
Mt
DT
D
mc

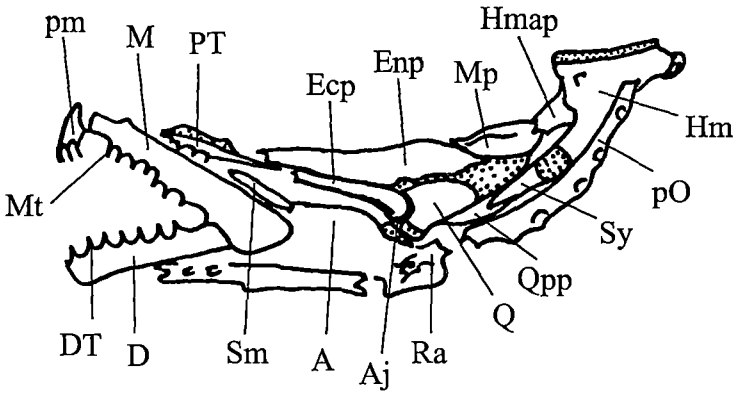
Figure 14. Camera lucida diagrams of the lateral view of the lower jaw, upper jaw and jaw suspensorium dissected from stained and cleared cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at 5 weeks (706° days) post-hatching. Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: A = angular, Aj = articulation joint, D = dentary, DT = dentary teeth, Ecp = ectopterygoid, Enp = endopterygoid, Hmap = hyomandibular anterior process, Hm = hyomandibular, M = maxilla, mc = Meckel's cartilage, Mt = maxilla teeth, mq = metapterygoid-quadrate plate, Mp = metapterygoid, pm = premaxilla and teeth, pO = preopercula, PT = palantine plus teeth, Qpp = quadrate posterior process, Q = quadrate, Ra = retroarticular, Sy = symplectic. Note: Preopercula bone shown in association with jaw suspensorium rather than with the opercula bones in Fig. 7 - 11, so as not to obscure details of the latter. Scale bar = 3 mm.

706° days

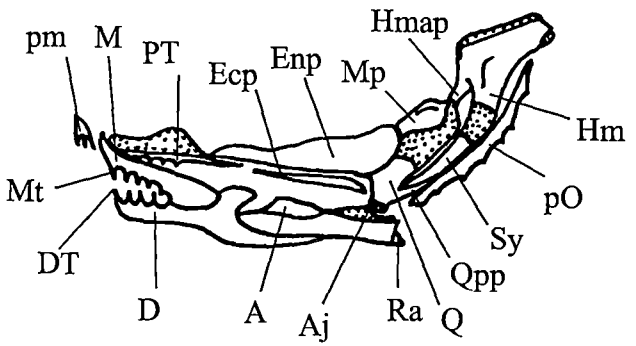
FD



FT



MD



MT

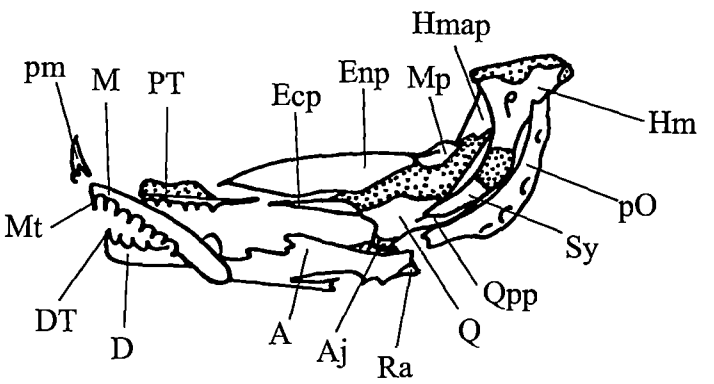
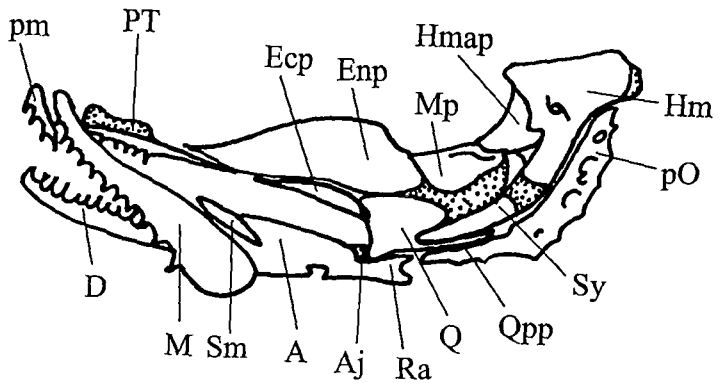


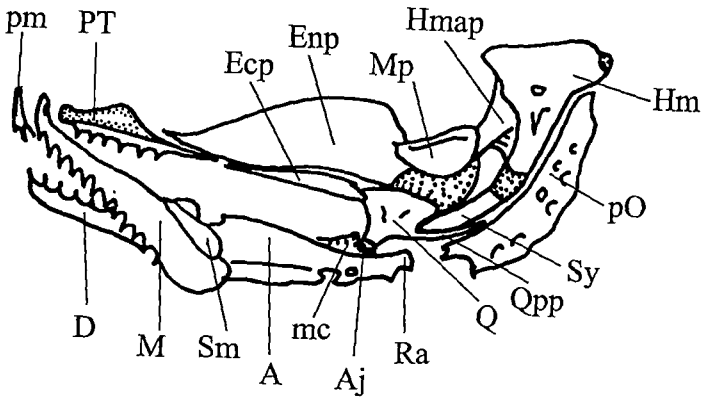
Figure 15. Camera lucida diagrams of the lateral view of the lower jaw, upper jaw and jaw suspensorium dissected from stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at 8 weeks (913° days) post-hatching. Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: A = angular, Aj = articulation joint, D = dentary, Ecp = ectopterygoid, Enp = endopterygoid, Hmap = hyomandibular anterior process, Hm = hyomandibular, M = maxilla, mc = Meckel's cartilage, mq = metapterygoid-quadrate plate, Mp = metapterygoid, pm = premaxilla and teeth, pO = preopercula, PT = palantine plus teeth, Qpp = quadrate posterior process, Q = quadrate, Ra = retroarticular, Sm = supramaxilla, Sy = symplectic. Note: Preopercula bone shown in association with jaw suspensorium rather than with the opercula bones in Fig. 7 - 11, so as not to obscure details of the latter. Scale bar = 3 mm.

913° days

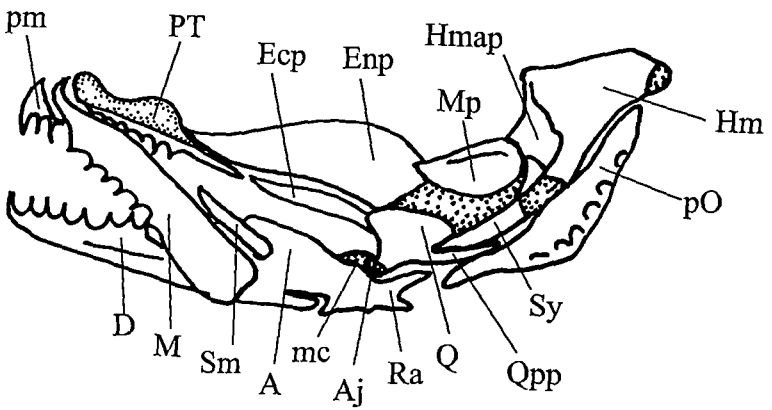
FD



FT



MD



MT

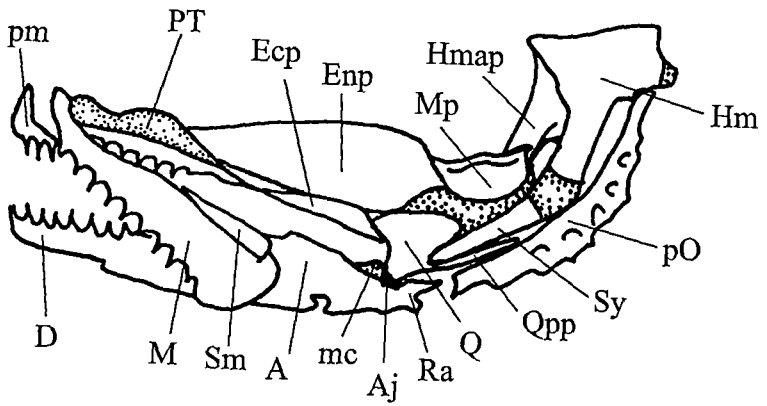
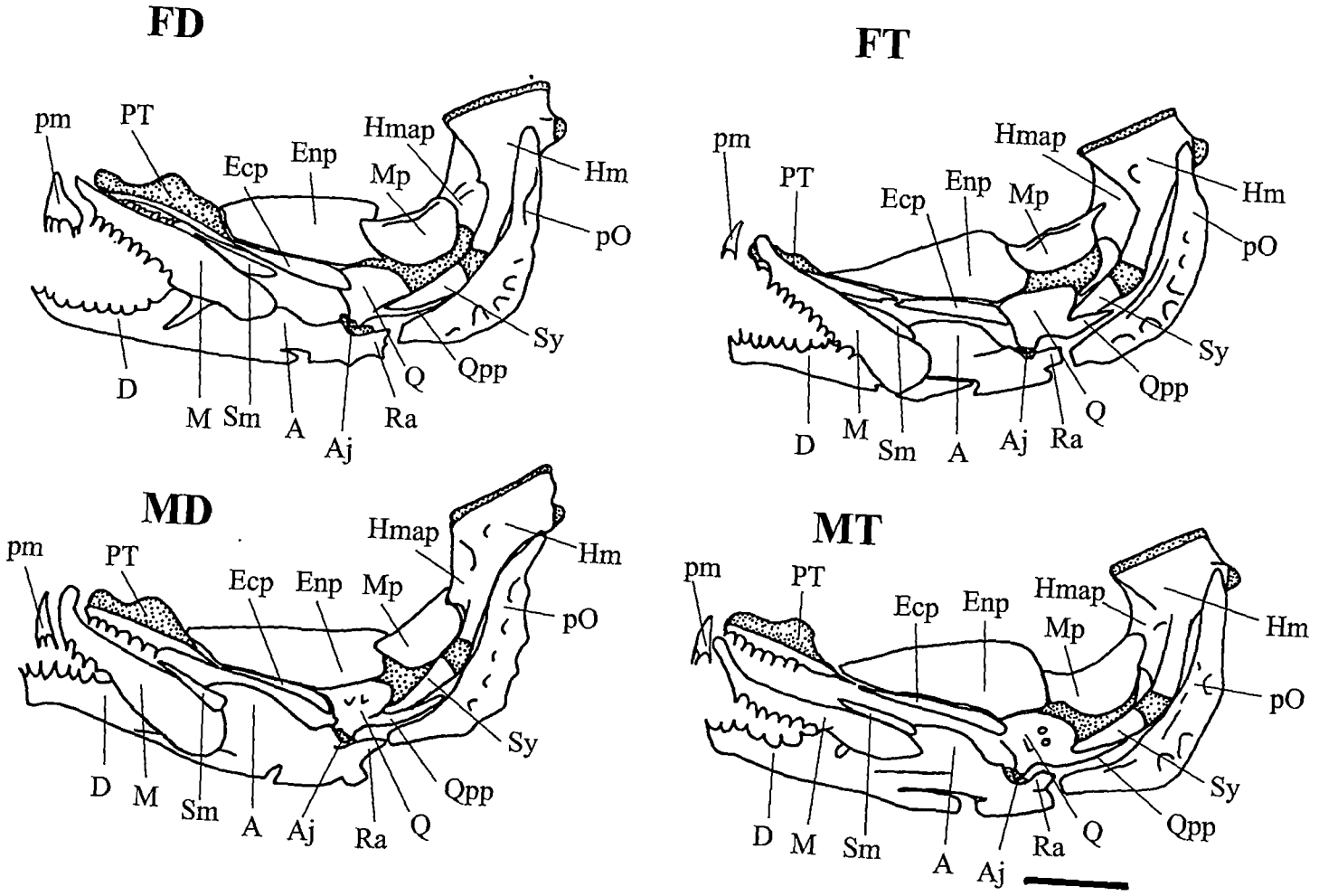


Figure 16. Camera lucida diagrams of the lateral view of the lower jaw, upper jaw and jaw suspensorium dissected from stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at 12 weeks (1510° days) post-hatching. Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: A = angular, Aj = articulation joint, D = dentary, Ecp = ectopterygoid, Enp = endopterygoid, Hmap = hyomandibular anterior process, Hm = hyomandibular, M = maxilla, mc = Meckel's cartilage, mq = metapterygoid-quadrate plate, Mp = metapterygoid, pm = premaxilla and teeth, pO = preopercula, PT = palantine plus teeth, Qpp = quadrate posterior process, Q = quadrate, Ra = retroarticular, Sm = supramaxilla, Sy = symplectic. Note: Preopercula bone shown in association with jaw suspensorium rather than with the opercula bones in Fig. 7 - 11, so as not to obscure details of the latter. Scale bar = 3 mm.

1510° days


FD
pm
PT
Ecp
Enp
Mp
Hmap
Hm
pO
Sy
Qpp
Q
Ra
Aj
A
Sm
M
D
FT
pm
PT
Ecp
Enp
Mp
Hmap
Hm
pO
Sy
Qpp
Q
Ra
Aj
A
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D
MD
pm
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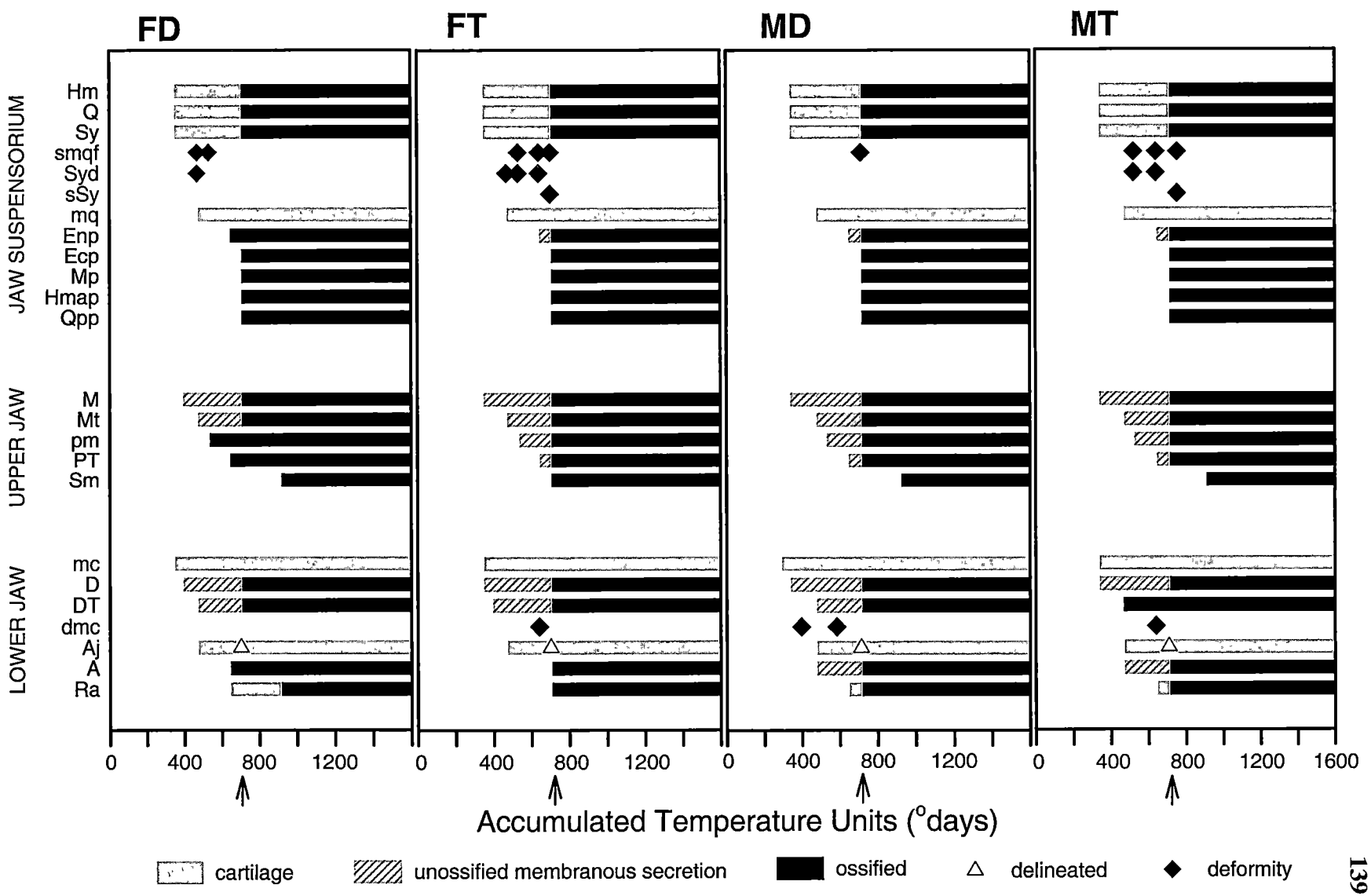
Enp
Mp
Hmap
Hm
pO
Sy
Qpp
Q
Ra
Aj
A
Sm
M
D
MT
pm
PT
Ecp
Enp
Mp
Hmap
Hm
pO
Sy
Qpp
Q
Ra
Aj
A
Sm
M
D

Generally, there was little to no difference in the timing of skeletal development of the lower jaw, upper jaw and jaw suspensorium in Atlantic salmon despite differences in ploidy and sex status (Fig. 17). The supramaxilla bone appeared at an earlier stage of development in the all-female triploid fish (706° days) compared to fish from the all-female diploid, mixed sex diploid and mixed sex triploid populations (913° days), but this was not consistent between fish of different ploidy or sex status (Fig. 17). There were a number of deformities detected in the jaw and jaw suspensorium of both diploid and triploid populations (Fig. 17), the morphology of which will be described in the next section (section 5.3.4). The incidence of these deformities was greater in triploid populations compared to diploid populations (Fig. 17).

Figure 17. Ontogeny of each cartilage and bone element of the lower jaw, upper jaw and jaw suspensorium of all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon as defined by the presence of the skeletal element in more than 50% of the fish examined (sample sizes as per Table 1). Stage of development is represented by accumulated temperature units post-fertilisation (ATU = ° days). Plain grey = initial appearance of cartilage structure, striped grey = initial appearance of structure formed by membranous secretion, black = ossification, white triangle = structure delineated in shape and/or number, black diamond = deformity observed. Arrow indicates time of first feeding.

Abbreviations:

	SUSPENSORIUM		UPPER JAW		LOWER JAW
Ecp	ectopterygoid	Mt	maxilla teeth	A	angular
Enp	endopterygoid	M	maxilla	Aj	articulation joint
Hmap	hyomandibular anterior process	PT	palantine plus teeth	dmc	deformed Meckel's cartilage
Hm	hyomandibular	pm	premaxilla and teeth	D	dentary
mq	metapterygoid-quadrate plate	Sm	supramaxilla	DT	dentary teeth
Mp	metapterygoid			mc	Meckel's cartilage
pO	preopercula			Ra	retroarticular
Qpp	quadrate posterior process				
Q	quadrate				
smqf	symplectic and metapterygoid-quadrate plate fused				
Syd	symplectic divided				
sSy	symplectic short				
Sy	symplectic				


FD
FT
MD
MT
JAW SUSPENSORIUM
Hm
Q
Sy
smqf
Syd
sSy
mq
Enp
Ecp
Mp
Hmap
Qpp
UPPER JAW
M
Mt
pm
PT
Sm
LOWER JAW
mc
D
DT
dmc
Aj
A
Ra
0
400
800
1200
1600
Accumulated Temperature Units (°days)
cartilage
unossified membranous secretion
ossified
delineated
deformity

5.4.4 Deformities of the lower jaw and jaw suspensorium

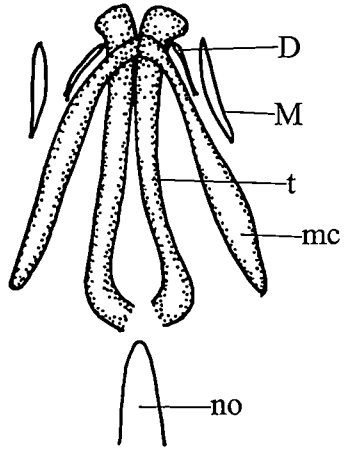
One in ten fish sampled from the all-female triploid, mixed sex triploid and mixed sex diploid populations between 340° and 642° days displayed abnormality of the Meckel's cartilage. A fish from the mixed sex diploid population sampled at 340° days, displayed irregular development of the Meckel's cartilage and dentary bones (Fig. 18b). Another mixed sex diploid fish sampled at 280° days, had a break in the Meckel's cartilage on one side of the body (Fig. 18c). In this fish the edges of the Meckel's cartilage on the right hand side of the body had patchy staining with alcian blue, irregular cartilage cell density, and showed symptoms of hyperplasia at the point of the break (Fig. 18c), whereas the Meckel's cartilage from the left hand side of the body appeared shorter and medially thicker with homogenous in cell structure (Fig. 18c). The dentary bones on each side of the body were different sizes in this fish, further indicating assymetrical development (Fig. 18c). One mixed sex triploid fish sampled at 280° days displayed abnormal dorso-ventral thickening of the Meckel's cartilage on one side of the body (Fig. 18d).

Up to fifty percent of fish sampled from each population between 340° and 772° days displayed abnormal fusion of the hyomandibular/symplectic to the metapterygoid-quadrate plate (Fig. 19b). In these fish the anterior medial edge of the hyomandibular/symplectic was fused to the median posterior edge of the metapterygoid-quadrate plate. In some cases this occurrence was coupled with the detachment of the symplectic from the hyomandibular or a division of the symplectic into two parts (= divided symplectic) (Fig. 20b). The fusion of the hyomandibular/symplectic to the metapterygoid-quadrate plate occurred in 50% of all-female diploids, 30% of mixed sex triploids and 20% of all-female triploids sampled at 470° days. Ten to twenty percent of these fish had a divided symplectic. In addition, 60% of mixed sex triploids and 10% of the all-female triploids sampled at 528° days had a symplectic fused with the metapterygoid-quadrate plate and of these 10% of fish had a divided symplectic.

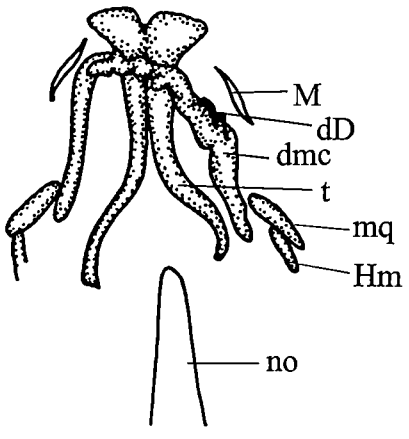
A small proportion of fish sampled from the all-female diploid, all-female triploid and mixed sex triploid populations, had a break in the symplectic bone (Fig. 20b), whereas other all-female triploid and mixed sex triploid fish sampled at 706° days and 772° days, respectively, had relatively short symplectic bones (Fig. 20c).

Figure 18. Camera lucida diagrams of the dorsal view of the lower jaw, upper jaw and cranial skeleton of stained and cleared (Taylor and Van Dyke, 1985, Appendix D) mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon with a) normal dentary bones and Meckel's cartilage, b) deformed dentary bones and deformed Meckel's cartilage, c) & d) deformed Meckel's cartilage. Stage of development is represented by accumulated temperature units post-fertilisation (ATU = ° days). Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: D = dentary, dD = deformed dentary bone, dmc = deformed Meckel's cartilage, Hm = hyomandibular, M = maxilla, mc = Meckel's cartilage, mq = metapterygoid-quadrate cartilage plate, no = notochord, t = trabecula. Scale bar = 1.5 mm.

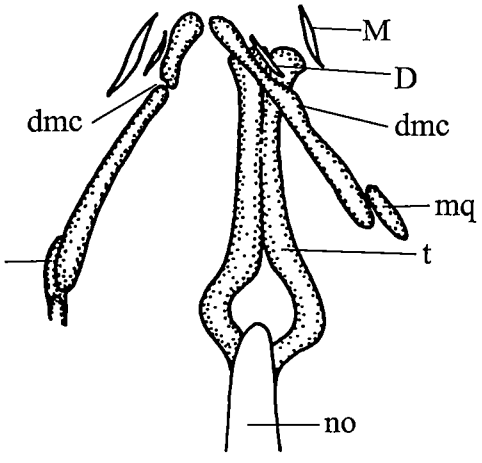
a) MT (280° days)



b) MD (340° days)



c) MD (280° days)



d) MT (280° days)

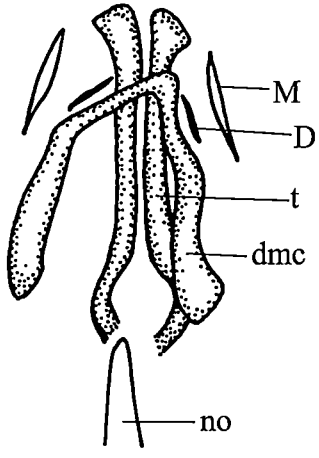
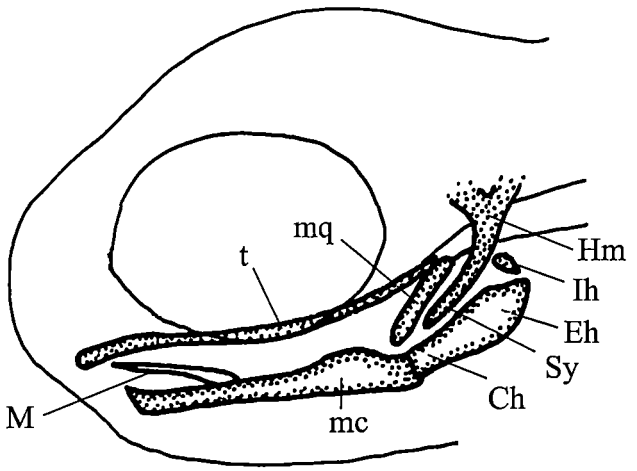


Figure 19. Camera lucida diagrams of the lateral view of the cranium, jaw suspensorium, lower jaw and hyoid arch of stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid Atlantic salmon (340° days) with a) a normal symplectic and metapterygoid-quadrate cartilage plate and b) a fused symplectic and metapterygoid-quadrate cartilage. Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: Ch = ceratohyal, Eh = epihyal, Hm = hyomandibular, Ih = interhyal, M = maxilla, mc = Meckel's cartilage, mq = metapterygoid-quadrate cartilage plate, smqf = symplectic and metapterygoid quadrate plate fused, Sy = symplectic, t = trabecula. Scale bar = 1.5 mm.

a)



b)

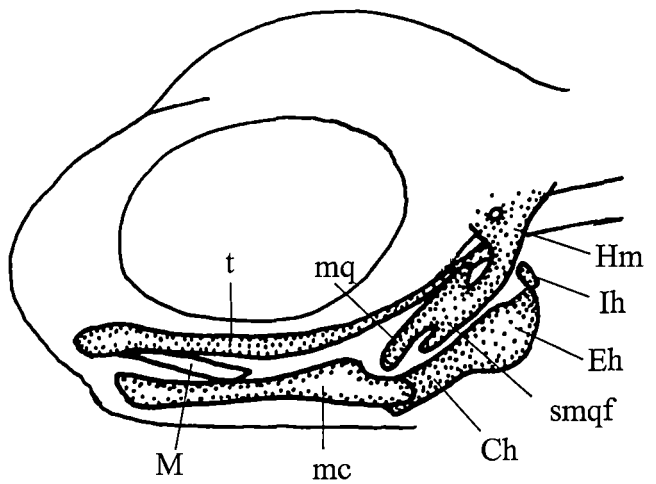
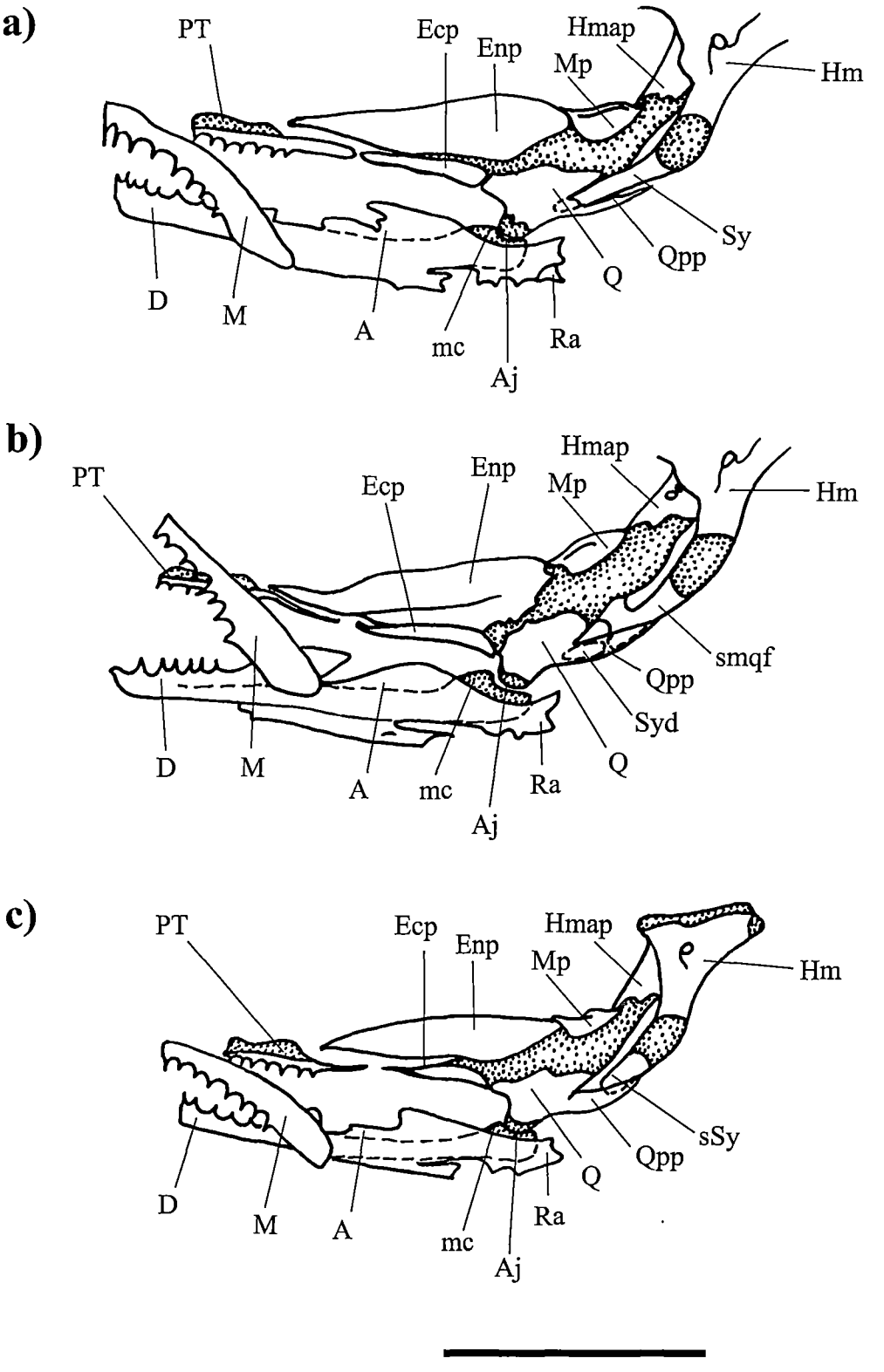


Figure 20. Camera lucida diagrams of the lateral view of the jaw suspensorium and lower jaw of stained and cleared (Taylor and Van Dyke, 1985, Appendix D) mixed sex triploid Atlantic salmon (706° days) with a) a normal symplectic and quadrate, b) a divided symplectic, the dorsal segment of which is fused to the metapterygoid-quadrate cartilage plate and c) a short symplectic. Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: A = angular, D = dentary, Ecp = Ectopterygoid, Enp = endopterygoid, Hm = hyomandibular, Hmap = hyomandibular anterior process, M = maxilla, mc = Meckel's cartilage, Q = quadrate, Qpp = quadrate posterior process, smqf = symplectic and metapterygoid- quadrate cartilage plate fused, Sy = symplectic, Syd = symplectic divided. Scale bar = 3 mm.


a)
PT
Ecp
Enp
Hmap
Mp
Hm
Sy
Qpp
Q
D
M
A
mc
Ra
Aj
b)
PT
Ecp
Enp
Hmap
Mp
Hm
smqf
Qpp
Syd
Q
D
M
A
mc
Ra
Aj
c)
PT
Ecp
Enp
Hmap
Mp
Hm
sSy
Qpp
Q
D
M
A
mc
Ra
Aj

5.4.5 Ontogeny of the fin skeleton

Caudal fin

Primordial rays of the caudal fin were evident as early as 280° days (Fig. 21) staining darkly blue with Alcian blue. At 390° days the caudal hypural cartilages started to form ventrally along the posterior tip of the notochord in fish from all populations, with the exception of the all-female triploids (Fig. 21). At this stage the soft rays of the caudal fin had started to form in fish from all populations. At one week post-hatching (470° days), six caudal hypural cartilages and approximately twelve soft rays were present in fish from each population type (Fig. 21). The neural and haemal spines had started to appear along the dorsal and ventral surface of the posterior tip of the notochord, respectively. The first caudal epiural cartilage had started to form in the all-female diploids at this stage. Two caudal epiural cartilages had formed in fish from all populations by 528° days and precaudal soft rays were evident in fish from the mixed sex populations (Fig. 21). The hypural cartilages of the caudal fin had started to ossify in all fish by 706° days, and the uroneural had formed by membranous secretion (Fig. 22). The epiural cartilages had started to ossify in fish from all populations with the exception of the all-female diploids (Fig. 22). An additional caudal hypural cartilage was present in mixed sex diploid and all-female diploid fish at 706° days (Fig. 22). By 913° days the uroneural had fused to adjacent vertebrae and the caudal epiurals were well ossified in fish from all populations (Fig. 23). An additional caudal hypural cartilage was present in all fish by 913° days (Fig. 23) and was ossified by 1510° days (Fig. 24). At 1510° days the distal tips of the caudal soft rays had divided to form two sub-branches (not shown in Fig. 24, see Fig. 33 for example of soft ray sub-branches).

Figure 21. Camera lucida diagrams of the lateral view of the caudal fin of stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at a) 5 weeks post-fertilisation (wpf) (280° days), b) 6 wpf (340° days), c) 7 wpf (390° days), d) 1 week post-hatching (wph) (470° days), and e) 2 wph (528° days). Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: Ce = caudal epiurals, CH = caudal hypural, Csr = caudal soft rays, Cpr = caudal primordial rays, hs = haemal spine, ns = neural spine, pCsr = precaudal soft rays. Scale bar = 3 mm.

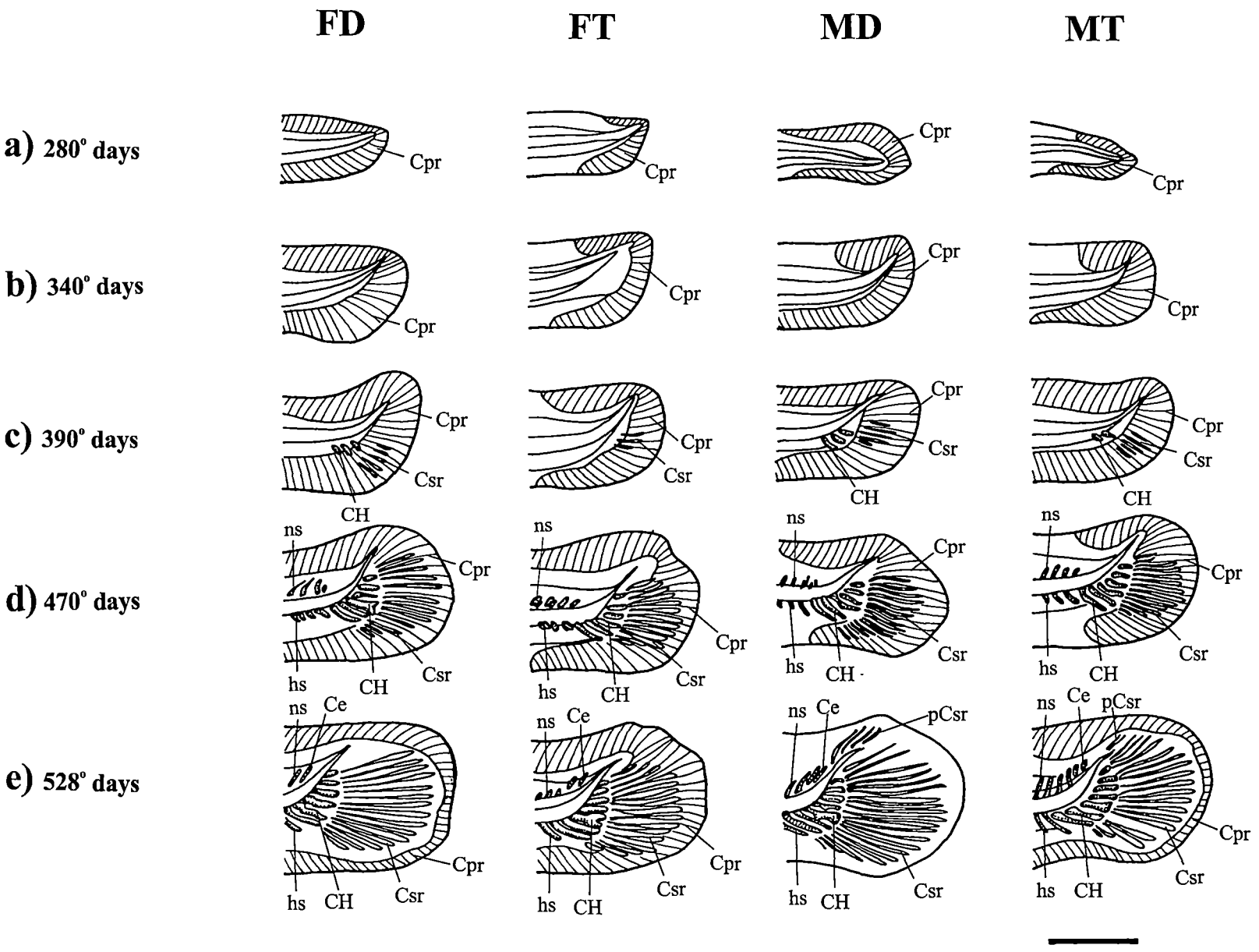

FD
FT
MD
MT
a) 280° days
b) 340° days
c) 390° days
d) 470° days
e) 528° days
Cpr
Csr
CH
ns
hs
Ce
pCsr

Figure 22. Camera lucida diagrams of the lateral view of the caudal fin of stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at 5 weeks post-hatching (706° days). Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: aCH = accessory caudal hypural, CH = caudal hypural, Csr = caudal soft rays, Ce = caudal epiurals, hs = haemal spine, ns = neural spine, pCsr = precaudal soft rays, Un = uroneural. Scale bar = 3 mm.

706° days

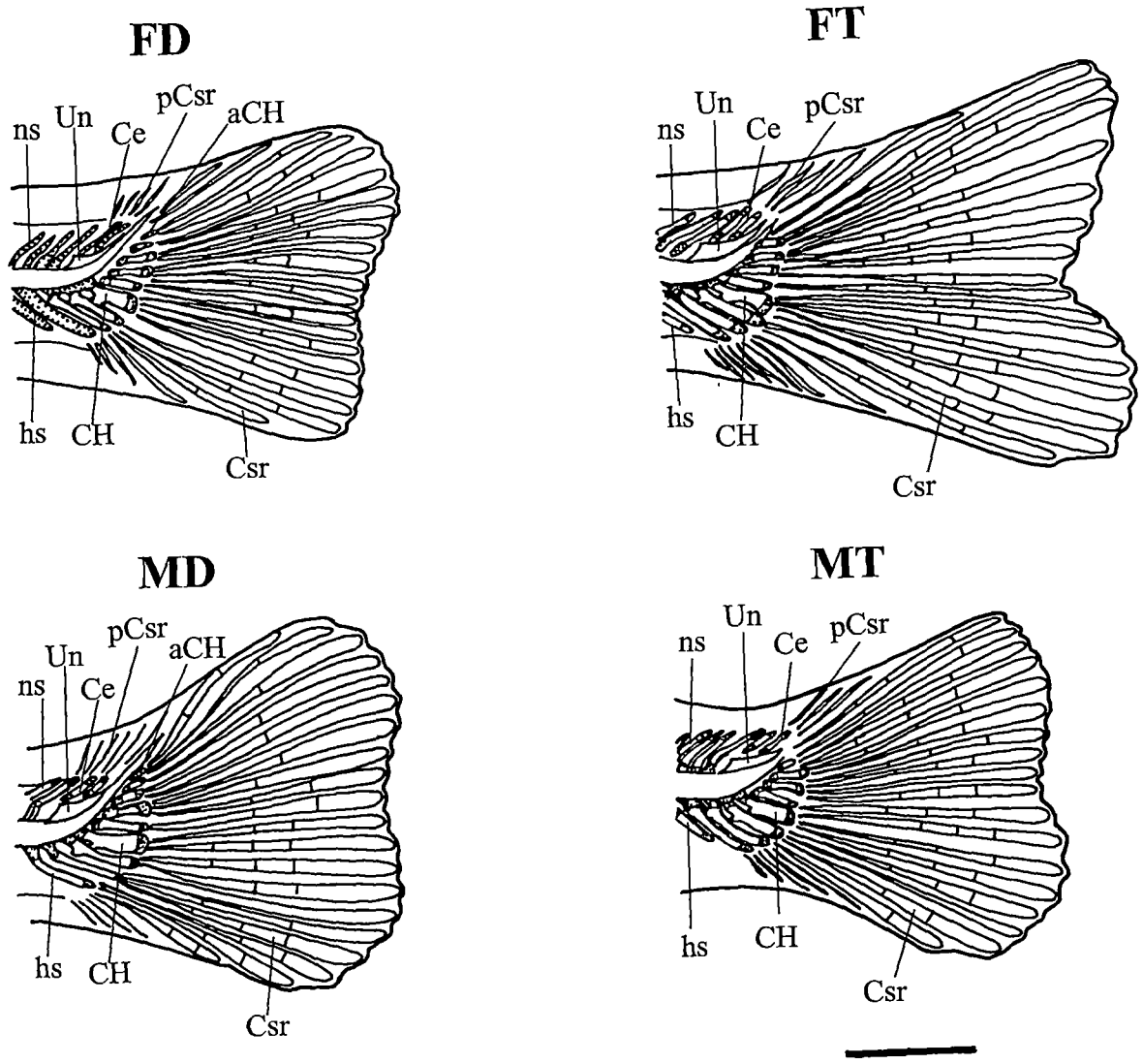
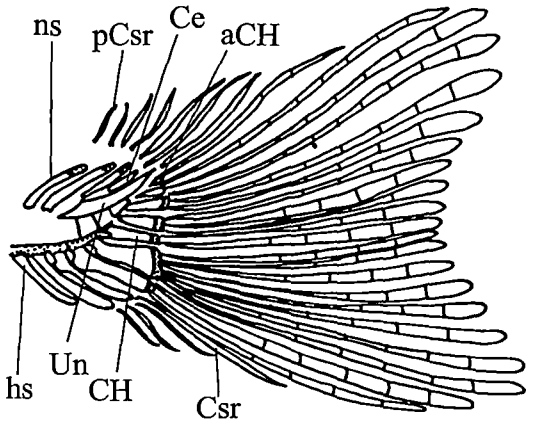

FD
ns
Un
Ce
pCsr
aCH
hs
CH
Csr
FT
ns
Un
Ce
pCsr
hs
CH
Csr
MD
ns
Un
Ce
pCsr
aCH
hs
CH
Csr
MT
ns
Un
Ce
pCsr
hs
CH
Csr

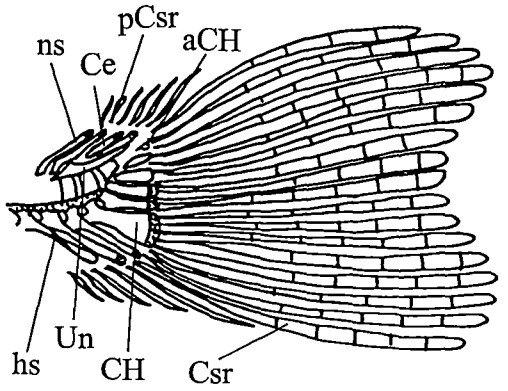
Figure 23. Camera lucida diagrams of the lateral view of the caudal fin of stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at 8 weeks post-hatching (913° days). Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: aCH = accessory caudal hypural, CH = caudal hypural, Csr = caudal soft rays, Ce = caudal epiurals, hs = haemal spine, ns = neural spine, pCsr = precaudal soft rays, Un = uroneural. Scale bar = 3 mm.

913° days

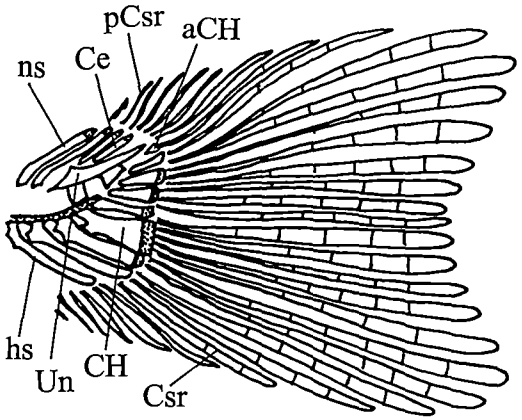
FD



FT



MD



MT

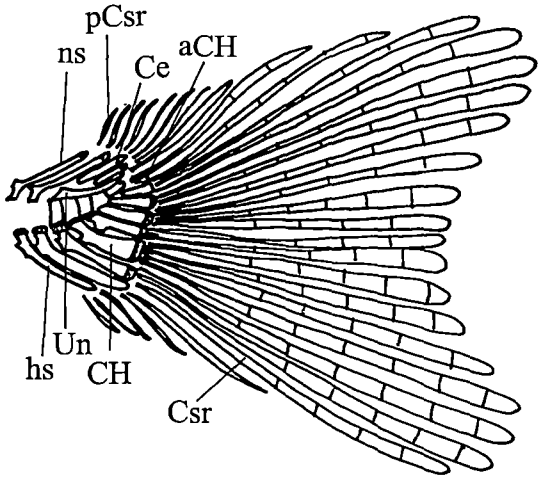
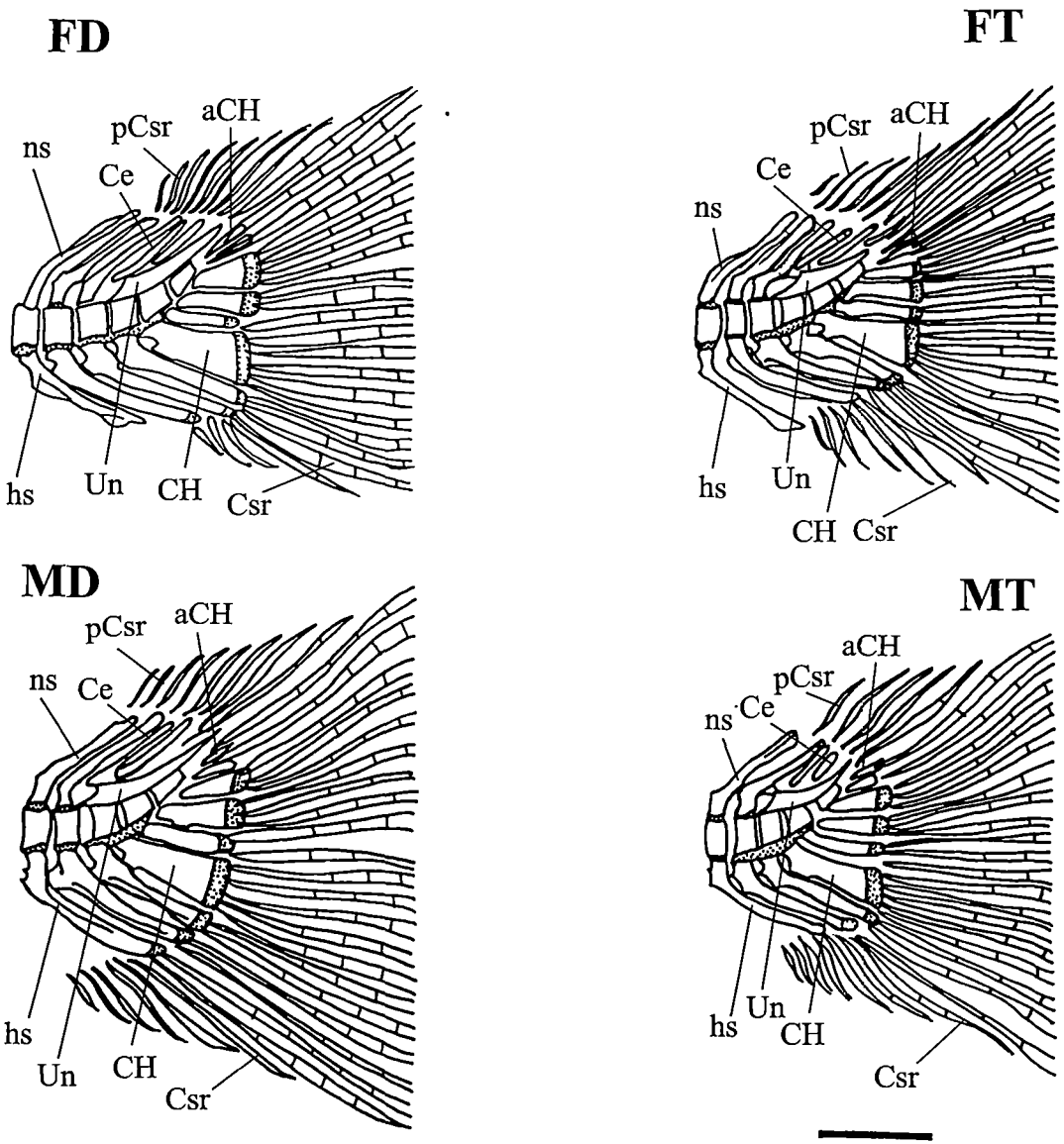


Figure 24. Camera lucida diagrams of the lateral view of the caudal fin of stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at 12 weeks post-hatching (1510° days). Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: aCH = accessory caudal hypural, CH = caudal hypural, Csr = caudal soft rays, Ce = caudal epiurals, hs = haemal spine, ns = neural spine, pCsr = precaudal soft rays, Un = uroneural. Distal tips of soft rays omitted. Scale bar = 3 mm.

1510° days


FD
ns
pCsr
aCH
Ce
hs
Un
CH
Csr
FT
ns
pCsr
aCH
Ce
hs
Un
CH
Csr
MD
pCsr
aCH
ns
Ce
hs
Un
CH
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aCH
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Ce
hs
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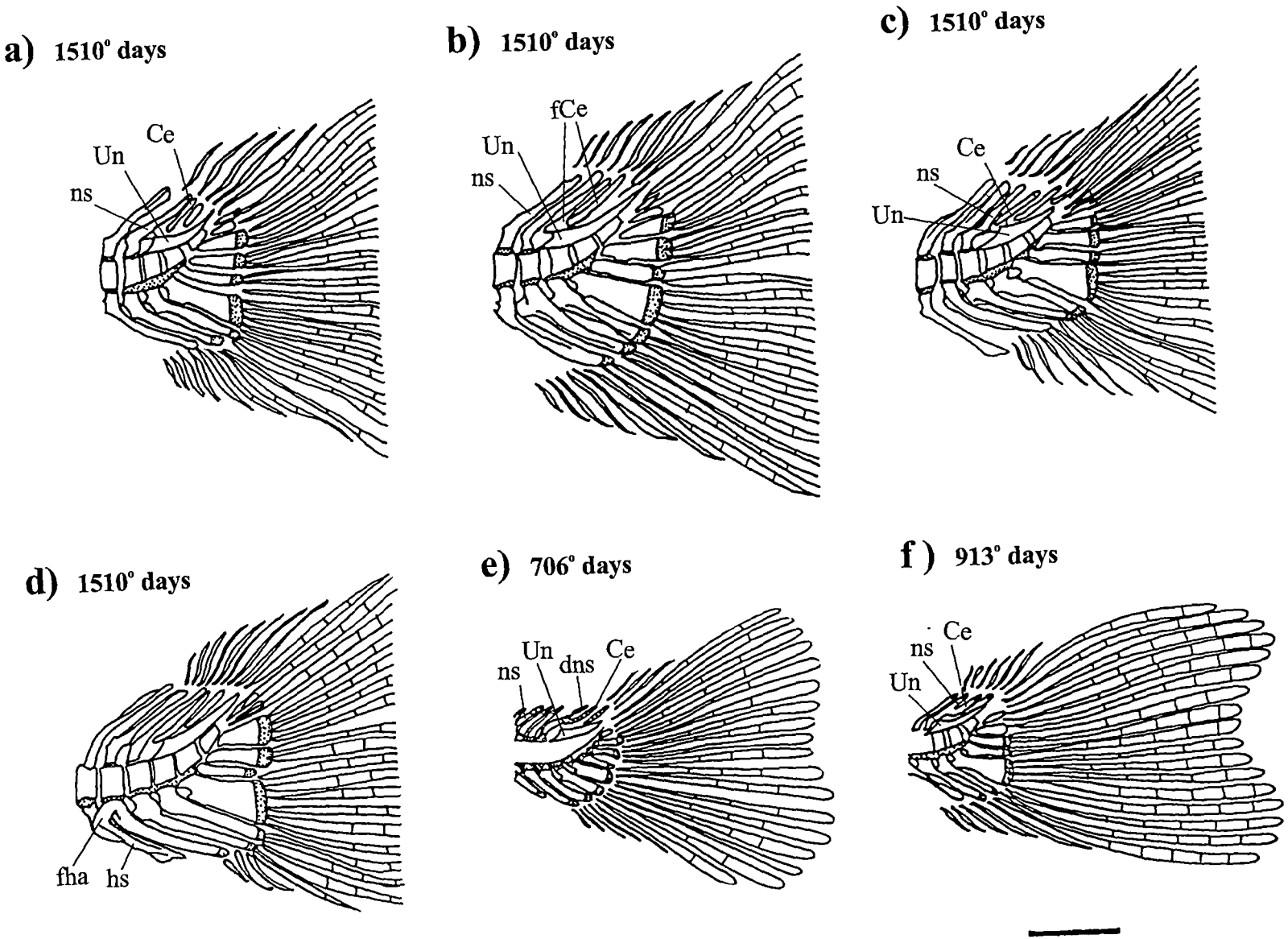
CH
Csr

Abnormal fusion of adjacent bones of the caudal fin were observed in fish from all populations at various stages of development (≥ 706° days). These abnormalities involved either the caudal epiurals, neural spines, haemal spines and/or the uroneural. In several fish between 706° and 1510° days, caudal epiurals were either fused to one another (Fig. 25b), fused to the uroneural (Fig. 25b), fused to the adjacent neural spine (Fig. 25e) or a combination of these (Fig. 25c). In some cases the neural spine was also fused to the uroneural (Fig. 25a & 25c). In other fish the base arches of either neural or haemal spines were staggered and fused to different vertebrae, rather than being bilaterally symmetrical and attached to one vertebrae only (Fig. 25d).

Figure 25. Camera lucida diagrams of the lateral view of the caudal fin of stained and cleared (Taylor and Van Dyke, 1985, Appendix D) Atlantic salmon with abnormal fusion of a) the neural spine and uroneural, b) adjacent caudal epiurals and the uroneural, c) neural spine, audal epiural and uroneural, d) haemal arches from adjacent haemal spines, e) divided neural spine and adjacent caudal epiural and f) neural spine and adjacent caudal epiural. Stage of development is represented by accumulated temperature units post-fertilisation (ATU = ° days).djacent bones. Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: Ce = caudal epiurals, dns = divided neural spine, fCe = fused caudal epiurals, hs = haemal spine, ns = neural spine, Un = uroneural. Distal tips of soft rays omitted in a, b, c & d. Scale bar = 3 mm.


a) 1510° days
Ce
Un
ns
b) 1510° days
fCe
Un
ns
c) 1510° days
Ce
ns
Un
d) 1510° days
fha
hs
e) 706° days
Un
dns
Ce
ns
f) 913° days
Ce
ns
Un

Pectoral fins

At 5 weeks post-fertilisation (280° days), fish from each population type had developed pectoral fins with primordial rays and a coracoid-scapula cartilage which extended from the cleithrum had formed by membranous secretion (Fig. 26). By 340° days a finplate cartilage supported the primordial rays of the pectoral fin and the coracoid-scapula cartilage was joined dorsally and ventrally to the cleithrum (Fig. 26). The pectoral soft rays, formed by membranous secretion amidst the primordial rays, started to appear by 470° days, regardless of fish sex or ploidy status. At this stage, the supracliethrum and post-temporal bones started to form dorsally to the cliethrum and a cartilagenous posterior process of the coracoid-scapula cartilage had also formed (Fig. 26). A cartilagenous scapula-cliethrum process, pectoral digitals and distal radials had developed in fish from all populations by 706° days (Fig. 27). In all-female triploids the scapula, coracoid, scapula-cleithrum process and pectoral digitals had started to ossify. In addition, the post-cliethrum bones (upper and lower) were evident in the all-female triploid fish at 706° days (Fig. 27). By 913° days the scapula, coracoid, scapula-cleithrum process and pectoral digitals had started to ossify in fish from all populations (Fig. 28). The upper and lower post-cliethrum bones were also evident in all fish at this stage, regardless of sex or ploidy status (Fig. 28). Bones of the pectoral fin had doubled in size during the four weeks prior to 1510° days (Fig. 29).

Figure 26. Camera lucida diagrams of the lateral view of the pectoral fin of stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at a) 5 weeks post-fertilisation (wpf) (280° days), b) 6 wpf (340° days), c) 7 wpf (390° days), d) 1 week post-hatching (wph) (470° days), and e) 2 wph (528° days). Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: Cl = cleithrum, cs = coracoid-scapula cartilage, cspp = coracoid-scapula cartilage posterior process, fp = fin plate cartilage, Ppr = pectoral primordial rays, Psr = pectoral soft rays, Pt = post-temporal, SCl = supracleithrum. Scale bar = 3 mm.

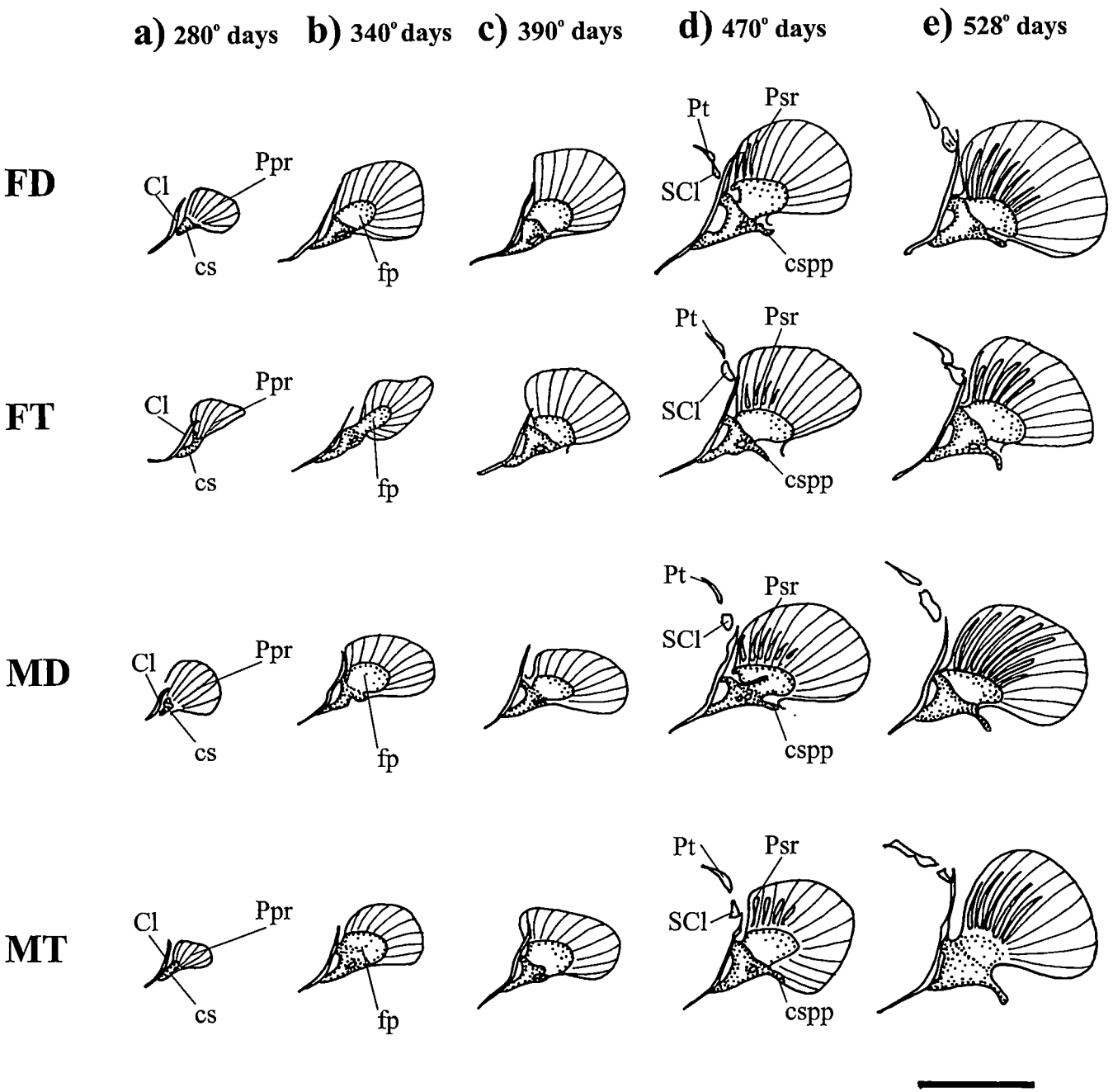
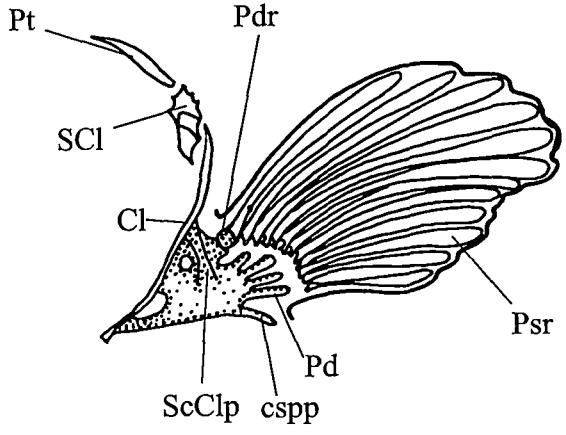

a) 280° days
b) 340° days
c) 390° days
d) 470° days
e) 528° days
FD
FT
MD
MT
Cl
Ppr
cs
fp
Pt
Psr
SCl
cspp

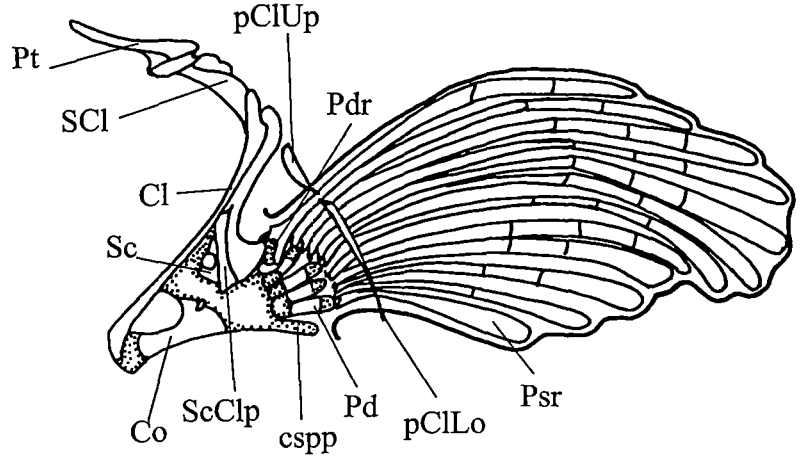
Figure 27. Camera lucida diagrams of the lateral view of the pectoral fin of stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at 5 weeks post-hatching (706° days). Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: Cl = cleithrum, Co = coracoid, cspp = coracoid-scapula cartilage posterior process, Pd = pectoral digitals, Pdr = pectoral distal radials, Psr = pectoral soft rays, Pt = post-temporal, pClUp = post-cleithrum upper, pClLo = post-cliethrum lower, ScClp = scapula-cleithrum process, Sc = scapula, SCl = supracleithrum. Scale bar = 3 mm.

706° days

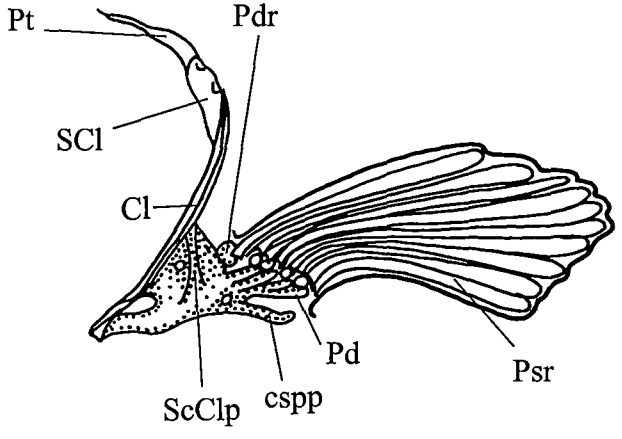
FD



FT



MD



MT

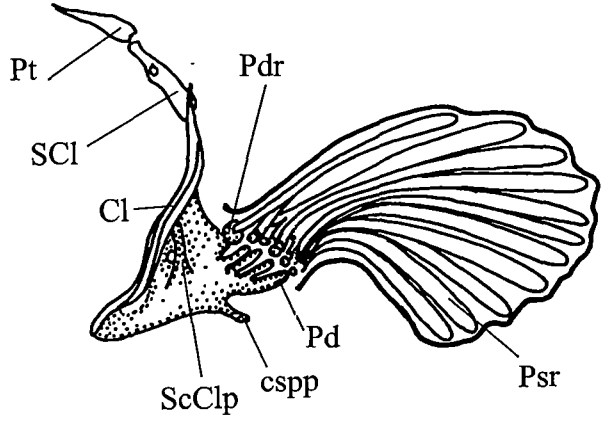


Figure 28. Camera lucida diagrams of the lateral view of the pectoral fin of stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at 8 weeks post-hatching (913° days). Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: Cl = cleithrum, Co = coracoid, cspp = coracoid-scapula cartilage posterior process, Pd = pectoral digitals, Pdr = pectoral distal radials, Psr = pectoral soft rays, Pt = post-temporal, pClUp = post-cleithrum upper, pClLo = post-cliethrum lower, ScClp = scapula-cleithrum process, Sc = scapula, SCl = supracleithrum. Scale bar = 3 mm.

913° days

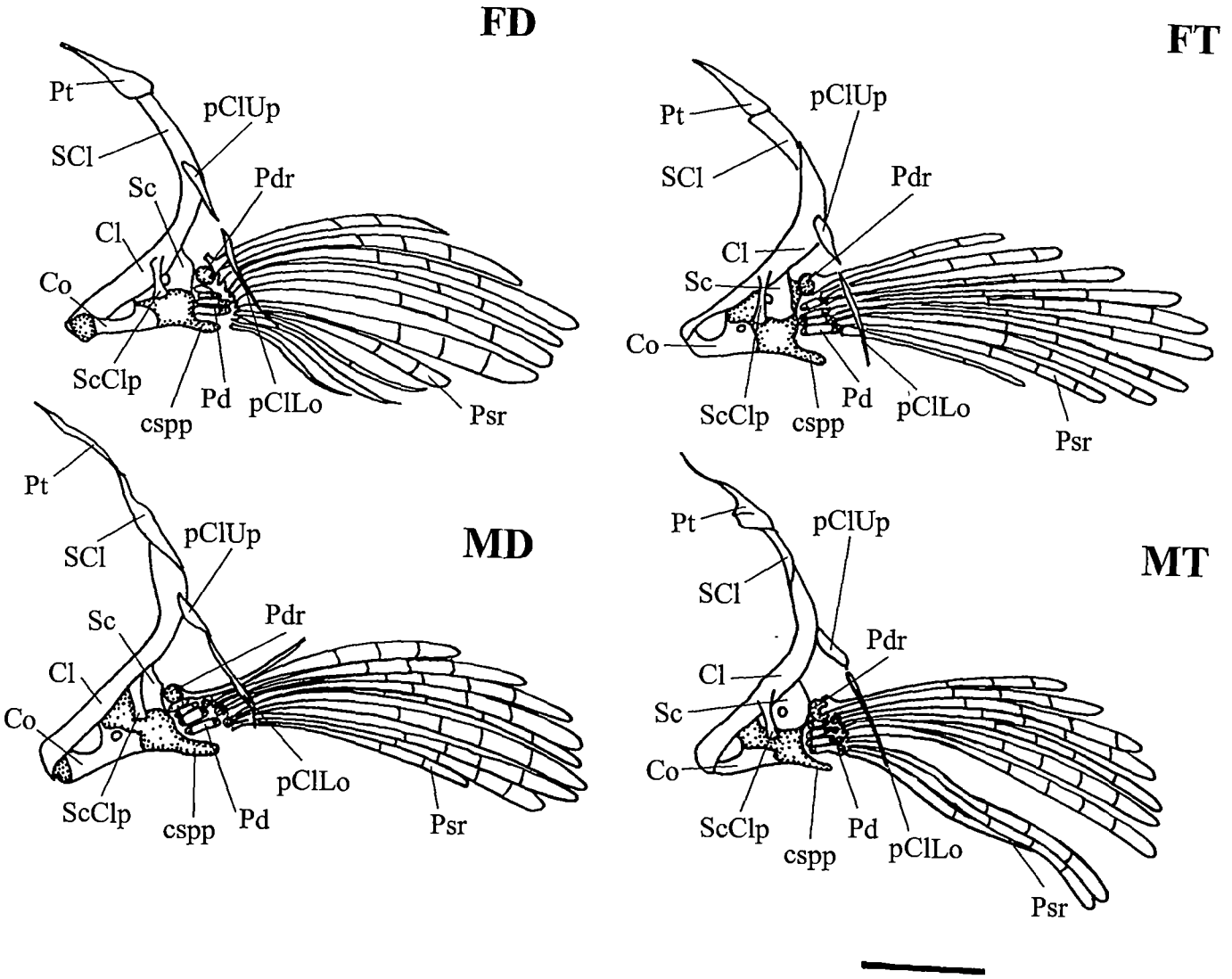
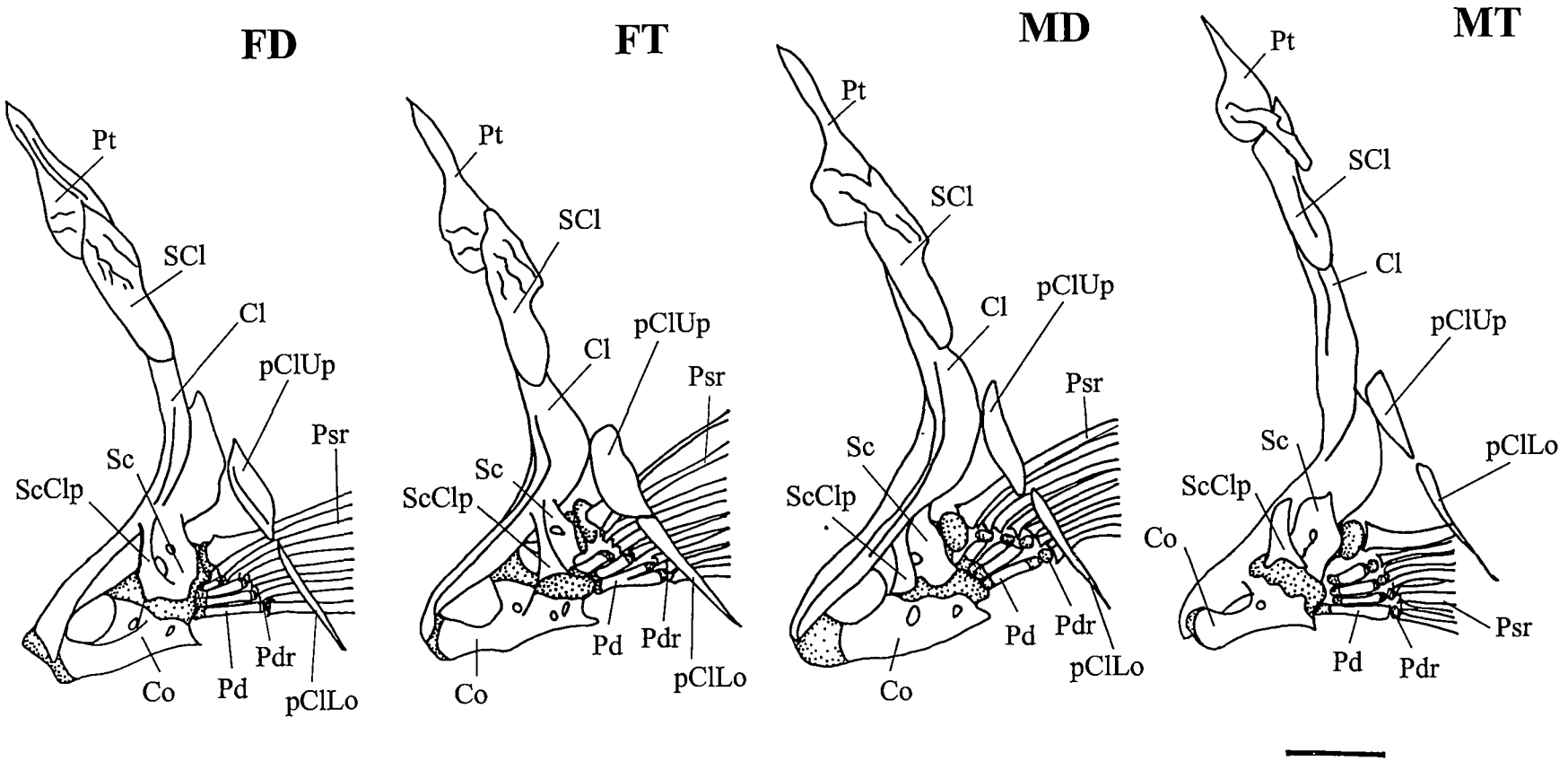

FD
Pt
pClUp
SCl
Sc
Pdr
Cl
Co
ScClp
cspp
Pd
pClLo
Psr
FT
Pt
pClUp
SCl
Pdr
Cl
Sc
Co
ScClp
cspp
Pd
pClLo
Psr
MD
Pt
SCl
pClUp
Sc
Pdr
Cl
Co
ScClp
cspp
Pd
pClLo
Psr
MT
Pt
pClUp
SCl
Pdr
Cl
Sc
Co
ScClp
cspp
Pd
pClLo
Psr

Figure 29. Camera lucida diagrams of the lateral view of the pectoral fin of stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at 12 weeks post-hatching (1510° days). Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: Cl = cleithrum, Co = coracoid, cspp = coracoid-scapula cartilage posterior process, Pd = pectoral digitals, Pdr = pectoral distal radials, Psr = pectoral soft rays, Pt = post-temporal, pClUp = post-cleithrum upper, pClLo = post-cliethrum lower, ScClp = scapula-cleithrum process, Sc = scapula, SCl = supracleithrum. Distal tips of pectoral soft rays omitted. Scale bar = 3 mm.

1510° days



Dorsal fin

The dorsal fin was delineated by primordial rays in embryonic fish up until 470° days, at which time the dorsal soft rays and the cartilagenous proximal radials had also appeared (Fig. 30). The primordial rays were no longer apparent in fish from the mixed sex diploid population at 470° days, and in fish from the other populations at 528° days (Fig. 30). By 706° days, the proximal radials and the soft rays of the dorsal fin had started to ossify in fish from the all-female triploid, mixed sex diploid and mixed sex triploid populations (Fig. 31). At this stage only the soft rays, and not the proximal radials of the dorsal fin, had started to ossify in fish from the all-female diploid population. In addition, the cartilagenous distal radials, the predorsal proximal radial and the dorsal stay, had developed in fish from all populations (Fig. 31). The predorsal proximal radial and predorsal soft rays were ossified in fish from all populations by 913° days (Fig. 32). The distal tip of the dorsal proximal radials had differentiated to form a second centre of ossification to support the dorsal distal radial cartilages at 1510° days (Fig. 33). At this stage the distal tips of the dorsal soft rays had divided to form two sub-branches (Fig. 33).

Figure 30. Camera lucida diagrams of the lateral view of the dorsal fin of stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at a) 5 weeks post-fertilisation (wpf) (280° days), b) 6 wpf (340° days), c) 7 wpf (390° days), d) 1 week post-hatching (wph) (470° days), and e) 2 wph (528° days). Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: Dpr = dorsal primordial rays, Dsr = dorsal soft rays, Dpxr = dorsal proximal radial, hs = arches of haemal spine, no = notochord, ns = arches of neural spine. Scale bar = 3 mm.

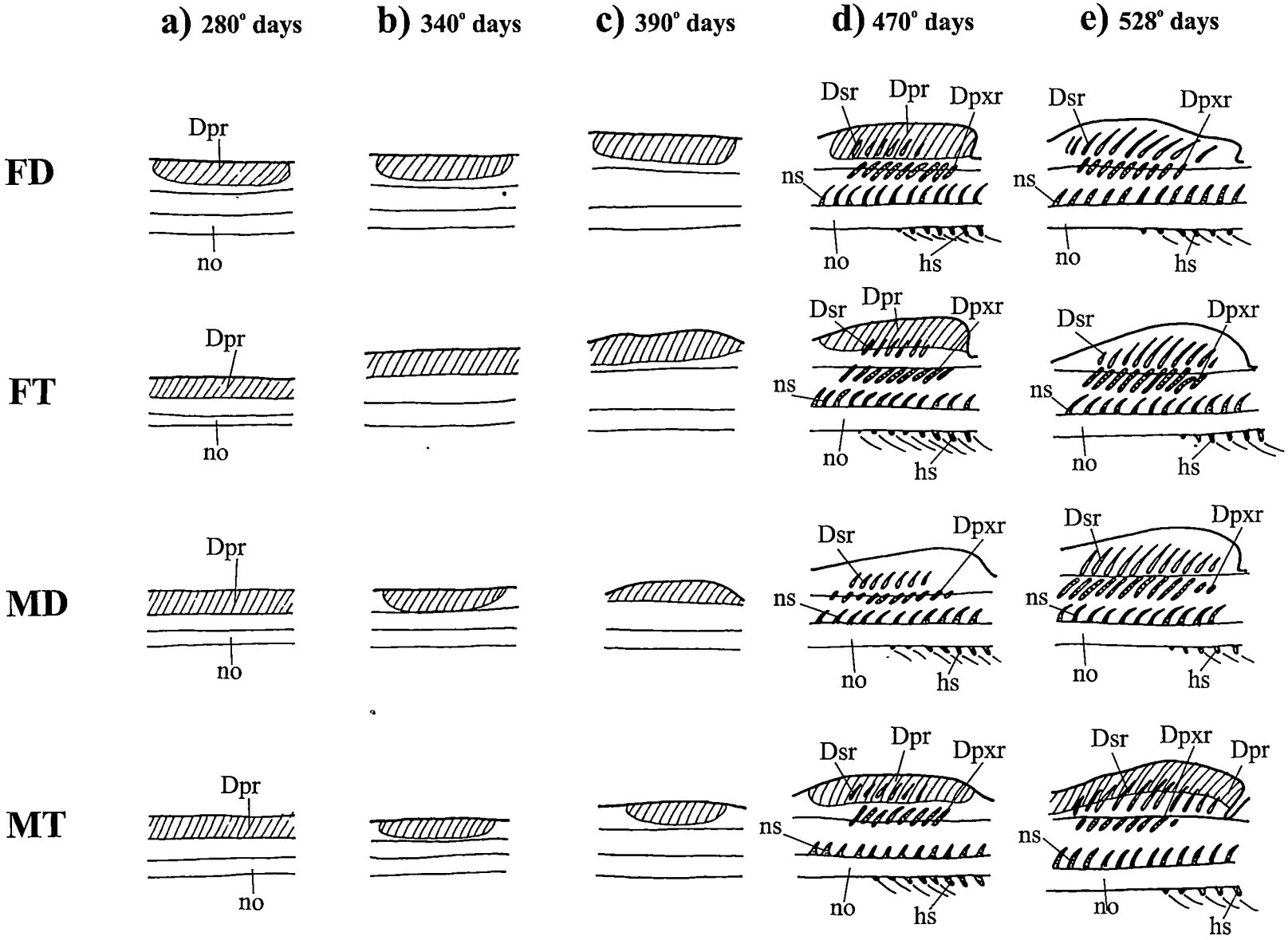
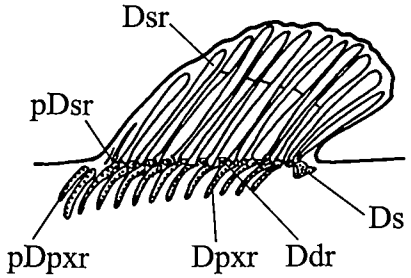

a) 280° days
b) 340° days
c) 390° days
d) 470° days
e) 528° days
FD
FT
MD
MT
Dpr
Dsr
Dpxr
ns
no
hs

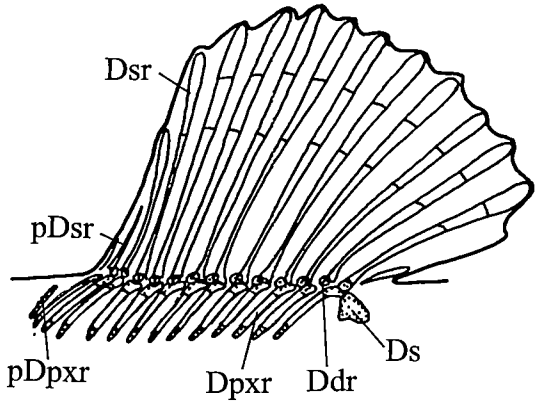
Figure 31. Camera lucida diagrams of the lateral view of the dorsal fin of stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at 5 weeks post-hatching (706° days). Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: Ddr = dorsal distal radial, Dsr = dorsal soft rays, Ds = dorsal stay, pDpxr = predorsal proximal rays, pDsr = predorsal soft rays, Dpxr = dorsal proximal radial. Scale bar = 3 mm.

706° days

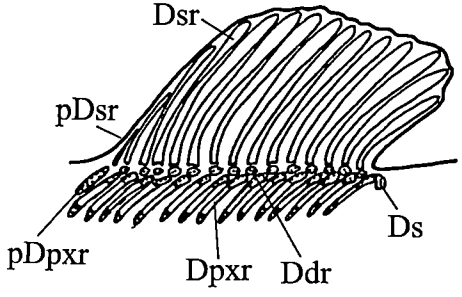
FD



FT



MD



MT

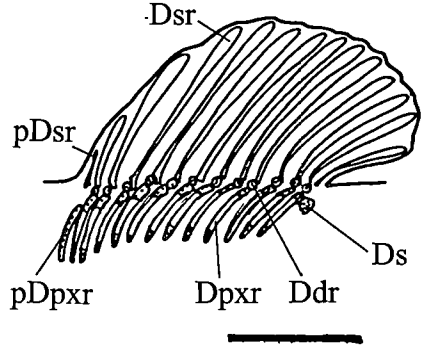


Figure 32. Camera lucida diagrams of the lateral view of the dorsal fin of stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at 8 weeks post-hatching (913° days). Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: Ddr = dorsal distal radial, Dsr = dorsal soft rays, Ds = dorsal stay, pDpxr = predorsal proximal rays, pDsr = predorsal soft rays, Dpxr = dorsal proximal radial. Scale bar = 3 mm.

913° days

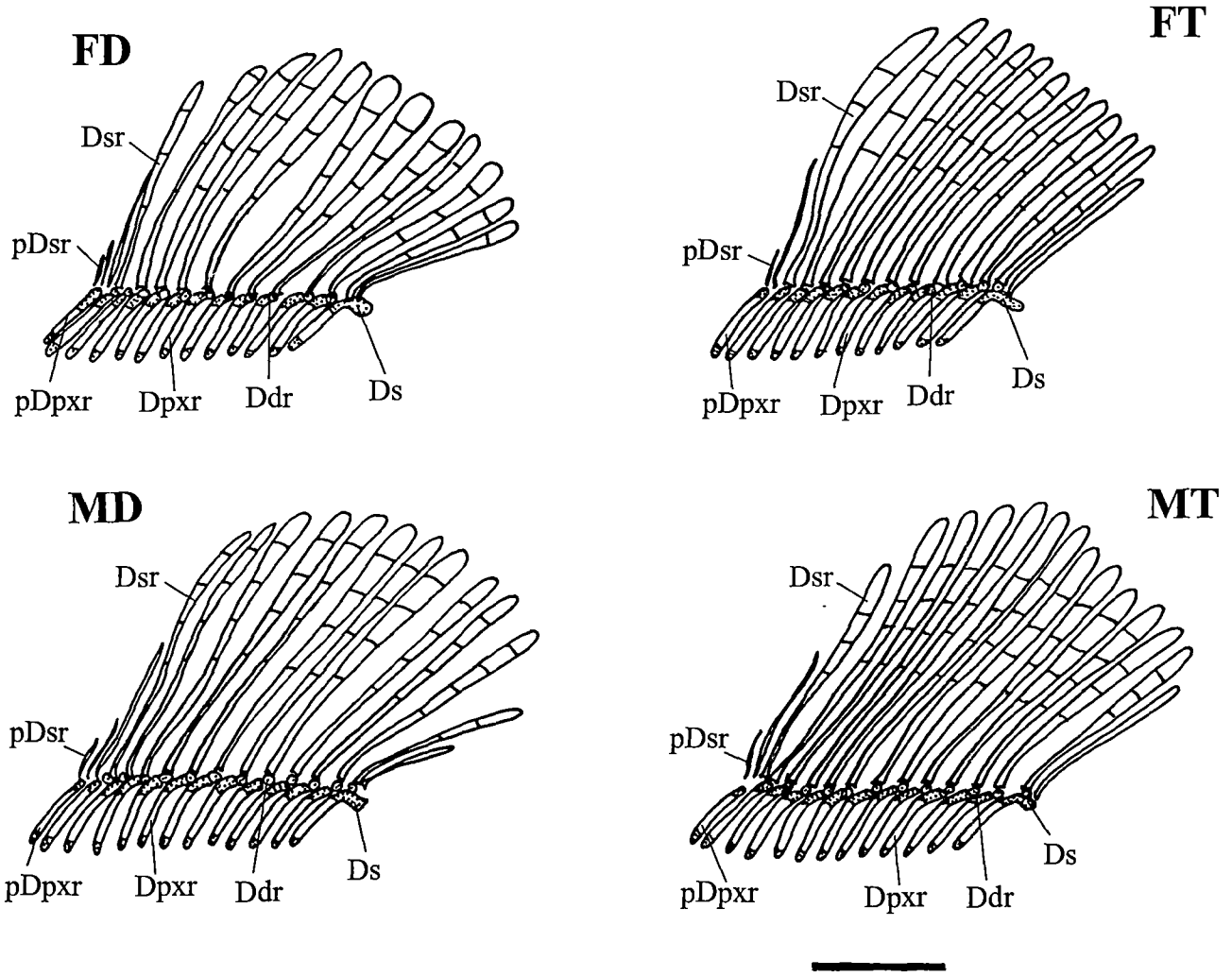
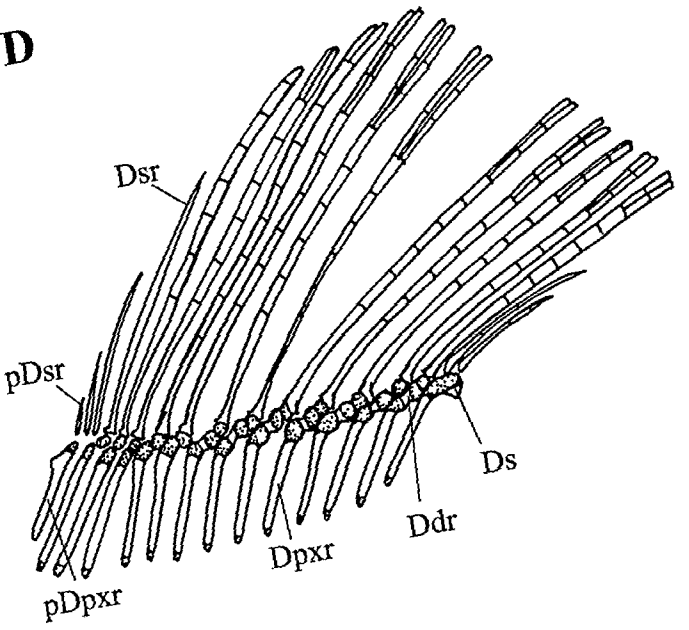

FD
Dsr
pDsr
pDpxr
Dpxr
Ddr
Ds
FT
Dsr
pDsr
pDpxr
Dpxr
Ddr
Ds
MD
Dsr
pDsr
pDpxr
Dpxr
Ddr
Ds
MT
Dsr
pDsr
pDpxr
Dpxr
Ddr
Ds

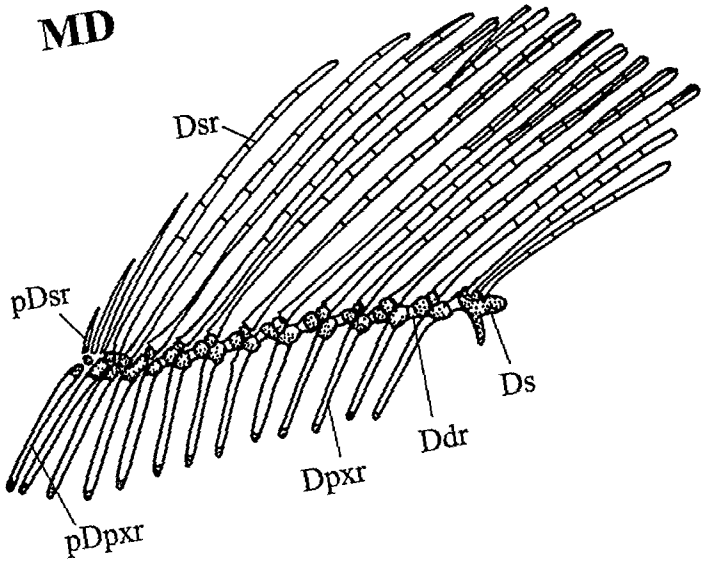
Figure 33. Camera lucida diagrams of the lateral view of the dorsal fin of stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at 12 weeks post-hatching (1510° days). Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: Ddr = dorsal distal radial, Dsr = dorsal soft rays, Ds = dorsal stay, pDpxr = predorsal proximal rays, pDsr = predorsal soft rays, Dpxr = dorsal proximal radial. Scale bar = 3 mm.

1510° days

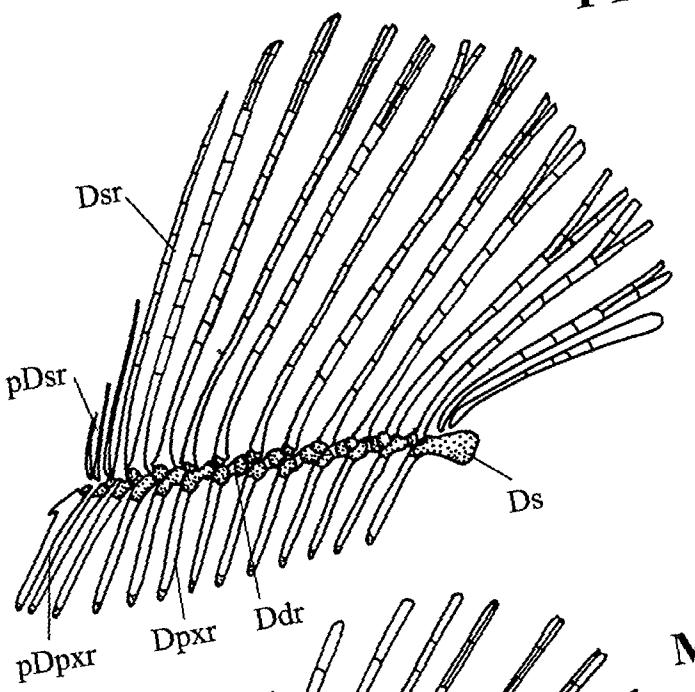
FD



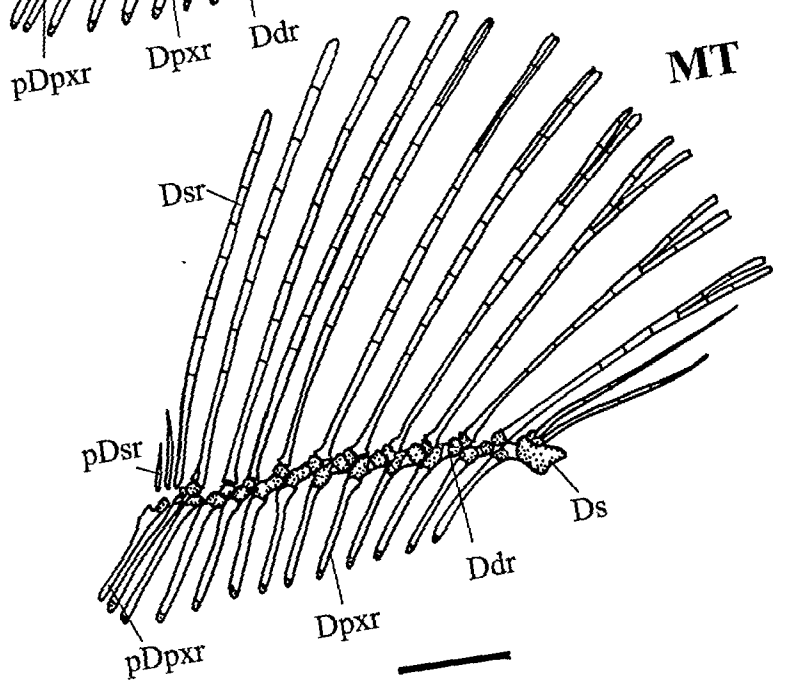
MD



FT



MT



Anal fin

The anal fin was delineated by primordial rays in embryonic fish up until 470° days, at which time the soft rays and the cartilagenous proximal radials of the anal fin had also appeared (Fig. 34). The primordial rays were no longer apparent in fish from the mixed sex diploid and mixed sex triploid populations at 528° days (Fig. 34), and in fish from the all-female populations at 706° days (Fig. 35). The cartilagenous distal radials and the anal stay had developed in fish from all populations at this stage (Fig. 35). By 706° days the proximal radials and the soft rays of the anal fin had started to ossify in fish from the all-female triploid and mixed sex diploid populations (Fig. 35). The proximal radials and soft rays of the anal fin had ossified in fish from all populations by 913° days (Fig. 36). At 1510° days the distal tips of the anal soft rays had divided to form two sub-branches (Fig. 37). The distal tip of the anal proximal radials had differentiated to form a second centre of ossification to support the anal distal radial cartilages at this stage (Fig. 37).

Figure 34. Camera lucida diagrams of the lateral view of the anal fin of stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at a) 5 weeks post-fertilisation (wpf) (280° days), b) 6 wpf (340° days), c) 7 wpf (390° days), d) 1 week post-hatching (wph) (470° days), and e) 2 wph (528° days). Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: Apr = anal primordial rays, Apxr = anal proximal rays, Asr = anal soft rays, hs = arches of haemal spine, no = notochord, ns = arches of neral spine. Scale bar = 3 mm.

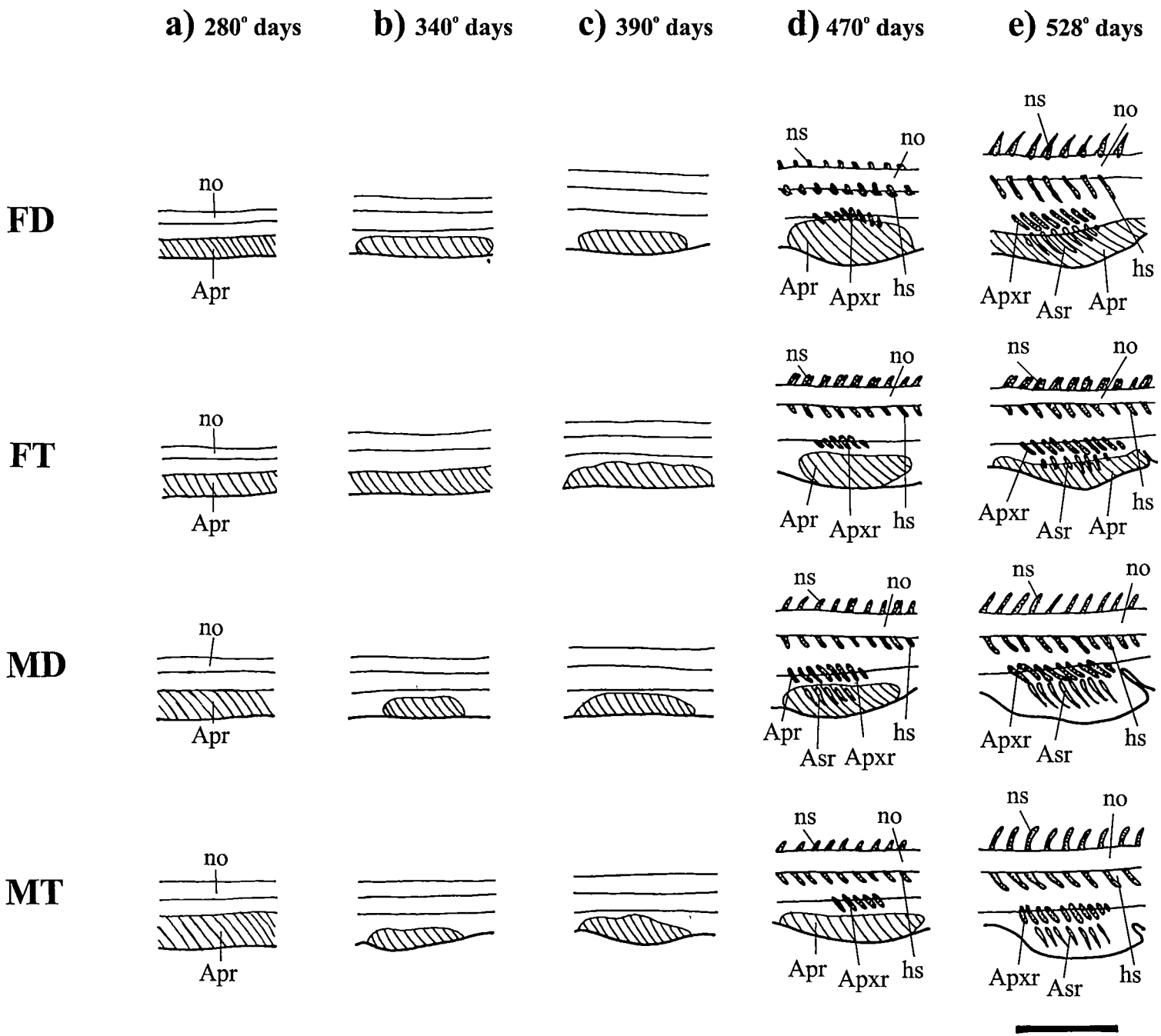
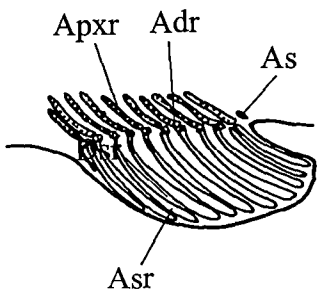

a) 280° days
b) 340° days
c) 390° days
d) 470° days
e) 528° days
FD
FT
MD
MT
no
ns
Apr
Apxr
Asr
hs

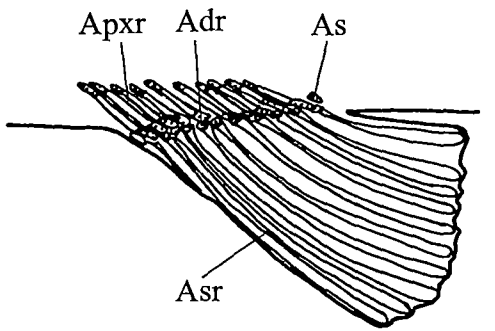
Figure 35. Camera lucida diagrams of the lateral view of the anal fin of stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at 5 weeks post-hatching (706° days). Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: Adr = anal distal radial, Apxr = anal proximal radial, Asr = anal soft rays, As = anal stay. Scale bar = 3 mm.

706° days

FD



FT



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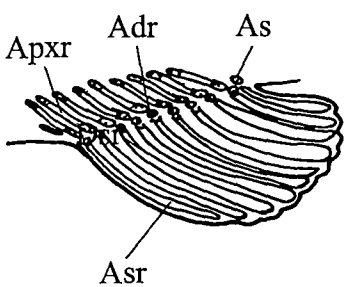


MT

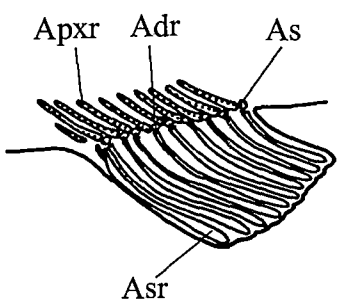
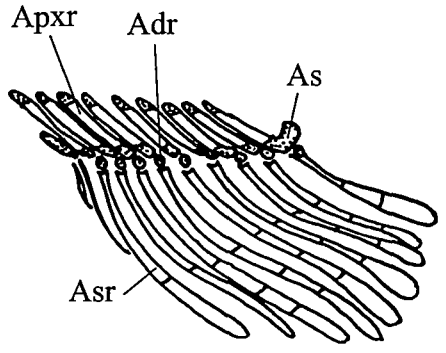


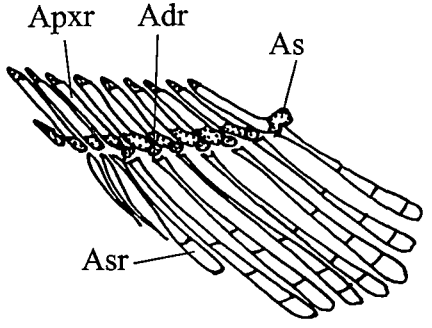
Figure 36. Camera lucida diagrams of the lateral view of the anal fin of stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at 8 weeks post-hatching (913° days). Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: Adr = anal distal radial, Apxr = anal proximal radial, Asr = anal soft rays, As = anal stay. Scale bar = 3 mm.

913° days

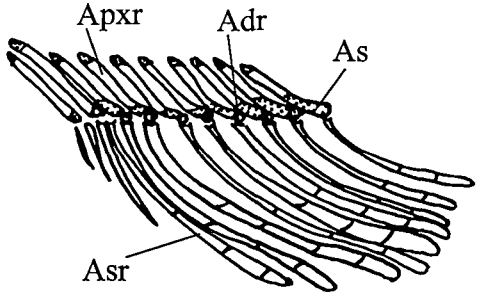
FD



FT



MD



MT

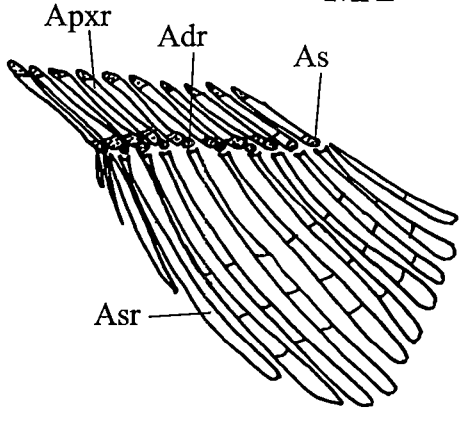
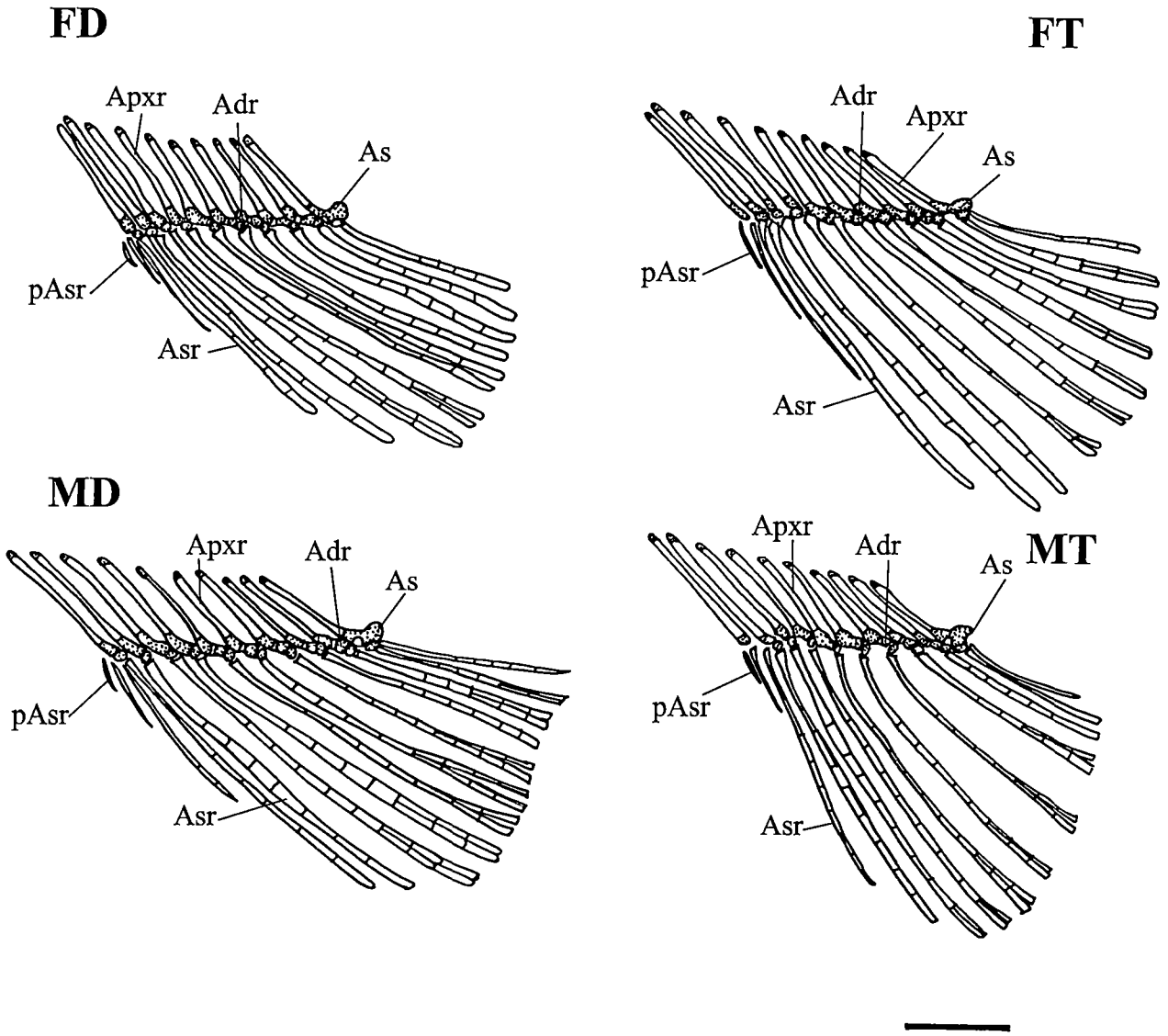


Figure 37. Camera lucida diagrams of the lateral view of the anal fin of stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at 12 weeks post-hatching (1510° days). Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: Adr = anal distal radial, Apxr = anal proximal radial, Asr = anal soft rays, As = anal stay, pAsr = preanal soft rays. Scale bar = 3 mm.

1510° days


FD
Apxr
Adr
As
pAsr
Asr
FT
Adr
Apxr
As
pAsr
Asr
MD
Apxr
Adr
As
pAsr
Asr
MT
Apxr
Adr
As
pAsr
Asr

Pelvic fins

Prior to 528° days the pelvic fins consisted of skin folds with primordial rays. At this time the basipterygium cartilages had started to form in all-female triploid, mixed sex diploid and mixed sex triploid fish (Fig. 38). The metapterygium cartilage was evident in all-female diploid, mixed sex diploid and mixed sex triploid fish at 640° days, but was not evident in all-female triploid fish until 706° days (Fig. 38). By 706° days the metapterygium and the distal radial cartilages of the pelvic fins were present in all fish and the basipterygium had started to ossify in the all-female triploid and mixed sex diploid fish. In addition, the all-female triploid fish had started to form a medial process along the basipteryium bones by membranous/fibrous ossification (Fig. 38). By 913° days the medial process of the basipterygium had formed in fish from all populations, with the exception of the all-female diploids (Fig. 39a). In addition, the metapterygium was ossified in all fish (Fig. 39a). The pelvic girdle had doubled in size by 1510° days, compared to the preceding stage (913° days), and the medial process of the basipterygium was evident in all-female diploid fish (Fig. 39b).

Generally, there were only very minor differences in the temporal development of the fin skeleton between fish from the different populations, but these differences were not consistent with either ploidy or sex status (Fig. 40). Deformities observed in the caudal fin at stages ≥ 706° days were observed in fish from all populations (Fig. 40).

Figure 38. Camera lucida diagrams of the dorsal view of the pelvic girdle of stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at a) 2 weeks post-hatching (wph) (528° days), b) 4 wph (583° days), and c) 5 wph (706° days). Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: Btg = basipterygium, Btp= basipterygium process, Vdr = pelvic digital radials, Mtg = metapterygium, Vpr = pelvic primordial rays, Vsr = pelvic soft rays. Scale bar = 3 mm.

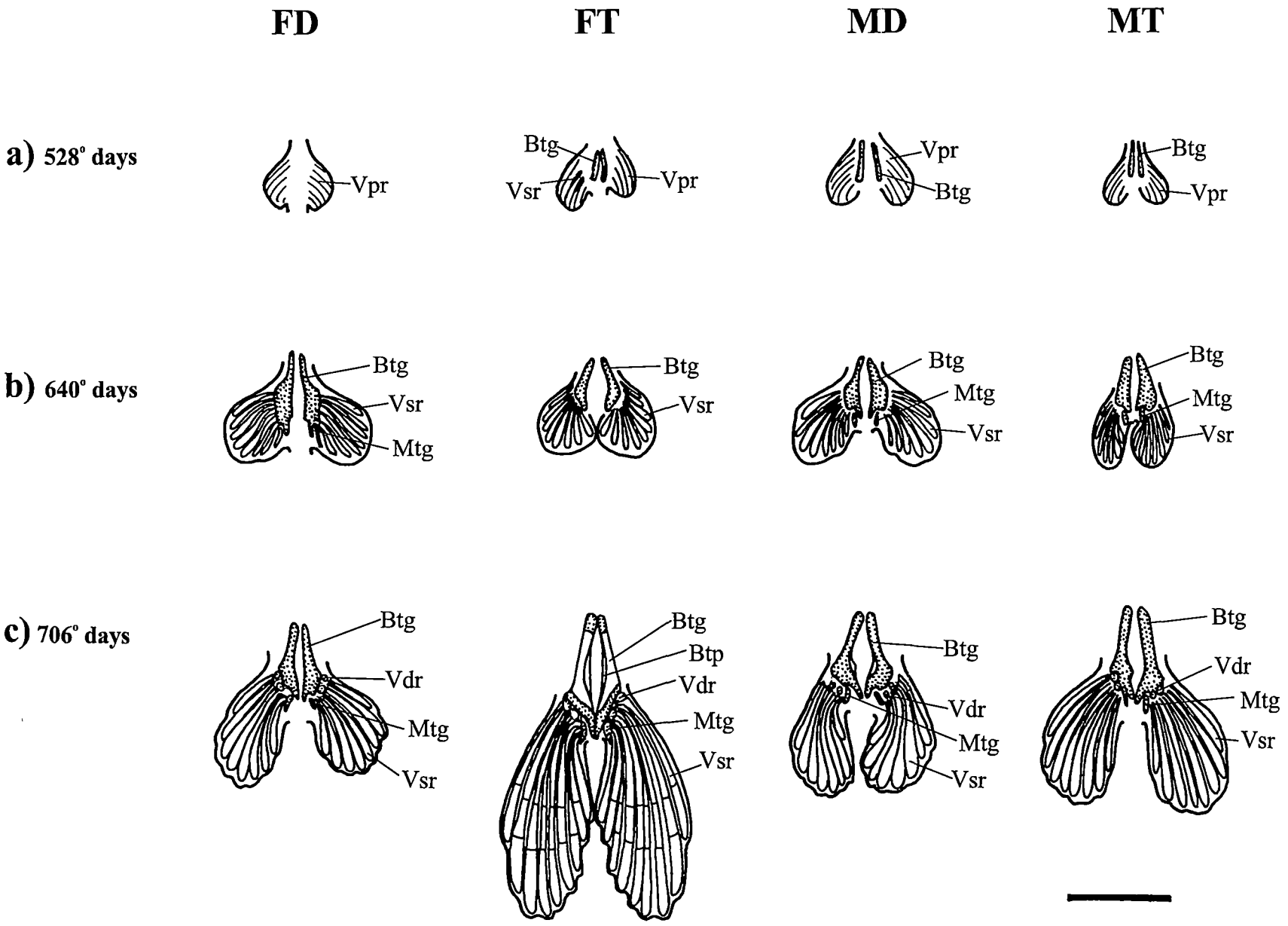

FD
FT
MD
MT
a) 528° days
b) 640° days
c) 706° days
Vpr
Btg
Vsr
Mtg
Vdr
Btp

Figure 39. Camera lucida diagrams of the dorsal view of the pelvic girdle of stained and cleared (Taylor and Van Dyke, 1985, Appendix D) all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon at 8 weeks (913° days) and 12 weeks (1510° days) post-hatching. Stippling denotes cartilage and unfilled areas denote bone. Abbreviations: Btp= basipterygium process, Btg = basipterygium, Vdr = pelvic digital radials, Mtg = metapterygium, Vsr = pelvic soft rays. Soft rays not shown on right hand side at 913° days and omitted at 1510° days. Scale bar = 3 mm.

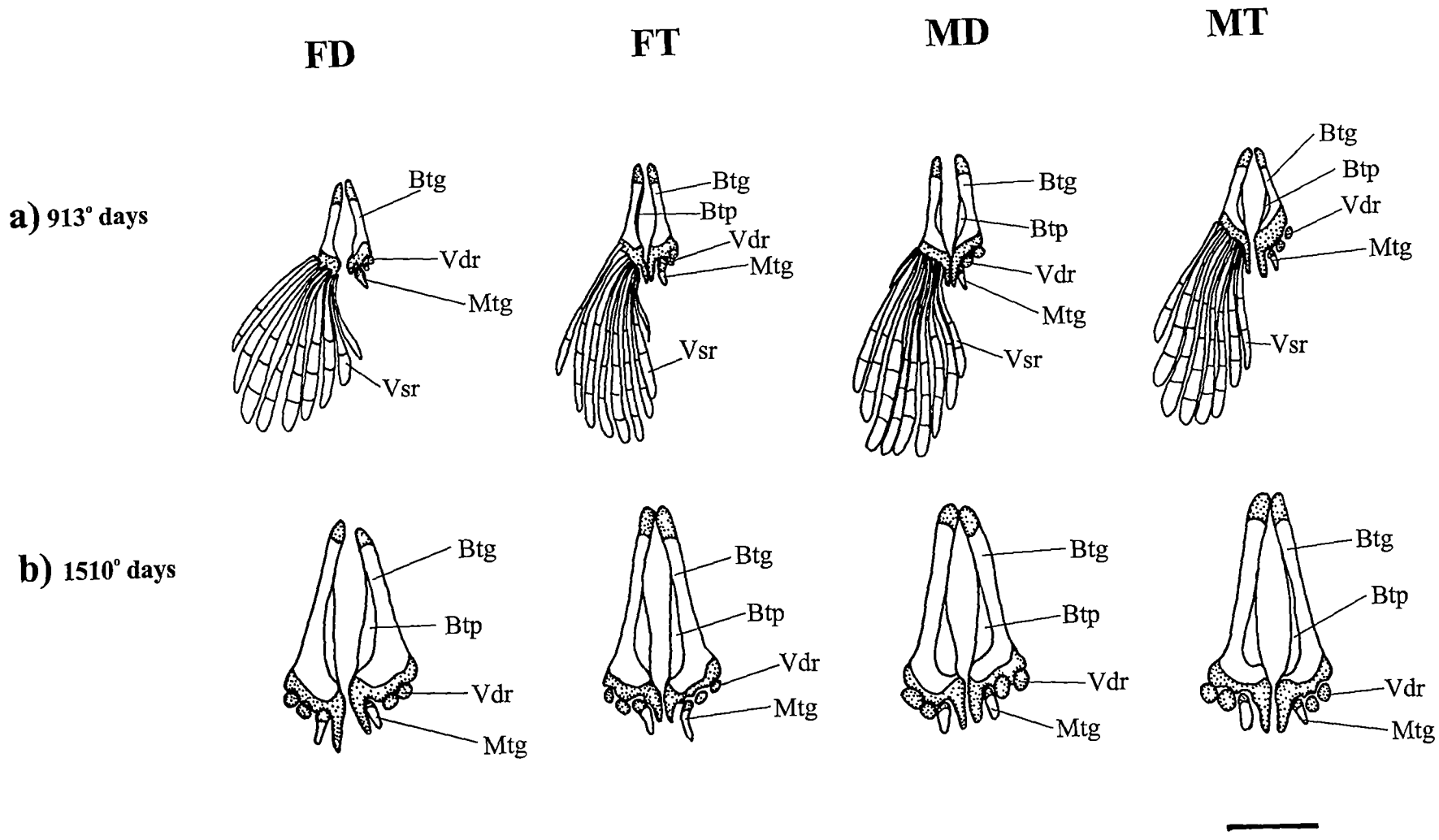

FD
FT
MD
MT
a) 913° days
b) 1510° days
Btg
Btp
Vdr
Mtg
Vsr

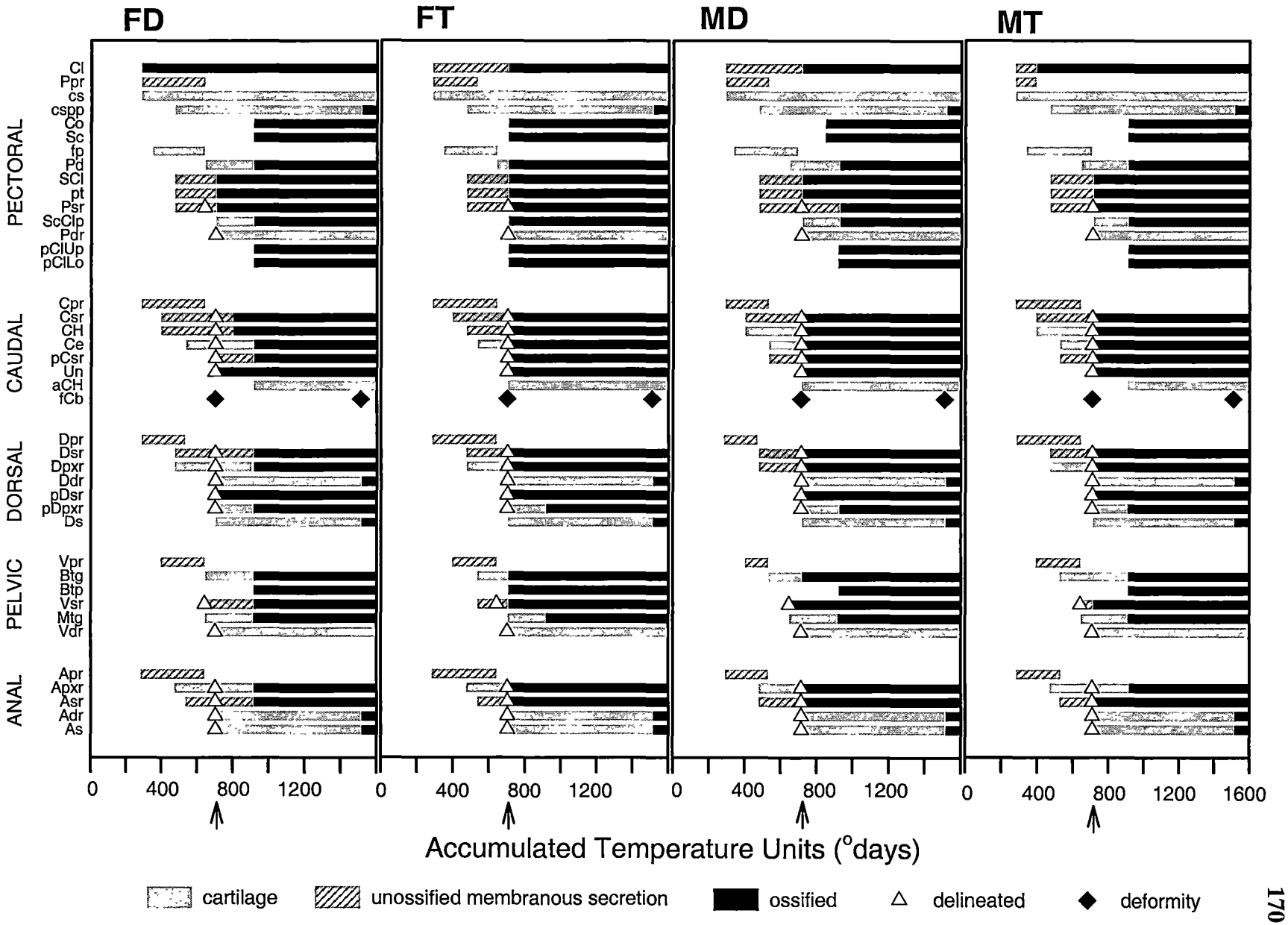
Figure 40. Ontogeny of each cartilage and bone of the fin skeleton of all-female diploid (FD), all-female triploid (FT), mixed sex diploid (MD) and mixed sex triploid (MT) Atlantic salmon populations as defined by presence of skeletal element in more than 50% of the fish examined (sample sizes as per Table 1). Stage of development is represented by accumulated temperature units post-fertilisation (ATU = ° days). Plain grey bar = cartilage structure observed, hatch grey bar = membranous secretion observed, black bar = structure ossified (stained red with alizarin red), triangle = structure delineated in shape and/or number, black diamond = deformity observed. Arrow indicates time of first feeding.

Abbreviations:

	PECTORAL FIN AND GIRDLE
Cl	cleithrum
Co	coracoid
cspp	coracoid-scapula cartilage posterior process
cs	coracoid-scapula cartilage
Pdr	distal radials
fp	fin plate cartilage
Pd	pectoral digitals
Ppr	pectoral primordial rays
Psr	pectoral soft rays
Pt	post-temporal
pClUp	post-cleithrum upper
pClLo	post-cliethrum lower
ScClp	scapula / cleithrum process
Sc	scapula
SCl	supra cleithrum
ns	neural spine
hs	haemal spine

	ANAL FIN
Adr	anal distal radial
Apr	anal primordial rays
Apxr	anal proximal radial
Asr	anal soft rays
As	anal stay
	CAUDAL FIN
aCH	accessory caudal hypural
CH	caudal hypural
Csr	caudal soft rays
Ce	caudal epiurals
Cpr	caudal primordial rays
fCb	fused caudal bones
Un	uroneural
pCsr	precaudal soft rays

	DORSAL FIN
Ddr	dorsal distal radial
Dpr	dorsal primordial rays
Dsr	dorsal soft rays
Ds	dorsal stay
pDpxr	predorsal proximal radial
pDsr	predorsal soft rays
Dpxr	dorsal proximal radial
	PELVIC FIN
Btp	basipterygium process
Bt	basipterygium
Vdr	pelvic digital radials
Mtg	metapterygium
Vpr	pelvic primordial rays
Vsr	pelvic soft rays


FD
FT
MD
MT
PECTORAL
Cl
Ppr
cs
cspp
Co
Sc
fp
Pd
SCl
pt
Psr
ScClp
Pdr
pClUp
pClLo
CAUDAL
Cpr
Csr
CH
Ce
pCsr
Un
aCH
fCb
DORSAL
Dpr
Dsr
Dpxr
Ddr
pDsr
pDpxr
Ds
PELVIC
Vpr
Btg
Btp
Vsr
Mtg
Vdr
ANAL
Apr
Apxr
Asr
Adr
As
0
400
800
1200
1600
Accumulated Temperature Units (°days)
cartilage
unossified membranous secretion
ossified
delineated
deformity

5.5 DISCUSSION

Notwithstanding deformities, there appears to be no marked difference in skeletal morphology between different populations of Atlantic salmon with ploidy or sex status, despite differences in cellular morphology between diploid and triploid fish. The results of the current study are unexpected because it has been suggested that the difference in cell size and cell surface to volume ratio in triploid fish may be reflected in differences in the processes of skclctal cell condensation, chondrification and/or ossification, due to potential differences in cellular energetics, ionoregulation and inter-cellular communication via cell surface receptors, thereby affecting skeletal morphology (Hall and Miyake, 1995). Further, it was expected that the increased heterozygosity of triploid cells (Allendorf and Leary, 1984) may lend itself to a higher degree of phenotypic variation in these fish (Schilling and Kimmel, 1997; Kimmel, 1998). There was a high prevalence of gross abnormality observed in triploid fish sampled from the populations examined in the current study (Chapter 6); however, although determination of 'abnormal' phenotypes within the 'normal' phenotypic range is difficult to quantify and therefore inherently subjective, there appeared to be no variation in the skeletal phenotype of normal triploids compared to normal diploids. In addition, it appears that the function of the pituitary and CS tissues, as well as the possible function of the ultimobranchial body in the hormonal regulation of calcium homeostasis and osteogenic activity in Atlantic salmon, does not appear to be affected during initial bone mineralisation by the differential cell morphology in triploid fish since the onset of initial bone mineralisation did not differ with ploidy status. The proportions of intracellular amorphous calcium phosphate and mitochondrial granules (Martin and Matthews, 1970; Mathews, 1970), most likely increase in proportion with cell size in triploid fish to compensate for a decrease in cell number at the tissue level. In a similar fashion, the number of cytoplasmic processes and the volume of extracellular deposition of calcium globules (Bonucci, 1967) or vessicles filled with amorphous calcium (Bonucci,

1969; Martin and Matthews, 1970; Mathews, 1970), may increase proportionately with cell size in triploid fish.

Although the morphology and ossification of the skeleton during development up until 2350° days did not reveal any differential osteogenic activity between diploid and triploid fish, this does not preclude possible differences occurring in later development due to the effects of estradiol, growth hormones and/or corticosteroids on osteogenic activity. Estradiol has been shown to induce hypercalcemia in sexually mature fish (Mugiya and Watabe, 1977); however, an increase in estradiol levels is unlikely in sterile triploid fish (Lincoln and Scott, 1984; Benfey *et al.,* 1990). Osteogenic activity of osteoblasts can be affected by an excess or deficit of growth and thyroid hormones or corticosteroids (Palmer, 1993) and sexually mature diploid female rainbow trout *Onchorhynchus mykiss* have been shown to have higher plasma levels of growth hormone than triploids (Sumpter *et al.*, 1991). Although the results of the current study (Chapters 3 & 4) indicate corticosteroid stress response following confinement does not differ with ploidy status, chronic sources of stress and their possible effects on osteogenesis in triploid fish are unknown.

Data for inter- and intra-specific comparison of larval ontogeny in fish are rare (Fuiman, 1997). Comparison between studies indicates that the order of appearance of bones within the cranium, jaw and/or branchial apparatus of Atlantic salmon in the current study is similar to that reported in previous studies of Atlantic salmon (Saunderson, 1935), other salmonids (De Beer, 1927; Jollie, 1984), and other species, including Atlantic Halibut *Hippoglossus hippoglossus* (Morrison and MacDonald, 1995), red sea bream *Pagrus major* (Matsuoka, 1985, 1987), red snapper, *Lutjanus argentimaculatus* (Doi *et al.*, 1997) and zebra fish *Danio rerio* (Schilling and kimmel, 1997). In addition, appearance of skeletal elements of the pectoral, caudal, dorsal, anal and pelvic fins with the development (present study), followed a similar sequential pattern to those of red sea bream *Pagrus major* (Matsuoka, 1985, 1987), sea bass *Dicentrarchus labrax* L. (Marino *et al.*, 1993), sea bream *Sparus aurata* (Koumoundouros *et al.*, 1997). However, Atlantic salmon were

more advanced in development at hatching and first feeding compared to the other species listed above.

In Atlantic salmon all the skeletal elements of the jaw, with the exception of the supra-maxilla, were present at the time of first exogenous feeding (≈ 720° days post-fertilisation), whereas only a few skeletal elements of the jaw, including the Meckel's cartilage, quadrate and hyomandibular were present by first feeding in red snapper (≈ 96° days post-fertilisation, Doi *et al.*, 1997), zebra fish (≈ 85.5° days post-fertilisation, Kimmel *et al.*, 1995; Schilling and Kimmel, 1997) and red sea bream (≈ 3.0 mm TL, Matsuoka, 1985, 1987). The development of skeletal structures in the jaw of Atlantic salmon preceded the development of feeding ability, whereas the development of skeletal structures in the jaw of red snapper and red sea bream occurred in conjunction with the development of feeding ability (Doi *et al.*, 1997; Matsuoka, 1985, 1987). In Atlantic salmon, the structure of the chondrocranium and various osseous structures of the cranium were well delineated by first feeding (≈720° days), although a few additional osseous structures differentiated following first feeding. By comparison, only a few cartilages of the head skeleton, including the trabeculae and rudimentary structures of the auditory capsules and parachordals were present by first feeding in red sea bream (Matsuoka, 1985, 1987) and zebra fish (Schilling and Kimmel, 1997), although other skeletal elements developed rapidly after feeding onset in red sea bream. Furthermore, Atlantic salmon displayed the ossified elements of the pectoral fin girdle, the delineated cartilage fin plate and soft fin rays of the pectoral fin, cartilage hypurals and soft fin rays of the caudal fin, the cartilaginous proximal radials and soft fin rays of the dorsal and anal fins, upon hatching. In comparison, sea bass, sea bream and red sea bream larvae displayed a continuous finfold upon hatching which extended dorsally from the otic capsule and ventrally from the posteroventral edge of the yolk sac (Matsuoka, 1985, 1987; Marino *et al.*, 1993; Koumoundouros *et al.*, 1997), whereas medaka *Oryzias latipes* displayed rudiments of the cartilagenous epiurals and some fin rays of the caudal fin (Ishikawa, 1990). By first feeding, the cliethrum and pectoral fin plate cartilages were present in red sea bream (Matsuoka, 1985, 1987), sea bass (Marino *et al.*, 1993) and sea bream (Koumoundouros *et al.*, 1997),

with the addition of a few of the caudal hypurals and fin ray structures in the latter species. In species other than Atlantic salmon, further differentiation and ossification of fin structures occurred following first feeding, improving the function of fins during active swimming (Matsuoka, 1987). It has been suggested that the completion of caudal fin development is associated with acquisition of swimming function during early development (Marino *et al.*, 1993), whereas development of the other fins, including the pelvic and anal fins, may facilitate more complex functions and advanced swimming ability (Matsuoka, 1985). Obviously there are selective advantages in the development of skeletal form preceding function (Fuiman, 1997; Matsuoka, 1985,1987).

It appears that the period of skeletal ontogeny is relatively long for Atlantic salmon compared to that of other species. The extent of jaw ontogeny over a period of ≈ 366° days in Atlantic salmon held at 8° C in the current study was similar to that reached over a period of ≈ 171° days in red snapper held at 30 - 32° C (Doi *et al.*, 1997). The difference in the period of ontogeny between these species may in part be due to differences in rearing temperature, since lower temperatures have been shown to retard development (Gray, 1928; Polo *et al.*, 1991). However, the period of ontogeny of the jaw in Atlantic salmon in the current study may be longer than that of Atlantic Halibut held at 5° C (Morrison and MacDonald, 1995), despite the higher rearing temperature of Atlantic salmon, as indicated by the later initial appearance of the Meckel's cartilage, hyomandibular and quadrate cartilages in the salmon (340° days) compared to the halibut (115° days). In addition, the time of first feeding in Atlantic salmon (≈ 720° days) is later than that in Atlantic halibut at 144 - 157° days (reviewed by Morrison and MacDonald, 1995), and although the time of first feeding does not necessarily reflect jaw ontogeny in Atlantic halibut, ontogeny of all major organs in Atlantic halibut is complete by ≈ 280° days (reviewed by Morrison and MacDonald, 1995). It has been suggested that fishes hatched from small pelagic eggs tend to be less advanced in ontogeny (Matsuoka, 1987) and flatfish, including Atlantic halibut, display delayed ontogeny compared to other pelagic marine species (Fuiman, 1997). In light of the above comparisons, it appears that Atlantic salmon have a relatively long period of ontogeny compared to other species. The difference

in the period of ontological development between species most likely reflects differences in physiological adaptation to their respective habitats and lifestyles (Fuiman, 1997).

Each step in skeletal development, from mesenchymal condensation onwards is susceptible to disruption, and abnormalities may occur in the absence of appropriate genetic determinants, hormonal environment, nutritional requirements and mechanical stimuli (Palmer, 1993). Deformities of the skeletal elements of the jaw suspensorium and caudal fin in the current study that were not apparent externally were most likely the result of abnormal processes during cell condensation and/or mutation of homeiotic genes (reviewed by Hall and Miyake, 1995; Kimmel *et al.*, 1998). The morphology of cell condensations in zebra fish (Schilling and Kimmel, 1997; Kimmel *et al.* 1998) and other vertebrates (Kessel and Gruss, 1990; Hall and Miyake, 1995; Matzuk *et al.*, 1995; Parr and McMahon, 1995) has been shown to be under genetic control in conjunction with the action of molecular factors, including growth factors. The abnormal division of the symplectic from the hyomandibular in Atlantic salmon in the current study was similar in appearance to the rudimentary cell aggregations for the symplectic and hyomandibular reported in zebra fish, which are separate prior to a normal fusion event during ontogeny (Kimmel *et al.*, 1998). Therefore it is possible that the latter abnormality represents an earlier stage of cartilage formation, however, it is likely that cell proliferation within the symplectic and hyomandibular cartilages of abnormal Atlantic salmon was interrupted during ontogeny, precluding the normal fusion of these processes. Abnormal development of skeletal structures of the caudal fin in zebrafish (Joly *et al.*, 1992) and medaka *Oryzias latipes* (Ishikawa, 1990), has been shown to be the result of genetic mutation.

Previous studies have described the development of the cranium, jaw and branchial skeleton of Atlantic salmon (Parker, 1873; Saunderson, 1935) and other salmonids (De Beer, 1927; Verraes, 1974a, 1974b; Jollie, 1984) up until 6 weeks post-hatching or 100 mm TL; however, these studies do not describe the development of the whole skeleton. Furthermore, the authors have used fish body length or days pre- and post-

hatching as a measure of the stage of development of the fish. The latter creates ambiguity as to the sequence of events and precludes inter-study comparisons, since it has been shown that fish size and/or age are not directly related to the state of maturity (Jollie, 1984; Fuiman, 1997). The present study is the most comprehensive to date as it spans the developmental period 0 - 2350° days (≈ days post-fertilisation) and is the first to report osteological development of Atlantic salmon in temperature adjusted units. This facilitates intra-species, inter-species, inter-population and intra-population comparisons. Further, it provides a definitive study for the evaluation of various developmental milestones for this species, according to inflection points of morphometric measurements. For example, in both diploid and triploid Atlantic salmon, the full complement of fin ray number and/or the segmentation of the principal rays in all fins were observed at 913° days, marking the shift between larva and juvenile stages of development (reviewed by Matsuoka, 1987). In diploid and triploid Atlantic salmon, the formation of caudal hypural accessory cartilages and ossification of the middle radials of the dorsal and anal fin supports occurred by 1510° days. The latter features have previously been used to distinguish the end of the juvenile phase in teleosts, at which point skeletal morphogenesis is complete and, with the exception of reproductive organs, rod photoreceptors, lateral line canals, scales and, in flatfish, complete migration of the eye (Fuiman, 1997), fish have obtained an adult state (Matsuoka, 1987). The present study provides intimate knowledge of normal skeletal structure of Atlantic salmon, which is a pre-requisite for proceeding to defining and examining abnormality.