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ZEBRAFISH AND MEDAKA: MODEL ORGANISMS FOR A COMPARATIVE DEVELOPMENTAL APPROACH OF BRAIN ASYMMETRY

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Se informa a la Escuela de Postgrado de la Facultad de Ciencias que la tesis de Magíster presentada por la candidata.

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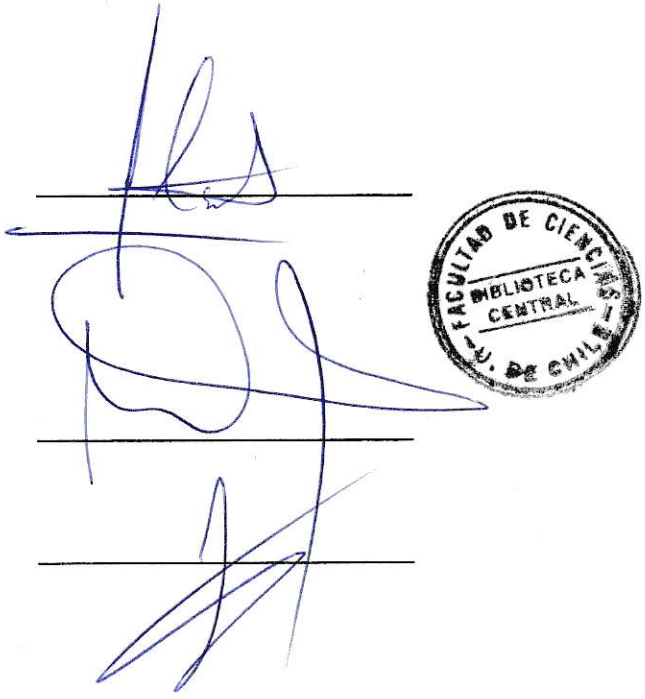

Todas las metas en mi vida siempre serán para mi Madre

Quiero agradecer

Al Laboratorio de Estudios Ontogénicos
A Alicia por su experiencia y cultura
A Karina por ser de verdad
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A Miguel por ser quien me hace construir cada vez más dimensiones para entender el Mundo

A.

TABLE OF CONTENTS

INDEX OF TABLE

INDEX OF FIGURES

ABREVIATIONS

L:	left
R:	right
TGFß:	transforming growth factor beta
LPM:	lateral plate mesoderm
IPN:	interpeduncular nucleus
GFP:	green fluorescent protein
DNA:	deoxyribonucleic acid
mRNA:	messenger ribonucleic acid
PFA:	paraformaldehyde
PBS:	phosphate buffered saline
TCA:	tricholoracetic acid
HPF:	hours post fertilisation
ST. :	stage of development

ABSTRACT

Comparison between related species is a successful approach to uncover conserved and divergent principles of development. Here we studied the pattern of epithalamic asymmetry in zebrafish (Danio rerio) and medaka (Oryzias latipes), two related teleost species with 115-200 million-years of independent evolution.

We found that these species share a strikingly conserved overall pattern of asymmetry in the parapineal-habenular-interpeduncular system. Nodal signalling exhibits comparable spatial and temporal asymmetric expression in the presumptive epithalamus preceding the development of morphological asymmetries. Neuroanatomical asymmetries consist of left-sided asymmetric positioning and connectivity of the parapineal organ, enlargement of neuropil in the left habenula compared to the right habenula and segregation of left-right habenular efferents along the dorso-ventral axis of the interpeduncular nucleus.

Despite the overall conservation of asymmetry, we observed heterotopic changes in the topology of parapineal efferent connectivity, heterochronic shifts in the timing of developmental events underlying the establishment of asymmetry, and divergent degrees of canalisation of embryo laterality. We offer new tools for developmental time comparison among species and propose, for each of these transformations, novel hypotheses of ontogenic mechanisms that explain inter-species variations that can be tested experimentally. Together, these findings highlight the usefulness of zebrafish and medaka as comparative models to study the developmental mechanisms of epithalamic asymmetry in vertebrates.

INTRODUCTION

Left-Right Asymmetry in Vertebrates

Most animals with few exceptions (sponges, cnidarians and comb jellies) are members of Bilateria and have a body-plan defined by anterior to posterior (AP) and dorsal to ventral (DV) axes. Geometrically, the intersection of these two axes results in a midline and, consequently, in two additional medio-lateral axes that lead to a bilaterally symmetric body.

However, bilateral symmetry is only conceptually true since the morphology of the two medio-lateral axes diverge during development. This phenomenon is known as left-right asymmetry and is classified into three types (Boorman & Shimeld 2002). *Fluctuating asymmetry* refers to a not heritable character that develops accordingly to differential environmental perturbation or/and behavioural stimuli. Asymmetry is absent if perturbation or behaviour is not present (Govind 1992; Palmer 2004). *Anti-symmetry* describes a morphological difference between left and right sides, which is heritable but has a random laterality (directionality). This random laterality determines that left and right phenotypes are equally frequent in the population. Finally, directional asymmetry refers to an inheritable asymmetric character with inheritable laterality that leads to a consistently prevailing laterality phenotype (left or right) at the population level (Boorman & Shimeld 2002). This study is framed within the third class of asymmetry.

Brain Asymmetry

In vertebrates, the heart and gut are asymmetrically distributed along the midline and the brain develops conspicuous differences between both hemispheres (Bisgrove et al. 2003; Burdine & Schier 2000; Capdevila et al. 2000; Hamada et al. 2002; Mercola & Levin 2001; Schier & Shen 2000; Schier 2003). Furthermore, asymmetry is a fundamental and conserved feature of the brain that is thought to enhance information processing and task performance in behaviours central for species survival, such as feeding and predator detection (Güntürkün et al 2000; Rogers 2000; Vallortigara & Rogers 2005). In addition, asymmetry has been proposed as the basis of speech and other behavioural traits (Hutsler & Galuske 2003; Rogers & Andrew 2002; Sherman et al 1982; Toga & Thompson 2003) and abnormal asymmetry appears to associate with several neuropathologies including schizophrenia (Li et al 2007), autism (Escalante-Mead et al 2003) and neuronal degenerative diseases (Toth et al 2004).

In the last decade, experimental studies have provided valuable insights into the developmental basis of brain asymmetry. Particularly helpful have been genetic model organisms that allow a comprehensive bottom-up (gene to behaviour) study of this phenomenon (Concha 2004). For example, recent work in the teleost zebrafish has unveiled genetic mechanisms that control the development of neuroanatomical asymmetries (reviewed in Concha 2004; Halpern et al 2003) and established the first operational links between genetics, asymmetric morphology and lateralised behaviours (Barth et al 2005).

Left–Right Asymmetry in the epithalamus of zebrafish

One of the best-studied cases of brain asymmetry is observed in the epithalamus of vertebrates (Concha & Wilson 2001). In zebrafish, epithalamic asymmetry is established through a sequence of developmental modules. Initially, asymmetry (structural differences between left and right sides) and laterality (directionality of asymmetry) are determined by the coordinated activity of members of the Fibroblast Growth Factor (Regan et al. submitted) and Nodal (Concha et al. 2000) signalling pathways, respectively. Then, a sequential program of asymmetric morphogenesis generates neuroanatomical asymmetries in the epithalamic pineal complex and habenulae.

Early asymmetric morphogenesis of the photoreceptive parapineal organ involves an initial phase of migration from the dorsal midline to the left side of the brain followed by development of efferent connectivity directed to the left habenula (Concha et al. 2003). Subsequent habenular asymmetries are characterised by differential growth of sub-nuclei (Aizawa et al. 2005; Aizawa et al. 2007; Gamse et al. 2005) and neuropil domains (Concha et al. 2000) between the left and right sides. Finally, the underlying habenular asymmetries result in segregation of left and right habenular efferents along the dorso-ventral axis of the interpeduncular nucleus in the ventral midbrain (Aizawa et al. 2005; Gamse et al. 2005).

Three main aspects are important to highlight about the development of epithalamic asymmetries. First, genetic pathways that establish asymmetry are autonomous from those that control the laterality (Concha et al. 2000). Such

independence in the developmental control of asymmetry and laterality makes the epithalamus of zebrafish an attractive vertebrate model to study the ontogenic mechanisms that underlie directional asymmetries, in which most individuals are asymmetrical in the same direction within the population (Palmer 2004). Second, laterality of epithalamic asymmetry is coupled to laterality of visceral asymmetry (Concha et al. 2000) in contrast to other structural and functional asymmetries of the vertebrate brain, e.g. asymmetries associated to speech and handedness (Kennedy et al. 1999; Tanaka et al. 1999; Torgersen 1950). This indicates that asymmetries controlled by independent mechanisms co-exist in the vertebrate brain. Finally, epithalamic asymmetries are immersed in an evolutionary conserved circuit involved in limbic-system related responses (Klemm 2004; Sutherland 1982) that has been implicated in the origin of neuropsychiatric disorders (Ellison 1994; Sandyk 1991).

Altogether, these observations underscore the relevance of understanding the evolutionary origin, genetic control, circuit configuration and behavioural correlates of epithalamic asymmetry to begin dissecting general principles of directional asymmetries and the specific role of the epithalamus and its associated asymmetric circuit in normality and pathology.

Epithalamic Asymmetry: a comparative approach

Recent comparative surveys reveal a striking conservation of epithalamic asymmetry among a wide range of vertebrate species (Concha & Wilson 2001; Guglielmotti & Cristino 2006). However, the lack of systematic comparative analyses addressing the genetic and developmental basis hampers the examination of general principles of epithalamic asymmetry development.

Teleosts have a unique potential in this context, since the number of teleost species far exceeds that of any other kind of vertebrate. In addition, teleost radiation suffered whole-mount genome duplication 345 million years ago (Christoffels et al. 2004). Consequently, duplicated genes could acquire new functions (neo-functionalisation) or share the original complex function (sub-functionalisation), complementing one another (Furutani-Seiki & Wittbrodt 2004).

In this context, the emergence of zebrafish and medaka as complementary models suitable for comparative developmental approaches (Furutani-Seiki & Wittbrodt 2004) offer a unique opportunity to start elucidating the conserved and divergent principles of epithalamic asymmetry development. As lineages of zebrafish (*Danio rerio*, Order Cypriniformes) and medaka (*Oryzias latipes*, Order Beloniformes) diverged 115-200 million years ago, comparison has the potential to unveil those aspects that represent the backbone of epithalamic asymmetry and those which are subjected to evolutionary variation.

From this background, we formulated the following hypothesis:

HYPOTHESIS

Zebrafish and medaka share general developmental mechanisms and at the same time diverge in species-specific traits during the establishment of epithalamic left-right asymmetry.

MAIN AIM

The main aim of this work is to carry out a first systematic inter-species comparison of brain asymmetry development in teleosts in order to identify shared and divergent traits between zebrafish and medaka.

SPECIFIC AIMS

1. Analyse and compare the morpho-topological organisation of epithalamic asymmetry in zebrafish and medaka, from molecular to connectivity level.

2. Propose a novel method for time normalisation based on the rate of somitogenesis in order to study and compare the temporal organisation of developmental modules involved in the establishment of epithalamic asymmetry in zebrafish and medaka.

MATERIAL AND METHODS

Fish lines

Zebrafish (Danio rerio) lines used in this study were wild type Tübingen and *Tg(foxD3::GFP)*. This transgenic line expresses green fluorescent protein (GFP) in the pineal complex (Gilmour et al. 2002).

Medaka (Oryzias latipes) lines were wild type Cab and two transgenic lines that express GFP in developmental epithalamus. *Tg(fRx2::GFP)* embryos show GFP expression in the pineal complex while in *Tg(fRx2/DE::GFP)* GFP is also expressed in habenulae (Grabher et al. 2003; Wittbrodt et al. 2002). Medaka strains were kindly provided by Dr. Jochen Wittbrodt Group, EMBL, Heidelberg, Germany.

Embryos and fry were obtained by natural spawning, raised at 26-28°C in Standard embryo medium (zebrafish: E3 medium; medaka: ERM medium), and staged according to morphology (Iwamatsu 2004; Kimmel et al. 1995) and age (hours post fertilisation (hpf).

Strains used in this study are routinely raised and maintained at the Aquarium facilities of Experimental Ontogeny Lab, Anatomy and Developmental Biology Program, ICBM, Universidad de Chile.

Embryos media:

E3: 5mM NaCl; 0.17mM KCl; 0.33mM $CaCl_2 \cdot 2H_2O$; 0.33mM $MgSO_4 \cdot 7H_2O$

ERM: 17.1 mM NaCl; 0.4mM KCl; 0.27mM $CaCl_2 \cdot 2H_2O$; 0.66mM $MgSO_4 \cdot 7H_2O$

Fixation protocol for zebrafish and medaka embryos.

For *in situ* hybridisation, embryos and larvae were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C, mechanically dechorionated, and stored in pure methanol at -20°C.

For Nissl and To-pro staining and labelling with lipophilic tracers, embryos were fixed in 4% PFA/PBS as described before. In this case embryos were not dehydrated with methanol and were stored in 0.5% PFA/PBS at 4°C up to two weeks.

For indirect immunofluorescence experiments, fixative agent was trichloroacetic acid. Embryos were immersed in fixative for 1.5 hours (medaka) or 3 hours (zebrafish) at room temperature. Embryos were washed in PBTr and chorion was mechanically removed. TCA fixed embryos were stored at 4°C in PBTr and darkness for two weeks.

Fixative and Buffer solutions:

PBS:	10mM Na2HPO4, 1.75mM KH2 PO4, 13.7mM NaCl, and 2.65mM KCl
PFA:	4% Paraformaldehyde in PBS
TCA:	2% Trichloroacetic acid in PBS
PBTr:	1x PBS, 0.5% Triton

Whole mount *in situ* hybridisation

We generated RNA antisense probes to detect expression of *Dr-lefty1* (Bisgrove et al. 1999; Thisse & Thisse 1999), *Dr-pitx2c* (Essner et al. 2000), *Ol-lefty1* (Soroldoni et al. 2007) and *Ol-pitx2* (Jaszczyszyn et al. 2007).

Linear DNA template for *in vitro* transcription was obtained by digestion of plasmids that are currently available in our lab. All targeted genes were cloned into pCRII (Invitrogen) which has a cloning site flanked by SP6 and T7 promoters. To obtain an antisense riboprobe, the plasmids were digested with a restriction enzyme that cut the vector near the 5' region of the gene but did not recognise any site within the insert or in the promoter sequence located upstream of 3' end of the gene.

In vitro transcription was performed at 37°C during 6 hours in the presence of digoxigenin-UTP using either SP6 or T7. The digoxigenin-labelled RNA was column-purified, re-suspended in nanopure water and stored in formamide at -20°C.

In situ hybridisation was performed according to standard protocols for medaka (Loosli et al. 1998) and zebrafish (Westerfield 1996). In order to permeabilise embryos, Proteinase K (10µg/mL) treatment was performed. Duration of enzymatic treatment was empirically determined and is showed in Table 1.

Table 1. Incubation Time in PK (10µg/mL) during permeabilisation step in whole mount- ISH

ZEBRAFISH		MEDAKA	
24 hpf	10 min	St.17 - St.20	10 min
30 hpf	30 min	St.21 - St.28	30 min
48 hpf	1 hour	St.28 - St.34	1 hour
96 hpf	3 hours	St.34 - St.37	2 hours
120 hpf	4 hours	St.34 - St.39	4 hours

Probe hybridisation step was carried out at 65°C overnight, and BM-Purple (Roche) was used as substrate for the coloured reaction of alkaline phosphatase. Embryos were mounted in glycerol for microscopic observation and photographed in a Nikon Eclipse 80i, 40x objective.

In vitro transcription and In situ hybridisation reagents

Probe Quant G-50 micro columns	--	Amersham Bioscience	(cat n° 275335)
DIG-RNA Labeling Mix	--	Roche	(cat n° 11277073910)
Proteinase K	--	Sigma	(cat n° P6556)
Blocking Reagent	--	Roche	(cat n° 11096176001)
Anti-Dig-AP Antibody	--	Roche	(cat n° 11093274910)
BMP Purple AP-substrate	--	Roche	(cat n° 11442074001)

In situ hybridisation solutions

PBT:	1x PBS, 0.1% Tween-20, pH 7.5
HYB- :	50% Formamide, 5xSSC 0.1%Tween-20, pH 6.0
HYB+ :	HYB- , 5mg/mL RNA de torula (yeast), 50µg/mL Heparin
SCC:	150mM NaCl , 15mM Sodium citrate, pH 7
MAB:	100mM Malic acid, 150mM NaCl, pH 7.5
BCL:	0.1M TrisCl -pH9.5, 100mM NaCl, 50mM $MgCl_2$, 0.1% Tween-20

Whole mount indirect immunofluorescence

Whole mount immunostaining was performed as described in Concha et al. 2003. Larvae fixed in TCA were permeabilised using a 0.25% trypsin in PBS solution. Reaction was allowed to proceed on ice for 12 minutes, and stopped with repeated

wash in cold PBS. Non-specific epitopes were blocked by incubation in IB solution for two hours, prior to overnight incubation with primary and secondary antibodies. After secondary antibody incubation, larvae were washed with PBTr and kept in darkness. Embryos were mounted in agarose-filled chambers for microscopic observation.

Indirect immunofluorescence solutions

PBTr:	1x PBS, 0.5% Triton
PBDTr:	1x PBS, 0.8% Triton, 1% Dimethyl sulfoxide (DMSO)
Tris/PBS:	1x PBS, 0.25% Trypsin
IB:	10% Goat serum in PBDTr

Primary antibodies

Rabbit Anti-GFP	(polyclonal, ABcam)
Mouse Anti-acetylated α-tubulin	(monoclonal, Sigma)

Work concentration for primary antibodies was 1:1000.

Secondary antibodies

Alexa 488_antirabbit IgG	(Molecular Probes)
Alexa 647_antimouse IgG	(Molecular Probes)

Work concentration for secondary antibodies was 1:200.

Fluorescent Nissl and To-Pro staining

The presence of Nissl substance is a characteristic feature of neuronal cell. These granular bodies are rough endoplasmic reticulum and can be seen in the soma and dendrites of neurons. Thus, Nissl staining allows the analysis of cytoarchitectonic organisation. Fluorescent Nissl stain comprised an overnight incubation of larvae with Neurotrace (1:200 in PBS) at 4°C.

To-Pro 3 iodide is a fluorescent dye for nuclear counterstaining due to its strong affinity for double strained nucleic acid. Larvae was incubated in 1:1000 To-Pro in PBS solution for 1 hour at room temperature, washed in PBTr and mounted for microscopic observation. No permeabilisation step was needed.

Labelling of habenular efferent projections

In order to analyse the organisation of habenular efferent connectivity at interpeduncular nucleus (IPN) level, differential labelling with lipophilic carbocianine dyes was performed. Larvae were fixed in PFA as described before, and the skin covering the brain and eyes were manually removed. Crystals of lipophilic dyes DiD and DiO were applied either in left or right habenula using tungsten needles connected to a micromanipulator (Aizawa et al. 2005). Labelled larvae were incubated in 0.5% PFA/PBS at 4°C for 2 days in darkness to allow lipophilic dyes reach the IPN trough fasciculus retroflexus.

Since dyes have a different emission spectra, efferent projection of each habenular nucleus can be unequivocally identified at IPN level.

Fluorescent Dyes

NeuroTrace red 530/615	--	Molecular Probes	(cat N-21482)
To-Pro-3 iodide red 642/661	--	Molecular Probes	(cat n° T3605)
DiO green, 488/510	--	Molecular Probes	(cat n° D275)
DiD red, 644/665	--	Molecular Probes	(cat n° D307)

Image acquisition, processing and 3-dimensional reconstruction

Fluorescent samples were imaged on either Zeiss LSM 5 Pascal confocal or UltraView RS spinning disk (Perkin Elmer) microscopes using an Achroplan 40x/0.8 W dipping objective or a Plan-Apochromat 40x/1.2 W objective. Images were deconvolved to reduce blurring and noise using Huygens Professional and Scripting Deconvolution Software (SVI). 3-dimensional image projections were obtained using the opacity reconstruction model in Volocity software (Improvision).

Microscopy and digital photography equipment

Nikon SMZ645 stereoscopic zoom microscope

Nikon SMZ1500 stereoscopic zoom microscope with epi-fluorescence attachment

Nikon Eclipse 80i DIC-Nomarski microscope

Nikon Coolpix 4500 Digital camara

Zeiss LSM 5 Pascal Confocal microscope

Perkin Helmer Ultraview RS Spinning disk microscope

Rationale and methodology for normalisation of developmental time

According to a hypothetical model of developmental time control, the overall rate of embryo development depends of both intrinsic clock and temperature sensitive mass-specific metabolic rate signals (Reiss 2003).

Zebrafish and medaka exhibit similar size of embryos, larvae and adults and likely share comparable mass-specific metabolic rates. To avoid the influence of temperature upon this variable, we considered the timing of onset and offset of developmental events at a single constant temperature (26°C). In zebrafish, developmental events were determined as hpf at 28°C and then scaled to hpf at 26°C according to Kimmel *et al* (1995). In medaka, timing of developmental events were expressed as hpf at 26°C using the Iwamatsu developmental stage table (Iwamatsu 2004).

To scale the influence of the internal clock we normalised absolute times based on the rate of somitogenesis. This periodic segmentation process is known to be controlled by a molecular clock linked to oscillatory gene expression (Freitas *et al.* 2005; Saga & Takeda 2001) that depends on the rates of transcription and translation (Giudicelli & Lewis 2004).

We considered the time needed for making a single somite during the linear phase of somitogenesis as a time normalising factor, and expressed the newly calculated normalised times in *somite time units* (s.t.u.). The calculation method used available data on the rate of somitogenesis at 26°C in zebrafish (Kimmel *et al.* 1995) and medaka (Iwamatsu 2004). Somite-number versus time was plotted using

OriginPro v.7.0220. The linear phase of somitogenesis extended between 4 and 30 somites for both species, and the total number of somites formed was 34 and 35 for zebrafish and medaka, respectively. Linear regression of the data revealed that zebrafish and medaka form 1.7 and 0.797 somites per hour, respectively. The reciprocal of the slope values indicated the time needed for making a single somite (*t-1som*) in both species. Normalised times of development were obtained by dividing absolute time by *t-1som*.

RESULTS

Morphological and topological organisation of epithalamic asymmetries

Asymmetric expression of Nodal signalling genes in the embryonic epithalamus

In zebrafish, several components of the Nodal signalling pathway are asymmetrically expressed in the epithalamus preceding the onset of asymmetric morphogenesis (Concha et al. 2000; Liang et al. 2000). For example, Nodal inhibitor *lefty1* (*Dr-lefty1*) and the downstream transcriptional effector *pitx2c* (*Dr-pitx2c*) define restricted dorsal domains of expression in the left side of the neural tube, posterior to the lateral flexure of the diencephalic ventricle (figure 1a,b).

We performed whole-mount *in situ* hybridisation to analyse the asymmetric activation of Nodal signaling pathway in the embryonic epithalamus of zebrafish and medaka. Our results show that *Ol-lefty1* and *Ol-pitx2* also display asymmetric expression in the dorsal diencephalon, in agreement with recent reports (Carl et al. 2007; Soroldoni et al. 2007; Jaszczyszyn et al. 2007). A close examination indicates that the extent and topology of expression of these genes are similar to zebrafish (compare figure 1a,a' and 1b,b').

Asymmetric positioning and connectivity of the embryonic parapineal organ

In zebrafish, asymmetric morphogenesis of the parapineal organ involves an initial phase of migration from the dorsal midline to the left side of the brain followed by development of efferent connectivity directed to the left habenula (Concha et al. 2003). Confocal imaging of transgenic *Tg(foxD3::GFP)* zebrafish embryos reveal that the parapineal organ is located on the left and ventral side of the pineal organ, and send axonal projections that distribute broadly in the left habenula (figure 1c).

In medaka, the parapineal organ of transgenic *Tg(fRx2::GFP)* embryos also becomes positioned and develops efferent connectivity directed to the left habenula (figure 1c'). However, the volume of the parapineal organ compared to the pineal organ appears to be considerably larger in medaka than in zebrafish (compare figure *1c,c'*).

To quantify and compare volumetric parapineal/pineal ratio, we used 3d-reconstruction from manually segmented confocal z-stacks of *Tg(fRx2::GFP)* and *Tg(foxD3::GFP)* embryos and measured that the relation in medaka is 0.6/1±0.16, n=3 embryos, (mean±s.d) and 0.1/1±0.02, n=3 in zebrafish.

In addition, parapineal efferents in medaka form a thick and long bundle of axons that make an orthogonal turn towards anterior, dorsal and the midline to end in a well-defined neuropil domain with the shape of a dandelion seed head (figure 1*c'*).

Asymmetric cytoarchitectonic organisation of the larval habenulae

In zebrafish, left and right habenular nuclei undergo distinct patterns of neurogenesis (Aizawa et al. 2007) that result in asymmetric growth of neuropil domains (Concha et al. 2003; Gamse et al. 2003). We performed immunostaining against acetylated α-tubulin to reveal the distribution of neuropil (figure 1*d*) and fluorescent-Nissl staining to expose the spatial organisation of neuronal cell bodies (figure 1*e*). We confirmed that neuropil asymmetries are limited to a dorso-medial region of the left habenula located in the vicinity of the habenular commissure (arrows in figure 1*d*).

In medaka, neuropil asymmetries define a compact and Nissl well-delimited neuropil domain situated in the most dorso-antero-medial aspect of the left habenula (arrowheads in figure 1*d',e'*). Such a singular domain is not observed in the right habenula of medaka and is absent from both left and right habenula of zebrafish (figure 1*d-e'*).

Dorso-ventral segregation of left-right habenular efferents in the larval midbrain

The target regions of habenular neurons can be determined by labelling left and right habenular nuclei with the lipophilic dyes DiD and DiO, respectively (Aizawa et al. 2005). In zebrafish larvae, efferent connectivity from left and right habenular nuclei forms distinct and segregated ring-shape domains within dorsal and ventral regions of the IPN, respectively (figure 1f,g) (Aizawa et al. 2005; Gamse et al. 2005).

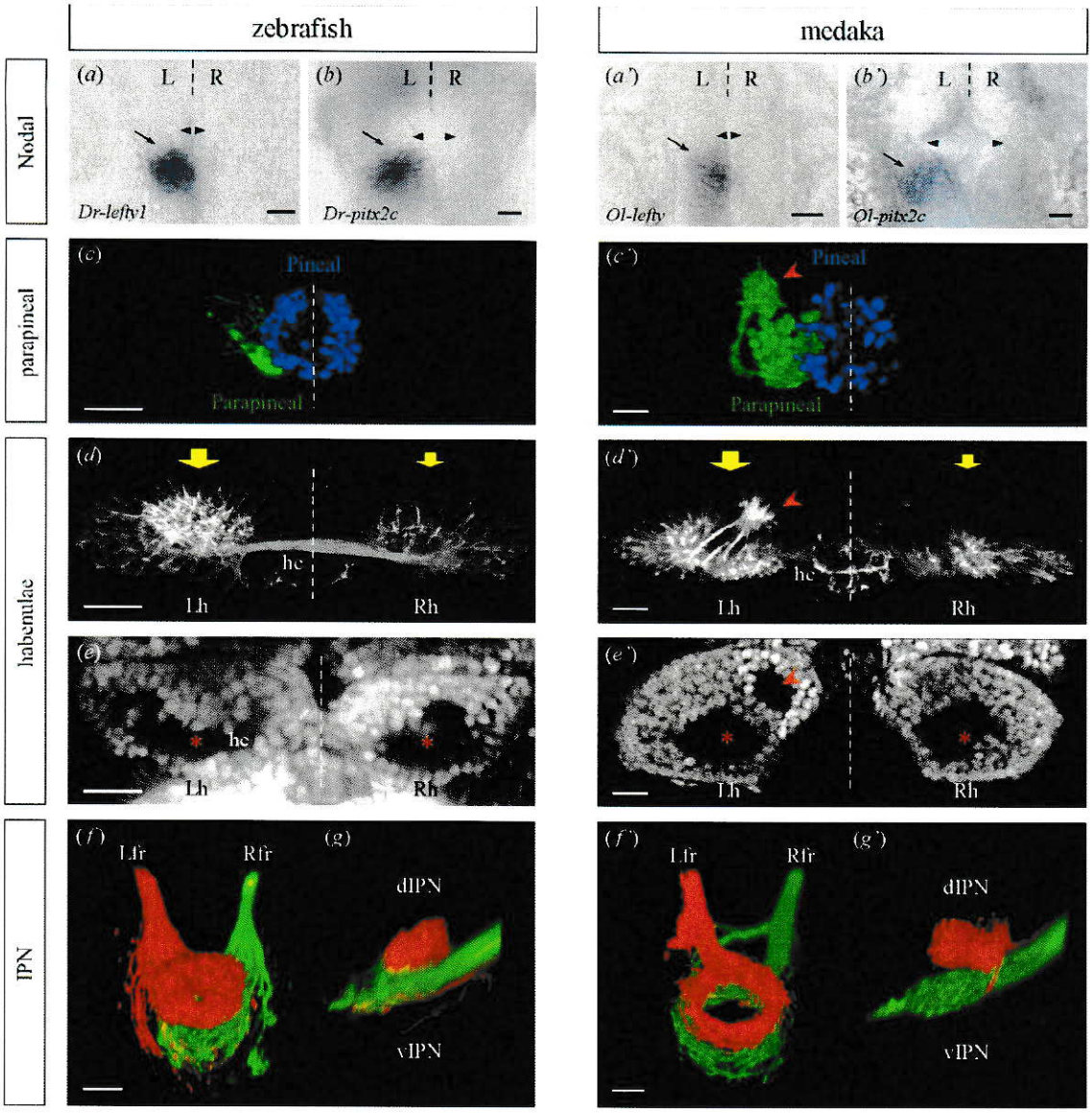
A similar pattern of habenular efferent connectivity is observed in the larval IPN of medaka (see figure 1f',g', and Carl et al. 2007). However, the central fibre-free region within IPN rings, compared to the total cross sectional area of the IPN, appear relatively larger in medaka (26.7%±3%, n=3 embryos, mean±s.d) than zebrafish (8.8±4.6%, n=3).

Figure 1 shows the compared pattern of epithalamic asymmetry in zebrafish and medaka. Images correspond to dorsal (a-f') and lateral (g, g') views with anterior and dorsal to the top, respectively. Stages of development correspond to Iwamatsu's stage 39 (medaka) and 120 hpf (zebrafish, at 26°C), unless otherwise stated. Images c to g' correspond to 3D projections from confocal z-stacks.

Following abbreviations are used : L (left), R (right), Lh (left habenula), Rh (right habenula), hc (habenular commissure), Lfr (left fasciculus retroflexus), Rfr (right fasciculus retroflexus), dIPN (dorsal domain of the interpeduncular nucleus), vIPN (ventral domain of the interpeduncular nucleus).

The scale bars used in figure 1 correspond to 20µm in (a-e, a'-e') and 30µm in (f, f', g, g').

Figure 1. Zebrafish and medaka share an overall pattern of epithalamic asymmetry.



(a-b') Asymmetric expression of Nodal signalling pathway detected by whole-mount *in situ* hybridisation (arrows) at normalised s.t.u. 35 (*Dr-lefty1*, *Ol-lefty1*) and 43 (*Dr-pitx2c*, *Ol-pitx2*) (see table 2). The lateral flexure of the third ventricle is indicated with arrowheads.

(c,c') Expression of GFP was detected in medaka *Tg(fRx2::GFP)* and zebrafish *Tg(FoxD3::GFP)* and pseudo-coloured in blue and green to label pineal and parapineal organs, respectively. The red arrowhead points to the dandelion seed head shaped domain of parapineal efferent connectivity.

(d,d') Immunostaining against acetylated α-tubulin. Arrows indicate regions of neuropil that exhibit dissimilar growth between the left and right habenula. Arrowhead points to a neuropil domain that is detected exclusively in the left habenula of medaka.

(e,e') Asterisks indicate equivalent regions of the left and right habenula. Arrowhead points to a domain devoid of fluorescent Nissl staining that is detected exclusively in the left habenula of medaka.

(f-g') Dorso-ventral segregation of left-right habenular efferents in the IPN. Left and right habenular projections were labelled with DiD (red) and DiO (green), respectively.

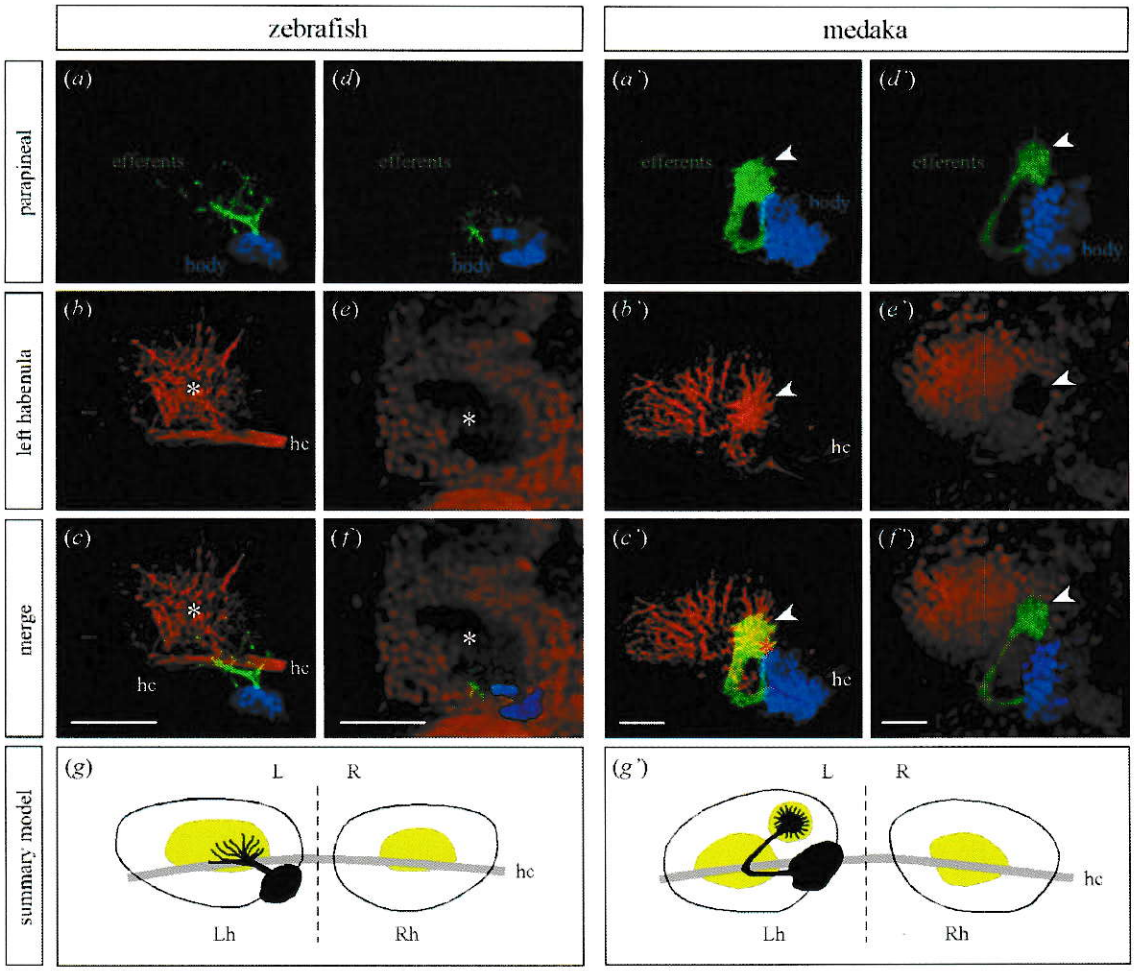
Contribution of parapineal connectivity to habenular asymmetry

Double immunostaining against acetylated α-tubulin (neuropil) and GFP (parapineal organ) in transgenic embryos reveal that parapineal efferents make a hidden morphological contribution to habenular asymmetry in zebrafish. Parapineal efferent connectivity blends into a neuropil domain situated immediately anterior to the habenular commissure that becomes asymmetrically enlarged in the left habenula compared to the right counterpart (asterisks in figure 2a-g) (Concha et al. 2003).

In contrast, parapineal efferents make a more explicit contribution to morphological asymmetry in medaka. Parapineal axonal terminals segregate from other sources of habenular neuropil to form a distinct dorso-antero-medial domain situated distant from the habenular commissure, which corresponds to the singular left-sided habenular neuropil domain defined by acetylated α-tubulin and Nissl staining (arrowheads in figure 2a'-g'; see also arrowheads in figure 1d',e').

Figure 2 show that Parapineal efferents blend into a dorso-medial neuropil domain of the left habenula in zebrafish whereas they segregate from other sources of habenular neuropil to form a distinct dorso-antero-medial in the left habenula in medaka. Panels correspond to dorsal views of the left habenula with anterior to the top. Images were obtained after 3D maximum-projections from confocal z-stacks.

Figure 2. Heterotopic parapineal efferent connectivity in the left habenula of zebrafish and medaka.



(a-f') The parapineal organ was pseudo-coloured in blue (parapineal body) and green (parapineal efferents) after immunostaining against GFP in 120 hpf zebrafish Tg(FoxD3::GFP) (a,d) and st.39 medaka Tg(fRx2::GFP) (a',d'). Distribution of neuropil and nuclei in the left habenula were detected by immunostaining against acetylated α-tubulin (b,b') and by fluorescent To-pro staining (e,e'), respectively. Merged images of double labelling are shown at the bottom panels (c,c',f,f'). Asterisks indicate nuclei-free domains of the left habenula where parapineal connectivity is distributed in zebrafish. Arrowheads point to the terminal dandelion seed head shaped domain of parapineal efferent connectivity in medaka. hc : (habenular commissure). Scale bars: 20μm.

(g-g') Summary model of parapineal efferent connectivity in zebrafish and medaka. In zebrafish, parapineal efferents distribute broadly within a large dorso-medial neuropil domain of the left habenula situated immediately anterior to the habenular commissure (g). In medaka, parapineal efferents form a thick bundle of axons that after entering the left habenula make a turn towards the midline to end in a well-defined dandelion seed head shaped neuropil domain situated in the most dorso-antero-medial aspect of the left habenula (g'). All diagrams correspond to dorsal views, with anterior to the top. The parapineal organ and its efferent connectivity are shown in black, the habenular commissure in grey, and neuropil domains in yellow. Abbreviations: L (left), R (right), Lh (left habenula), Rh (right habenula), hc (habenular commissure).

Laterality of epithalamic asymmetries and its correspondence to organ laterality

In zebrafish, the development of parapineal and habenular asymmetries is interdependent and result in larvae showing concordant laterality of epithalamic asymmetries (Concha et al. 2003; Gamse et al. 2003). In addition, laterality of epithalamic and visceral asymmetries are coupled as both depend on left-sided Nodal signalling emerging from a common symmetry-breaking event (Concha 2004; Levin 2005).

In medaka, we scored the laterality of parapineal (GFP), habenular (acetylated α-tubulin) and organ (heart looping) asymmetries and found a similar concordant laterality pattern (table 2). Surprisingly, we were unable to find a single case of reversal from normal heart laterality in three different strains of medaka (Tg(fRx2::GFP), n=317 embryos; Tg(fRx2/DE::GFP), n=153; cab, n=148). It has been reported that in other teleost species, spontaneous *situs inversus* phenotype is a common situation (5% of individuals in average) (Palmer 2004).

Table 2. Concordant laterality of brain and organ asymmetry in zebrafish and medaka

	habenular laterality *			heart jog/loop laterality #		
parapineal laterality	left (%)	right (%)	no.	normal (%)	reversed (%)	no.
zebrafish (Danio rerio)						
left	100	0	48	98	0	306
right	0	0	0	0	2	6
no.	100	0	48	98	2	312
medaka (Oryzias latipes)						
left	100	0	98	100	0	470
right	0	0	0	0	0	0
no.	100	0	98	100	0	470

* Concordant laterality of parapineal and habenular asymmetries was analysed by immunostaining against acetylated α-tubulin (habenulae) and GFP (parapineal) *in Tg(Foxd3::GFP)* (zebrafish, 140 hpf at 26°C) and *Tg(fRx2::GFP)* (medaka Iwamatsu St.39, 216 hpf at 26°C).

Concordant laterality of brain and organ asymmetries was analysed in living embryos by scoring the orientation of heart jog/looping, and the position of the parapineal organ in *Tg(Foxd3::GFP)* (zebrafish, 56 hpf at 26°C), *Tg(fRx2::GFP)* and *Tg(fRx2/DE::GFP)* (medaka, Iwamatsu St.27, 58 hpf at 26°C).

Temporal analysis of epithalamic asymmetry development

To analyse how developmental time of epithalamic asymmetry has changed during the evolution of zebrafish and medaka we compared three main aspects: *sequence* (temporal arrangement of developmental modules), *relative timing* (onset/offset of events with respect to some intrinsic time scale) and *duration* (overall rate of development).

We found that all main developmental modules were present in both species and temporally arranged in a similar *sequential* manner (figure 3*a*). For example, asymmetric Nodal expression preceded left-sided positioning of the parapineal organ, which in turn was followed by establishment of habenular asymmetry and segregation of habenular efferents in the IPN. We also found that the onset of asymmetric Nodal expression occurred at a later absolute time in medaka (36±2 hpf) than zebrafish (19.9±1 hpf), and that morphological asymmetries took longer to develop in medaka than zebrafish (figure 3*a*), in agreement with the sluggish pace of embryo development reported for medaka (Furutani-Seiki & Wittbrodt 2004).

As analysis of absolute time hampered a meaningful comparison of *relative timing* and *duration* across species (Reiss 2003), we proceed to scale absolute times to the duration of a conserved developmental process that depends on intrinsic embryo dynamics (e.g. somitogenesis) and produced a normalised time that could be compared among taxa (figure 3*b* and table 3; see description of methodology in Material and Methods).

Table 3. Comparison of developmental times of brain asymmetry between zebrafish and medaka

Developmental event		zebrafish (*Danio rerio*)			medaka (*Oryzias latipes*)		
		time (hpf)*		s.t.u.#	time (hpf)*		s.t.u.#
epithalamic Nodal expression							
lefty1	- onset	19.8±1	[17±1]	34.4±2	36±2	[St.21-22]	28.7±2
	- offset	28.5±1	[24.5±1]	49.4±2	52±2	[St.25-26]	41.4±2
	duration	8.7±1	[6.5±1]	13.4±2	16±2		12.7±2
pitx2	- onset	19.8±1	[17±1]	34.4±2	36±2	[St.21-22]	28.7±2
	- offset	54.7±1	[47±1]	94.4±2	103±3	[St.32-33]	82.5±3
	duration	34.9±1	[21.5±1]	58.4±2	67.5±3		53.8±3
parapineal and habenular asymmetry							
onset of axonal projection		57±1	[49±1][a]	98.2±2	66±8	[St.27-29]	52.6±7
habenular efferents in IPN							
initiation of connectivity Ψ		64±6	[74±7]	128±12	111±10	[St.32-33]	88.5±8

* Staging is expressed in hours post fertilisation (hpf) at 26°C (Iwamatsu 2004; Kimmel et al. 1995). Corresponding times at 28°C (zebrafish) and Iwamatsu stages (St) (medaka) are indicated in brackets.

Normalised times are expressed in somite time units (s.t.u.) (see Materials and Methods).

[a] Taken from (Concha et al. 2000; Concha et al. 2003)

Ψ Initiation of connectivity between the habenula and IPN is defined by the initial axonal branching pf left and right fasciculus retroflexus within the IPN, prior to the establishment of dorsal and ventral ring shaped domains.

Comparison of normalised developmental times among zebrafish and medaka uncovered three main groups of developmental events that reveal unexpected similarities/differences in the *relative timing*.

A first group comprised early embryonic processes whose timing of onset became highly coordinated after time normalisation. Within this group we found epiboly, gastrulation (shield formation), onset of expression of hatching enzymes genes (Inohaya et al. 1997; Inohaya et al. 1995), and somitogenesis (figure 3b).

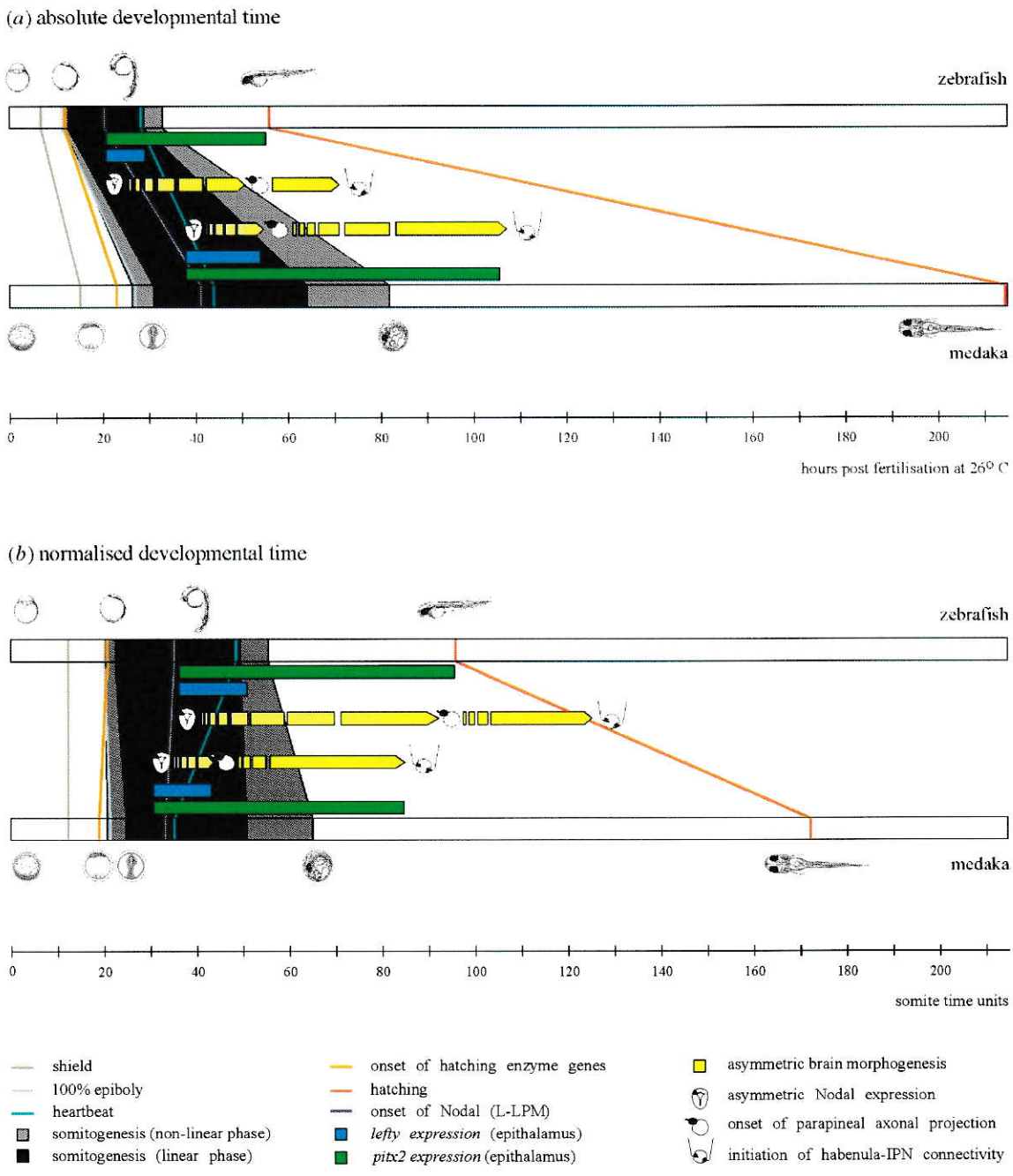
A second group included developmental events whose absolute differences in timing become inverted after time normalisation. Important examples within this group were the onset of asymmetric epithalamic Nodal signalling, onset of parapineal axonal projection, and the initiation of habenula-IPN connectivity (figure 3b). The onset of asymmetric epithalamic Nodal expression exhibited a delay of around 6 somite time units (s.t.u) toward later developmental times after normalisation, when compared to medaka (table 2 and figure 3b). Interestingly, the magnitude of this delay was comparable to the delay in the onset of heart beating (13 s.t.u.) but was considerably smaller than the temporal shift in the initiation of both parapineal axonal projection (50 s.t.u.) and habenula-IPN connectivity (40 s.t.u.) (figure 3b).

Finally, a third group included developmental events whose differences in timing were conserved after time normalisation. The single example of this group corresponded to hatching, which occurred at an earlier developmental time in zebrafish than medaka (figure 3b).

A last step of comparison concerned the *duration* of developmental events. We focused our analysis on the expression of Nodal signalling genes as they were transient

and could be determined with accuracy. Absolute duration of expression of *Ol-lefty1* (16 h) and *Ol-pitx2* (67,5 h) was considerably larger than of *Dr-lefty1* (8.7 h) and *Dr-pitx2c* (34.9 h), respectively (table 3 and figure 3*a*). However, the ratio between the lengths of *lefty1* and *pitx2* expression was equivalent in both species (zebrafish=0.25; medaka=0.24) suggesting that absolute differences in the length of gene expression could result from variations in the intrinsic speed of embryo development. To test this hypothesis we compared normalised lengths of gene expression and found them strikingly similar for each pair of orthologue genes - differences represented less than 15% for *lefty1* and 10% for *pitx2* when calculating the ratio zebrafish/medaka (figure 3*b*).

Figure 3. Comparison of *sequence*, *relative timing* and *duration* of developmental events during the establishment of epithalamic asymmetry in zebrafish and medaka.



The diagrams show the temporal occurrence of main embryonic events, the relative timing and duration of asymmetric Nodal expression in the brain and the principal steps of asymmetric morphogenesis expressed in absolute (a) and normalised (b) times. The colour codes shown at the bottom of the figure indicate the different developmental events (lines) and periods (boxes or bars) analysed in the temporal plots of a and b. For clarity, equivalent events in medaka and zebrafish are joined. Diagrams of developmental stages were obtained from the literature (Iwamatsu 2004; Kimmel et al. 1995). Schematic representations of epithalamic asymmetry events (bottom right) correspond from top to bottom to: a frontal view of the epithalamus depicting in black the asymmetric Nodal expression, a dorsal view of the pineal complex showing the left-sided location of the parapineal organ as a black circle, and the asymmetric organization of the parapineal-habenular-IPN system depicting the segregation of left right habenular efferents along the dorso-ventral axis of the IPN. The scale was maintained in both plots to emphasize the effect of time normalisation. Medaka and zebrafish show a conserved sequence of developmental events of brain asymmetry although they exhibit distinct relative timing (heterochrony).

DISCUSSION

Overall conservation of asymmetry in the parapineal-habenular-IPN system of teleosts

In this study we compared the main developmental modules of epithalamic asymmetry in two related teleost species with 115-200 million years of independent evolution. Our findings reveal a striking conservation of both the overall spatial organisation of brain asymmetry and the temporal sequential arrangement of development modules underlying to the formation of the parapineal-habenular-IPN system. Such conservative ontogenetic trajectory suggests a causal dependency between the different asymmetry modules.

This idea is supported by recent experimental evidence showing that habenular asymmetry is affected by physical removal of the parapineal organ (Bianco et al. 2008; Concha et al. 2003; Gamse et al. 2003). In addition, segregation of habenular efferents in the IPN depends on the proper development of asymmetry in the habenulae (Aizawa et al. 2005; Bianco et al. 2008; Carl et al. 2007; Gamse et al. 2005; Kuan et al. 2007).

Evolutionary conservation also suggests that the overall pattern of asymmetry in the parapineal-habenular-IPN axis is plesiomorphic to teleosts. Indeed, habenular and parapineal asymmetries are described in a number of teleost species (Concha & Wilson 2001) and recent observations extend these findings to the IPN of the southern flounder *(Paralichthys lethostigma)* (Kuan et al. 2007) and guppy *(Poecelia reticulata)* (Villalón & Concha, unpublished). Interestingly, despite the overall conservation of habenular asymmetry among a wide range of vertebrate groups (Concha & Wilson 2001) the segregation of left-right habenular efferents along the dorso-ventral axis of the IPN

appears unique to teleosts as it is absent in frogs (Rana clamitans), salamanders (Ambyostoma maculatum) and mice (Kuan et al. 2007). Whether or not this peculiar asymmetry trait represents a variation of form evolved exclusively by the teleost lineage will need further experimental testing.

Heterotopic parapineal efferent connectivity suggests divergent principles of development between zebrafish and medaka

Our results support the notion that left-sided positioning of the parapineal organ is a shared feature of asymmetric brain morphogenesis within the teleost group (Borg et al. 1983; Concha & Wilson 2001). However, the relative size of the parapineal organ (compared to the pineal organ) and its pattern of efferent connectivity greatly differ between zebrafish and medaka.

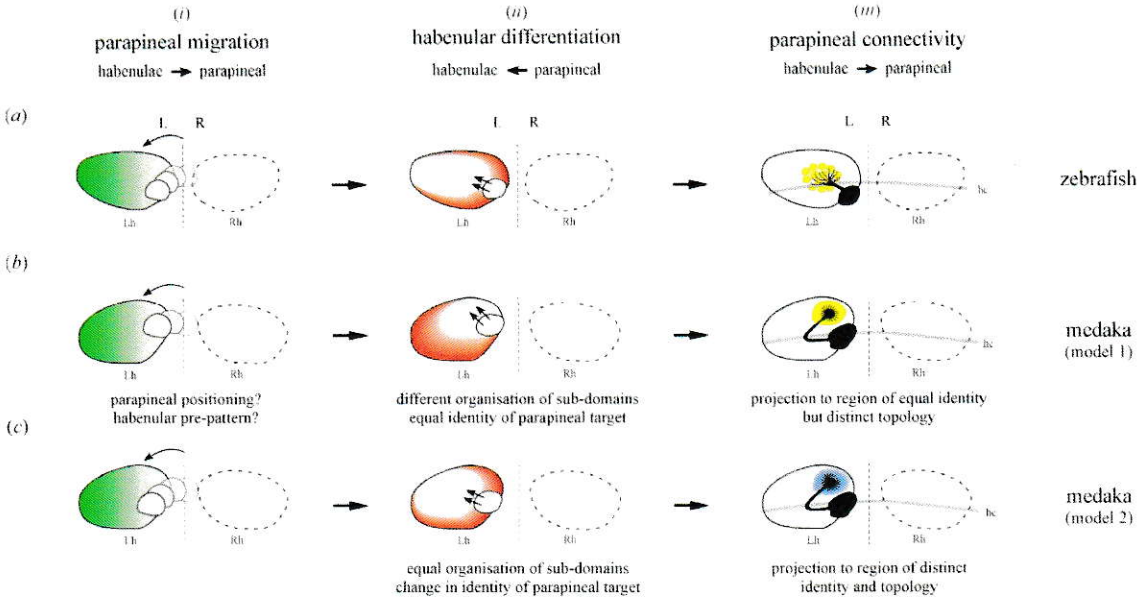
In zebrafish, the body of the parapineal organ is relatively small in size (±10% of the pineal) and its efferent connectivity distribute broadly in the left habenula. In contrast, the parapineal organ of medaka is larger (±60% of the pineal) and its efferent connectivity forms a large and well-defined antero-dorso-medial neuropil domain within the left habenula (see figure 2). Although the number and nature of parapineal-habenular synapses remains unknown, our results suggest that divergent principles of development and circuit configuration emerged during the independent evolution of zebrafish and medaka lineages.

Previous results suggest that the spatial organisation of parapineal efferents depends on a bi-directional interaction established between the parapineal organ and

habenulae during development (see figure 4). Initially, early asymmetry in the presumptive habenular region is though to guide asymmetric parapineal migration (Concha et al. 2003). Subsequent left-sided positioning of the parapineal organ is required for the amplification (and perhaps the topological setting) of distinct differentiation programs in the left and right habenula (Bianco et al. 2008; Gamse et al. 2003). Finally, parapineal axons distribute in regions of the left habenula that exhibit enlarged neuropil (Concha et al. 2003) and asymmetric leftover expression (Gamse et al. 2003) therefore linking the topology of parapineal efferent connectivity to the underlying organisation of differentiation domains within the left habenula.

Based on these observations, we propose two developmental models to explain the different topologies of parapineal efferent connectivity observed in zebrafish and medaka (figure 4). In the first model, the molecular/connectional identity of parapineal target cells is conserved in the two species but the topological organisation has diverged due to changes in the spatial and/or temporal organisation of a shared set of signals that pattern the habenulae (model 1; figure 4b). In the second model, the molecular/connectional identity of parapineal target cells has diverged as a result of divergent signalling mechanisms involved in either guiding parapineal connectivity or patterning the habenulae (model 2; figure 4c).

Figure 4. Developmental models for the emergence of heterotopic parapineal efferent connectivity during the evolution of zebrafish and medaka lineages.



(a) Zebrafish: asymmetries of the parapineal organ and habenulae interact in three consecutive steps during development. Initial left-right biases in the presumptive habenular region guide parapineal migration to the left side (i). Subsequent left-sided positioning of the parapineal organ induces a distinct pattern of sub-nuclear organisation in the left habenula (ii). Finally, the emerging topology of habenular sub-nuclear organisation determines the target region of parapineal efferent connectivity (iii) (for more details see text).

(b) Medaka (model 1): heterotopic parapineal efferent connectivity emerged from topological reorganisation of habenular sub-nuclei. In this model, differences in the spatial route of parapineal migration and/or heterochronic shifts in the timing of migration result in a parapineal organ that position differently in zebrafish and medaka with respect to the left habenula at the time of induction (i). Such differences lead to induction of distinct patterns of asymmetric habenular differentiation and as a consequence, a topological reorganisation of habenular sub-nuclei (ii). As identity of parapineal target cells is unaffected, function of the parapineal-habenular-IPN system is likely maintained.

(c) Medaka (model 2): heterotopic parapineal efferent connectivity emerged as a consequence of redefinition of parapineal target cells within the habenulae. In this model, the initial phases of parapineal migration and habenular sub-nuclear organisation are unaffected. Instead, the identity of parapineal target cells changes during the evolution of both species. Such changes may result, or be a consequence, of concomitant acquisition/loss of complementary axon guidance cues in parapineal and habenular cells. As identity of parapineal target cells changes, this evolutionary transformation likely provokes functional

changes in the parapineal-habenular-IPN system.

In column (i), shaded green regions depict molecular left-right biases within the presumptive habenulae. The movement of the parapineal organ from the midline to the left side (arrows) is represented as partially overlapping drawings of parapineal outlines. In column (ii), white and red regions illustrate the sub-nuclear organisation of the habenulae. Arrows illustrate the direction of the inductive properties of the parapineal organ. The domain of the left habenula that receives parapineal induction is shown in white. In column (iii), the topological pattern of parapineal efferent connectivity (black) and the location of parapineal target cells (colours) within the habenula are shown (see also figure 2). Equal and dissimilar colours represent equal and dissimilar identities, respectively. All diagrams correspond to dorsal views, with anterior to the top. For clarity, only the changes occurring to the left habenula are illustrated, and the location of the right habenula is depicted with dotted lines. Abbreviations: L (left), R (right), Lh (left habenula), Rh (right habenula), hc (habenular commissure).

The proposed models have potential dissimilar implications in the function of the parapineal-habenular-IPN system. Whereas solitary changes in the topology of parapineal target cells likely represent no major functional modification of the system (model 1; figure 4b) transformations in the identity of parapineal target cells might result in distinct neurotransmitter and/or connectional influences of the habenulae upon the IPN (model 2; figure 4c).

Given the overall morphological and connectional conservation of the parapineal-habenular-IPN circuit it seems reasonable to expect a conservation of parapineal functionality in the circuit (model 1). Nevertheless, it is possible that the parapineal organ plays no major role in this asymmetric circuit and that the observed phenotypic variation in the topology of parapineal efferent connectivity is a direct consequence of this feature (Hallgrímsson 2003). To date, we have no sufficient data to either sustain or discard this possibility. As the parapineal organ contains both photoneuroendocrine cells and projection neurons it is possible that circadian variations

of light influences the neuroendocrine activity of the parapineal organ and consequently the function of the habenular-IPN system (Concha & Wilson 2001). However, it has also been reported that parapineal photoreceptors are rather rudimentary (Ekstrom et al. 1983; Rudeberg 1969; van Veen 1982) and that in many species the parapineal organ appears to have regressed in the adulthood (Borg et al. 1983).

Heterochronic shifts and the ontogeny of epithalamic asymmetry

The dimension of time is critical for development and a key factor in the generation of evolutionary diversity (Gould 1977). The examination of the temporal dimension of development among species allows the study of developmental trajectories, the detection of heterochronies (shifts in timing), the making of inferences about the coupling/uncoupling of developmental modules, and the reconstruction of the ancestral sequence of developmental events (Reiss 2003; Zelditch 2003).

In the present study we searched for events of conservation and variation in each of the three main aspects of time underlying the development of epithalamic asymmetries. We found a major conservation in the *sequence* of developmental modules of brain asymmetry (see above). For a proper examination of *relative timing* and *duration* we developed a method to normalise the intrinsic time scale of zebrafish and medaka development based on the clock properties of somitogenesis. Using this normalisation method we could synchronise the *relative timing* of early embryonic events. In addition, we found that *duration* of expression of genes involved in the control of brain laterality matched after time normalisation.

This finding provides support to the utility of this normalisation method for comparison of developmental time among related species, compared to the utility of alternative methods (Chipman *et al.* 2000; Clancy *et al.* 2001; Dettlaff & Dettlaff 1961; Reiss 1989). Moreover, this observation suggests that both species share a similar *tempo* of Nodal-dependent laterality determination, and that absolute differences in the duration of Nodal signalling depend primarily on the intrinsic rate of embryo development of each species.

The normalisation method also allowed the distinction of inter-species changes in the *relative timing* of epithalamic asymmetry events. Three main heterochronic shifts involved the onset of epithalamic Nodal signalling, the onset of parapineal axonal efferent projection, and the initiation of habenula-IPN connectivity expressed as the initial axonal branching of left and right fasciculus retroflexus within the IPN (figure 3).

The direction of these shifts is consistent with previous reports suggesting that brain development is delayed relative to somitogenesis in zebrafish compared to medaka (Wittbrodt *et al.* 2002). More recent data adds extra support to this general concept as it reveals a reversal in the *relative timing* of asymmetric Nodal signalling in the brain with respect to the lateral plate mesoderm (LPM) in the two species, e.g. brain precedes LPM in medaka whereas it follows LPM in zebrafish (figure 3*b*) (Bisgrove *et al.* 2000; Rebagliati *et al.* 1998; Soroldoni *et al.* 2007). Unexpectedly, the onset of parapineal axonal projection and the initiation of habenula-IPN connectivity exhibited a pronounced heterochronic shift with respect to the onset of Nodal signalling, being largely delayed in zebrafish with respect to medaka (figure 3*b*). As parapineal connectivity appears to be linked to the program of habenular differentiation, it is possible that the latter is delayed in zebrafish, and that the more dispersed distribution

of parapineal target cells of the zebrafish larvae represents a transitional state towards a more segregated distribution reached in the habenulae at post-larval stages.

Consistent with the idea of a shift in the timing of habenular differentiation, we observed that the onset of axonal branching of habenular efferents within the IPN is also delayed in zebrafish compared to medaka (figure 3*b*). Further experimental testing of this hypothesis might provide a causal link between the heterotopic and heterochronic changes described in this study.

It is important to notice that aspects of organogenesis such as the onset of heart beating are shifted in the same temporal direction as shifts in brain development. This observation opens the possibility that organogenesis as a whole has undergone a heterochronic shift during the evolution of medaka and zebrafish lineages. In this respect, it is intriguing that hatching shows a reversed heterochronic shift to that observed for organogenesis, e.g. it is delayed in medaka compared to zebrafish. As the onset of expression of hatching enzyme genes is comparable in zebrafish and medaka (figure 3*b*) it is likely that differences in hatching time are a result of dissimilar chorion composition and thickness between the two species (Hart & Donovan 2005; Hart *et al.* 1984). Regardless of the underlying developmental mechanism, a main consequence of the heterochronic shift in hatching is the definition of zebrafish as altricial (immature) and medaka as precocial (more developed) species (MacArthur & Wilson 1967).

Is the laterality of asymmetry canalised in medaka?

Although left-sided laterality of heart asymmetry is a well-conserved trait of vertebrates, a small percentage of individuals in the population shows spontaneous reversal of this asymmetry. Incidence of heart reversals have declined during vertebrate evolution from fish (around 5%) through amphibians and birds (1-2%) to mammals (less than 0.1%), indicating a canalisation of heart laterality during vertebrate evolution (Palmer 2004). Our finding that medaka showed 0% of heart reversals indicates that this species deviates from the expected teleost pattern (e.g. zebrafish, trout and salmon).

Although we can not discard that the inbreeding nature of the medaka strains (Wittbrodt *et al.* 2002) reduces the normal fluctuation of individual laterality, it is possible that symmetry-breaking mechanisms are more robust and resistant to genetic and environmental perturbations in medaka than in other teleosts. A major mechanism of vertebrate laterality determination involves the generation of extracellular leftward fluid flow (the so-called Nodal flow) within the ventral node of mice (Hirokawa *et al.* 2006) and the Kupffer's vesicle (KV) of teleosts (Essner *et al.* 2005; Kramer-Zucker *et al.* 2005; Okada *et al.* 2005).

Recent reports reveal that the KV of medaka shares more similarities to the mammalian node than to the zebrafish KV, when considering the cytoarchitectonic organisation of ciliated cells and the robustness of the Nodal flow (Essner *et al.* 2005; Hirokawa *et al.* 2006; Kramer-Zucker *et al.* 2005; Okada *et al.* 2005; Oteiza *et al.* 2008). Hence, we propose that canalisation of embryo laterality may be linked to the morphology of laterality organs and consequently the nature of the Nodal flow they

produce. In this context, other developmental conditions that have been proposed to make laterality decisions more predictable (e.g. placental environments; Palmer 2004) would play only additive roles.

CONCLUSIONS

Since the initial proposal of medaka and zebrafish as an evolutionary twin for comparative developmental biology (Furutani-Seiki & Wittbrodt 2004), several reports have made use of the experimental and evolutionary advantages of these genetic organisms to start revealing conserved and species-specific principles of vertebrate development (e.g. Gajewski et al 2006; Lynn Lamoreux et al 2005).

The present study brings additional support to this notion, offers new tools for time comparison between these species, and provides novel comparative data and hypothesis to start addressing the ontogenic mechanisms that explain inter-species variations of epithalamic asymmetry.

Together, these findings highlight the usefulness of zebrafish and medaka as comparative tools of brain asymmetry development and function.

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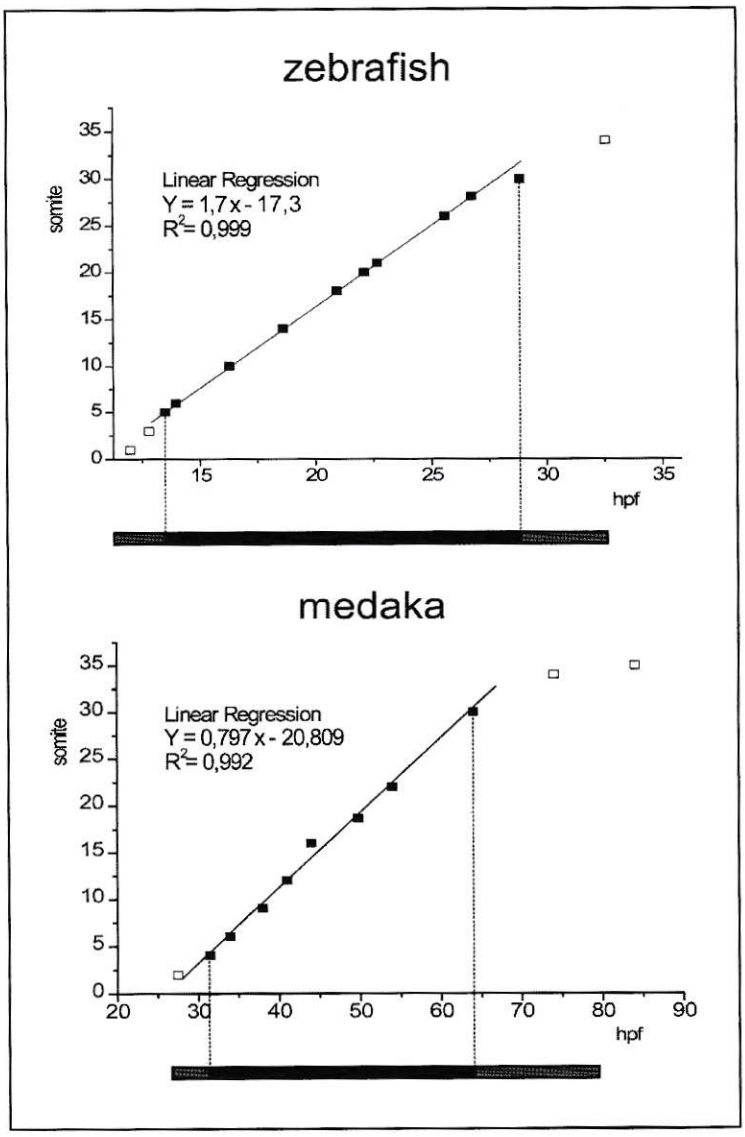
SUPPORTING INFORMATION

Linear regression data for temporal normalisation in zebrafish and medaka.



Black dots and bars correspond to the linear phase of somitogenesis. In each case, the reciprocal of the slope corresponds to the length (in hours) of one somite time unit (s.t.u).