TABLE OF CONTENTS

SOMMARIO .......................................................................................... 5
ABSTRACT ........................................................................................... 9
1. INTRODUCTION .............................................................................. 13
  1.1. Vertebrate visceral and neuroanatomical asymmetries .............. 13
   1.1.1. Visceral asymmetries ......................................................... 13
   1.1.2. Neuroanatomical asymmetries ......................................... 15
  1.2. The epithalamus and diencephalic asymmetries ...................... 17
  1.3. Epithalamic asymmetries in fish, amphibians and reptiles .......... 18
   1.3.1. Fish ................................................................................. 19
   1.3.2. Amphibians ................................................................. 21
   1.3.3. Reptiles ........................................................................... 22
   1.3.4. Birds and mammals ..................................................... 23
  1.4. Behavioral lateralization ......................................................... 24
  1.5. Development of asymmetries in the vertebrate body plan ......... 24
   1.5.1. First step: breaking symmetry ......................................... 25
   1.5.2. Second step: activation of asymmetrical gene expression cascade ... 29
   1.5.3. Third step: asymmetrical organogenesis ............................ 32
  1.6. Zebrafish as a model species .................................................. 34
  1.8. Early steps in zebrafish L-R development ................................ 39
  1.9. Zebrafish functional asymmetries ......................................... 43
2. METHODS ....................................................................................... 45
  2.1. Abbreviations .......................................................................... 45
  2.2. Methods for keeping and raising zebrafish ............................. 46
  2.3. Recipes .................................................................................... 48
   2.3.1. Whole-Mount in situ Hybridization Solutions .................. 49
   2.3.2. Solutions for bacteria transformation and RNA probe synthesis ...... 51
  2.4. Transformation of E. coli ......................................................... 52
  2.5. Purification of plasmid DNA ..................................................... 52
  2.6. Anti-sense probe synthesis ....................................................... 53
  2.7. Single-probe Whole-Mount In Situ Hybridization (WISH) ........ 55
  2.8. Mounting and imaging ........................................................... 58
  2.9. Fluorescent beads injections ................................................... 59
  2.10. Bright field screening of Kupffer’s vesicles ............................ 60
  2.11. Behavioral tests ...................................................................... 60
    2.11.1. Test subjects .................................................................. 60
L'organizzazione corporea dei vertebrati è caratterizzata da simmetria bilaterale esterna, mentre le strutture interne non presentano la stessa organizzazione ed è possibile distinguere tre tipi di asimmetrie che caratterizzano gli organi interni: asimmetrie viscerali, neuroanatomiche e funzionali. Le asimmetrie viscerali fanno riferimento ad organi impari, come il cuore o gli organi dell’apparato digerente, che sono posizionati asimmetricamente nella cavità corporea (Capdevila et al., 2000) o ad organi pari come i polmoni che, pur essendo strutture bilaterali, presentano asimmetrie morfologiche. Accanto alle asimmetrie viscerali è possibile identificare asimmetrie neuroanatomiche. Nell’uomo una struttura localizzata nell’area di Wernicke, il planum temporale, è significativamente più grande nell’emisfero sinistro rispetto all’emisfero destro (Geschwind and Levitsky, 1968). Asimmetrie neuroanatomiche sono state riscontrate anche negli uccelli, nei topi e negli scimpanzè (Cantalupo and Hopkins, 2001).

Nei vertebrati inferiori un’importante asimmetria neuroanatomica si osserva nell’epitalamo. Questa regione è costituita da due strutture: l’abenula e il complesso pineale (Concha and Wilson, 2001). L’abenula è una struttura pari, ed è formata da due nuclei bilaterali. Il complesso pineale è una struttura impari formata dall’organo pineale e dall’organo parapineale (Concha and Wilson, 2001). In molte specie di vertebrati inferiori i nuclei dell’abenula e il complesso pineale sono asimmetrici nelle dimensioni, nell’organizzazione neurale e nell’espressione genica (Gamse et al., 2005; Gamse et al., 2003). L’organo pineale non presenta rilevanti asimmetrie mentre l’organo parapineale mostra un’organizzazione asimmetrica delle sue connessioni ed è posizionato asimmetricamente all’interno dell’epitalamo (Concha and Wilson, 2001).

Numerosi studi hanno indagato i meccanismi di sviluppo delle asimmetrie corporee e i modelli proposti in letteratura suddividono il processo di formazione delle asimmetrie corporee in tre fasi principali: la prima delle quali è l’alterazione della simmetria bilaterale al termine della gastrulazione (Hamada et al., 2002). Si suppone che la prima fase sia la comparsa di un
organo transiente ciliato che nell’embrione di topo è chiamato nodo embrionale e si trova nella parte posteriore della notocorda, nello Xenopus è il tetto del gastrocele (Essner et al., 2002; Schweickert et al., 2007), e in zebrafish è chiamata vescicola di Kupffer (Brummett and Dumont, 1978; Essner et al., 2002). Le cilia di questa struttura generano una corrente di fluido direzionale che si muove verso il lato sinistro dell’embrione ed è stato ipotizzato che questo flusso nodale sposti correnti ioniche o morfogeni che vengono accumulati a sinistra dell’embrione. Lo spostamento e il conseguente aumento di concentrazione di questi determinanti attiva una cascata di espressione genica asimmetrica unicamente nel lato sinistro dell’embrione che caratterizza la seconda fase del modello proposto per la specificazione dell’asse sinistro-destro. Nella terza fase, questo segnale asimmetrico attiva la morfogenesi asimmetrica degli organi e delle strutture cerebrali (Hamada et al., 2002).

Questo lavoro di tesi si propone di caratterizzare una mutazione spontanea alla base dell’aumento nella frequenza di individui con asimmetrie cerebrali rovesciate, e che è stata isolata in seguito ad uno studio di lateralizzazione comportamentale nell’organismo modello zebrafish (Facchin et al., 2009a). I risultati dimostrano che questa mutazione riguarda un allele ad effetto materno e le femmine portatrici sono state identificate osservando la posizione sinistra (normale) o destra (rovesciata) dell’organo parapineale nella loro prole. Ulteriori analisi hanno permesso di ipotizzare che l’allele analizzato sia a dominanza incompleta perché le femmine classificate come eterozigoti presentano un fenotipo intermedio. Successive analisi hanno messo in luce che la mutazione compromette le primissime fasi dello sviluppo delle asimmetrie, e in particolare della formazione della vescicola di Kupffer, necessaria perché si stabilisca il flusso asimmetrico di morfogeni che attiva la cascata di segnale Nodal sul lato sinistro dell’embrione. Embrioni figli di femmine mutanti hanno in media vescicole più piccole rispetto a embrioni derivati da femmine normali, di conseguenza hanno una riduzione dell’espressione a sinistra dei geni della via di Nodal e un aumento di espressione alterata (bilaterale o destra) di tali geni. Embrioni derivati da femmine considerate eterozigoti hanno in ogni caso fenotipo intermedio.
Questo lavoro di tesi si propone inoltre di suggerire un possibile significato ecologico alla presenza in popolazioni naturali di zebrafish di un allele ad effetto materno che controlla lo sviluppo delle asimmetrie viscerali e anche neuroanatomiche, indagando anche la possibile correlazione tra asimmetrie cerebrali e lateralizzazione comportamentale. È stato ipotizzato che gli stadi iniziali di sviluppo delle asimmetrie possano determinare anche differenze precoci nello sviluppo anche della lateralizzazione cerebrale e che differenze precoci nelle asimmetrie epitalamiche possano determinare variazioni individuali nel temperamento degli animali. Per testare queste ipotesi femmine putative mutanti sono state incrociate con maschi transgenici della linea foxD3::GFP, in cui la proteina fluorescente verde sotto il controllo del promotore del gene foxD3 è espressa nell’organo pineale e nella parapineale, ed è stato possibile distinguere pesci con la parapineale in posizione normale (sinistra L-PPO) e destra (R-PPO). I soggetti sono stati testati per la lateralità motoria e visiva e per tre misure di temperamento. Differenze significative in pesci con parapineale opposta sono state riscontrate in tutti i test di lateralità (mirror test, rotazione in ambiente familiare, attività anti-predatoria e direzione di svolta in assenza di luce visibile) mentre il ruolo delle asimmetrie diencefaliche sul temperamento è più complesso. Pesci con parapineale rovesciata sono più coraggiosi quando osservano un predatore, passano meno tempo nella zona periferica di un ambiente aperto e in assenza di luce visibile coprono una distanza minore.
ABSTRACT

In vertebrates, the bilateral symmetry of the external body plan conceals consistent left-right (LR) asymmetries in the disposition, morphology and function of internal organs. Genetics and molecular mechanisms that establish LR identities in the two halves of the developing embryo act between late gastrulation and early somitogenesis and are known to be conserved amongst the different classes of vertebrates (Levin, 2005).

In the vertebrate embryo the event responsible for breaking of the initial body symmetry occurs during late gastrulation at the posterior end of the notochord (the node of mammalian embryos) where a transient ciliated structure becomes evident. In zebrafish, this structure is identified as the Kupffer’s vesicle (Essner et al., 2005; Kupffer, 1868; Okada et al., 2005). Cilia in this structure posses a rotating beating movement that generates a leftward flow of extracellular fluid that triggers the asymmetrical transcription of Nodal and Lefty genes in the left lateral plate mesoderm, and the transcription factor Pitx2 during early somitogenesis (Blum et al., 2009). Disturbance or absence of nodal leftward flow results in laterality defects and randomization of left-right asymmetries (Essner et al., 2005).

Recently it has been reported first evidence of nodal and Pitx orthologues isolated in two species of snails with opposite body handedness and direction of shell coiling. Authors found that nodal and Pitx are both expressed in the right side of the embryo in the dextral species and in the left side in the sinistral species. These results suggest that the asymmetrical expression of nodal and Pitx may represent an ancestral conserved feature in the evolution of Bilateria (Grande and Patel, 2009).

Furthermore, from previous studies it is known that in snails, body handedness is controlled by a maternal effect gene that determines the direction of shell coiling in the offspring (Boycott and Diver, 1923).

Here in this study we report the first evidence of a maternal effect gene controlling the establishment of L-R body asymmetries in a vertebrate embryo suggesting that other mechanisms are evolutionary conserved from snails to vertebrates.
Abstract

In a recent study Facchin et al. (2009a) showed that the progeny of lines of zebrafish artificially selected for the right eye preference in scrutiny a mirror had a significant increase in the frequency of reversed left-right asymmetry in the epithalamus. At the beginning of the present study it has been proposed that Facchin's selection for behavioral lateralization could have lead to the isolation of a spontaneous mutant allele responsible for the alteration of normal left-right patterning in zebrafish neuroanatomical structures. Based on a preliminary mendelian analysis of this trait, we performed selective crosses on females identified as mutant carriers to test the hypothesis of a recessive maternal effect mutation. We identified three different classes in the group of analyzed females according to the percentage of reversed brain asymmetries in their offspring. Females generating a frequency of 0-5%, between 5 and 16% and females generating more than 16% of progeny with reversed asymmetries. Females from the last group were considered as putative mutants, according to Facchin's data (2009a). We then decided to investigate in this strain the expression of members of signaling pathways responsible for the establishment of visceral and diencephalic left-right asymmetries. Single probe in situ hybridization analyses with *lefty1* and *ndr2* (*cyclops*) revealed that approximately 50% of embryos from putative mutant females showed altered -bilateral or right sided- expression of these markers that are normally expressed on the left side of the dorsal diencephalon and in the left lateral plate mesoderm (LPM). A third Nodal-related gene, *southpaw* (*ndr3*), is asymmetrically expressed upstream cyclops and *lefty1* in the left LPM at early somitogenesis stages. Also *southpaw* is expressed bilaterally or right-sided in approximately 50% of putative mutant’s progeny, suggesting that the mutation could act upstream the activation of the asymmetrical Nodal pathway.

We therefore decided to investigate the effect of the analyzed gene on the Kupffer's vesicle (KV) structure and morphogenesis. *In vivo* observations revealed that putative mutants’ offspring develop a smaller KV. We measured AP diameter and LR diameter and area of KV in embryos derived from the three groups of females. Progeny from females that generate 5-16% offspring with right parapineal display an intermediate phenotype in the size of KV.
Our hypothesis suggests that smaller size of KV can reduce the amount of morphogens accumulated by the leftward flow, thus leading to a randomization of the expression of genes of the Nodal pathway. *In vivo* observation are going to be supported by *in situ* hybridization data performed using probes specific for genes expressed in the cluster of cells (dorsal forerunner cells) that aggregate to form KV. Furthermore it was posited that this initial symmetry-breaking event determines the development of lateralized brain functions and early differences in epithalamic left–right asymmetry give rise to individual variation in coping styles and personality. We tested these two hypotheses by sorting zebrafish with left or right parapineal at birth using a *foxD3:GFP* marker and by measuring visual and motor laterality and three personality dimensions as they become adults. Significant differences between fish with opposite parapineal position were found in all laterality tests while the influence of asymmetry of the habenulae on personality was more complex. Fish with atypical right parapineal position, tended to be bolder when inspecting a predator, spent less time in the peripheral portion of an open field and covered a shorter distance when released in the dark. Activity in the open field was not associated to anatomical asymmetry but correlated with laterality of predator inspection that in turn was influenced by parapineal position. One personality dimension, sociality, appeared uncorrelated to both anatomical and functional asymmetries and was instead influenced by the sex of the fish, thus suggesting that other actors, i.e. hormonal, may be implicated in its development.
1. INTRODUCTION

1.1. Vertebrate visceral and neuroanatomical asymmetries

1.1.1. Visceral asymmetries

Vertebrate body plan is externally symmetric conversely, it shows distinct left-right (L-R) asymmetries that characterize internal structures. Visceral, neuroanatomical and functional asymmetries can be described.

Two types of visceral asymmetries can be distinguished. The first type refers to unpaired organs such as the heart, liver, spleen and digestive organs that display highly conserved left-right positions with respect to the body axis. The embryonic primordia of these organs are formed as midline structures and follow complex patterns of movements resulting in a consistent positioning of these organs along the L-R axis. The second type of visceral asymmetries concerns the generation of asymmetrical paired organs, specifically the lungs, the bronchi and the cardiac atrial appendages. These organs are present in both sides of the body but they acquire distinct left and right morphologies. For instance in human body the left lung has two lobes while the right lung has three lobes regardless of their symmetric position along the L-R axis. Moreover, asymmetry of unpaired and paired organs is handed or directional. This means that the global asymmetric L-R organization is consistently orientated with reference to the antero-posterior and the dorso-ventral axis in all members of a species. The handed asymmetry is known as situs and the normal disposition of organs is called situs solitus. The failure to generate normal L-R asymmetries and/or to orientate the situs results in laterality disturbances. This suggests that it is crucial for proper organ morphogenesis to keep a correct relative position of organ with respect to each other (Peeters and Devriendt, 2006).

Disturbances in the generation of normal L-R asymmetries and organ situs result in heterotaxia, that comprises a spectrum of laterality disorders. In humans these defects involve the situs orientation as well as the asymmetry of both paired and unpaired organs (Fig. 1.1).
Situs orientation defects result in the so called situs inversus totalis, a complete mirror image of the normal positioning of organs and structures along the L-R body axis. The frequency of this syndrome is 1 out of 6000-8000 newborns. Organs maintain their relative position with respect to each other but, in individuals with situs inversus totalis the risk of congenital hearth defects is more frequent (3-9%) than in individuals with normal organ situs (0.6%) (Merklin and Varano, 1963; Peeters and Devriendt, 2006).

![Figure 1.1](image.jpg)

**Figure 1.1:** Schematic representation of main laterality defects in the disposition and morphology of internal organs in humans. Adapted from Capdevila et al., 2000.

Between the situs solitus and the complete situs inversus conditions there is a wide spectrum of conditions in which only some organs fail to achieve their proper L-R position in the body plan, these cases are summarized as situs ambigus condition. Situs ambiguus is often associated with malformation in organ morphology and/or function, as cardiovascular defects and anomalies in the spleen and gastrointestinal apparatus (Kathiriya and Srivastava, 2000; Kosaki and Casey, 1998; Peeters and Devriendt, 2006).

Another class of laterality disorders is called isomerism and involves defects in the asymmetries of paired organs. In this derangement paired organs, that normally possess distinct L-R morphologies, develop as mirror images. A left and a right isomerism can be distinguished so the body does not develop visceral asymmetries. The left isomerism, in fact, defines a condition in which the body is formed by a mirror image of its left-side, and the reversal situation is represented by right isomerism (Peeters and Devriendt, 2006).
The mechanisms that underlie the development of left-right asymmetries are known to be evolutionary conserved among vertebrates and they will be discussed during this dissertation.

1.1.2. Neuroanatomical asymmetries

Together with visceral asymmetries, the comprehension of neuroanatomical asymmetries is notably important.

First evidences of neuroanatomical asymmetries came from the 60's. In humans the *planum temporale*, a structure that belongs to the Wernicke area, is significantly larger in the left hemisphere than in the right hemisphere (Geschwind and Levitsky, 1968). The horizontal segment of the Sylvian fissure in the left hemisphere results larger in right-handed males compared to left-handed males and to right and left-handed females (Geschwind and Galaburda, 1985). Finally the Heschl gyrus is larger in the left hemisphere temporal lobe than in the right hemisphere temporal lobe (Penhune et al., 1996).

Structural asymmetries of the brain in non-human species have already been evidenced since the beginning of the twentieth century (Braitenberg and Kemali, 1970). As an example in 1904 Gierse (quoted in Shanklin, 1935) reported that in the fish *Cyclotheta acclinidens* right habenular nuclei were bigger that left nuclei, while in the *Geotria australis* the right habenulo-peduncolar trait was larger than the left trait (Roethig, 1923 quoted in Frontera, 1952).

Since these first evidences the study of neuroanatomical asymmetries in vertebrates had no more development. But in the 70's and 80's, together with the study of functional asymmetries, also anatomical asymmetries have been investigated and striking structural asymmetries have been evidenced in the visual system of birds (Rogers, 1996). Birds show two distinct visual pathways; the thalamofugal and the tectofugal pathway. Briefly the thalamofugal projects from the retina to the contralateral side of the dorsolateral thalamus, specifically to the *nucleus opticus principalis thalami* (OPT), and from OPT there are projections to the hyperstriatal regions in each side of the forebrain. In the domestic chick it has been found a greater
number of contralateral projections from the left side of the thalamus to the right hyperstriatum than the opposite from the right side of the thalamus to the left hyperstriatum (Boxer and Stanford, 1985). It should be noted that the left side of the thalamus receives input from the right eye, and the right side is reached by input from the left eye. The tectofugal pathway projects from the retina to the optic tectum on the contralateral side of the brain, from this region to the ipsilateral and contralateral nucleus rotundus and from there to the ipsilateral ectostriatal region of the forebrain. In both males and females adult pigeon there are significantly larger cells with more dendrites in the deeper layers of the right optic tectum than in the left optic tectum (Dharmaretnam and Andrew, 1994).

In mammals, important studies have been performed by Collins in the mouse in which structural asymmetries can be associated to paw preference. At a population level, mice are equally distributed between left-handed and right-handed in the use of their anterior paw (Collins, 1977). Collins and colleagues found that, when crossing mice with the same preference in the use of anterior paw, they obtained progeny with the same preference of their parents. Further investigations demonstrated that this motor preference is associated to an anatomical asymmetries at the level of the orbito-frontal cortex and the hippocampus (Lipp et al., 1984).

An important asymmetry has also been documented in primates, *Pan troglodytes*, *Pan paniscus* and *Gorilla gorilla*. Using magnetic resonance imaging analysis, it has been found that in this species, the corresponding Broca's (Brodman's area 44) area is larger in the left hemisphere than in the right, according to what already known in the human species (Cantalupo and Hopkins, 2001).

Several recent studies have investigated on the association between structural asymmetries and manual preference (handedness) in the chimpanzee. These studies evidenced that left or right hand preference is related to brain asymmetries as in the corresponding Broca's and Wernicke's areas, in the motor primary cortex, in the corpus callosum and in the inferior frontal gyrus (Dadda et al., 2006; Hopkins et al., 2007a; Hopkins et al., 2007b; Taglialatela et al., 2006).
1.2. The epithalamus and diencephalic asymmetries

Many lower vertebrates display a consistent and important neuroanatomical asymmetry other than those previously described. This asymmetry is found in the region of the epithalamus. The epithalamus is a major subdivision of the diencephalon and it forms a functional cluster neuroanatomically distinct in all vertebrates. The epithalamus is constituted by two sets of neuronal conglomerates with strikingly dissimilar cytoarchitectonic organization: the habenula and the pineal complex (Concha and Wilson, 2001).

The habenula is a paired structure, lying adjacent to the dorsal midline of the diencephalon (Hendricks and Jesuthasan, 2007), and it is formed by a bilateral set of nuclei surrounding the lateral walls of the third ventricle. The habenula is part of an evolutionary conserved conduction pathway of connections between telencephalon and midbrain. Habenular nuclei relay impulses from the limbic area of the telencephalon via the stria medullaris to an unpaired midbrain nucleus, the interpeduncular nucleus (IPN) via the fasciculus retroflexus, integrating the neural and endocrine activities (Sutherland, 1982).

The habenular commissure divides the diencephalic roof plate into a larger rostral and a smaller caudal part. The rostral part gives rise to the saccus dorsalis, a membranous evagination that reaches the posterior end of the velum transversum. The caudal part of the diencephalic roof plate gives rise to a pair of saccular evaginations known as pineal organ or epiphysis cerebri, and parapineal organ or parietal eye. Both the pineal and the parapineal organs contain photoreceptors and express a similar subset of neurotransmitters. Globally, these two structures constitute the so-called pineal complex, which is an unpaired structure. (Concha et al., 2003; Concha and Wilson, 2001).

The pineal organ is present in almost all vertebrates as a medial structure that shows no evident asymmetry. The parapineal organ seems to be less conserved in the evolution of the vertebrate lineage and groups such as such as hagfish, cartilaginous fish, amphibians, birds and mammals lack this structure. On the other hand, when present, the parapineal organ shows
markedly asymmetry in connection and, in some cases, this epithalamic structure is asymmetrically positioned (Concha and Wilson, 2001).

From a functional point of view the epithalamic structures have been proposed to play different roles. The habenular nuclei coordinate the functional interaction among structures in the limbic forebrain and mesencephalon in olfactory responses and in mating and feeding behaviors. Moreover the habenula controls hormones secretion (epinephrine, norepinephrine and corticosterone) in response to stress conditions and in avoidance learning behavior (Sandyk, 1991). Moreover, playing a role in the control of reproduction and mating behavior, the habenula displays sex- and seasonal- related variations in some species (Concha and Wilson, 2001).

Despite the detailed characterization of habenular neural functions, the role of the pineal organ is less defined. However, the pineal organ controls melatonin synthesis and regulates the circadian activity. Conversely the function of the small parapineal organ is still unknown (Halpern et al., 2003). Many studies, in literature, have reported investigations on epithalamic asymmetries and, in particular, habenular asymmetries, mostly in fish, amphibians and reptiles.

### 1.3. Epithalamic asymmetries in fish, amphibians and reptiles

Several studies have reported that, in many species of amphibians and reptiles as well as in the teleost zebrafish, the left and right dorsal habenular nuclei display striking asymmetries in their size, structure and molecular composition. These ultrastructural L-R asymmetries include differences in cell morphology and, synaptic vesicles specialization, neuropil organization and gene expression profile (Aizawa et al., 2005; Barth et al., 2005; Braitenberg and Kemali, 1970; Gamse et al., 2005; Gamse et al., 2003; Wehrmaker, 1969). In two species of frogs, *Rana esculenta* and *Rana temporaria*, for example, the left medial habenula is consists of two compartments while the right nucleus shows a single compartment (Kemali and Guglielmotti, 1977; Morgan et al., 1973). Moreover in frogs variations in
the degree of habenular asymmetry are season-dependent, and this is probably correlated with the mating period (Kemali et al., 1990).

In most species of fish, amphibians and reptiles the pineal complex is formed by the medial-located pineal organ (also called epiphysis) and by the asymmetrical unpaired parapineal organ, which is an accessory structure also known as parietal eye or frontal eye. The parapineal organ is frequently situated on the left side of the brain. Moreover, in several species of fish, including stickleback, lamprey an trout, the parapineal organ innervates the dorsal portion of the left habenula (Van Veen et al., 1980; Yañez and Anadón, 1994; Yañez and Anadón, 1996).

1.3.1. Fish

In Mixinoidea, the habenula originates as a bilateral structure (Conel, 1931) but in adult hagfish it is composed of a single body located at the midline of the brain. Left and right habenular nuclei can be identified only at a microscopic observation showing a larger and hypertrophic right corpus habenularis (Jansen, 1930; Wicht and Northcutt, 1992). In adult hagfish a parapineal organ has not been described (Concha and Wilson, 2001).

In the vertebrate family of Petromizontoidea, the habenula of lampreys displays evident asymmetries both in size and neuronal organization. The right habenular portion is considerably larger and than the left one. Moreover neurons in the right habenula are larger in number and arranged in various superficial cell layers while in the left habenula neurons are restricted in a smaller periventricular area (Nieuwenhuys, 1977; Yañez and Anadón, 1994). The presence of asymmetries in the habenulae of primitive vertebrates, as in the group of jawless fish, suggests that this feature of the nervous system appeared very early in the evolutionary history of the vertebrate class.

With respect to the pineal complex, in lampreys, pineal and parapineal organs are both well developed and located at the dorsal midline of the head beneath a patch of translucent skin (Cole and Youson, 1981; Eddy and Strahan, 1970; Meiniel and Collin, 1971). Cells in the parapineal organ project to the left habenula and the interpeduncular nucleus (IPN), thus connecting telencephalic nuclei with the in the ventral midbrain (Yañez et al., 1999).
Moreover the parapineal ganglion and the left habenula reveal similar neurochemical and ultrastructural features. This suggested that probably the parapineal was a component of the left habenula that subsequently underwent migration to a novel location in the lamprey brain (Meiniel and Collin, 1971; Yañez et al., 1999).

In almost all species of Chondrichthyes the habenula is larger on the left side (Kemali and Miralto, 1979; Smeets et al., 1983). This asymmetry also extends to neuronal organization and fiber myelination (Kemali et al., 1980; Miralto and Kemali, 1980). Cartilaginous fish lack a parapineal organ (Concha and Wilson, 2001).

The class of bony fish (Osteichthyes) is the larger group of extant vertebrates. Osteichthyes, compared to Chondrichthyes, possess a bony skeletal structure that forms by substitution of the embryonic cartilaginous skeleton. The class of Osteichthyes comprises the two main subclasses of ray-finned fish Actinopterygii and fleshy-finned fish Sarcopetergygii (Meek and Nieuwenhuys, 1998). In almost all class of the Actinopterygii the habenula shows marked asymmetry in size, as the right portion is larger than the left side, and neuronal organization, as the right side also contains a wider layer of densely packed neurons compared to neurons of the left habenular portion (Braford M.R and Northcutt, 1983; Nieuwenhuys, 1998a; Nieuwenhuys and Bodenheimer, 1966). An exception is represented by teleost fish, in fact in eels (Anguilla anguilla), zebrafish (Danio rerio) and silver salmons (Onchorhynchus kisutch) an opposite asymmetry has been described, with the left habenular nucleus that is larger than the right (Braitenberg and Kemali, 1970; Concha et al., 2000; Ekström and Ebbesson, 1988).

With respect to the group of Saccopeterigii, in the subdivision of crossopterigian fish, it is important to notice that the asymmetry of the habenula is clearly observed in the coelacanth Latimeria chalumnae. In fact in this species the left side the habenula is enlarged compared to the right side (Nieuwenhuys, 1998b).

In the group of bony fish, a pineal complex has been described in the bowfish Amia calva, in teleosts and in the coelacanth Latimeria chalumnae. The pineal complex of Amia calva and of teleosts is located beneath the roof of the skull,
even if in some extant species it emerges from a foramen to localize underneath the skin (Concha and Wilson, 2001; Steyn, 1960). An asymmetrical parapineal organ has also been described in the bowfin and in many teleost species (Borg et al., 1983; Concha et al., 2000; Vigh-Teichmann et al., 1991). During the development, the pineal and parapineal organs originate as evaginations of the diencephalic roof plate (Eycleshymer and Davis, 1897; Hill, 1891), but while the pineal maintains its medial position in the diencephalon, the parapineal undergoes a lateral migration toward the left side of the brain, to reach a final position behind the left habenular nucleus (Borg et al., 1983; Concha et al., 2000; Holmgren, 1965). Moreover, nerve fibers from the parapineal evagination project toward the left medial habenula (Concha et al., 2000; Van Veen et al., 1980).

In the coelacanth *Latimeria chalumnae*, pineal and parapineal constitute a pair of saccular vesicles located deep into the head, covered by adipose tissue and protected by the roof of the skull. In contrast to what is seen in teleosts, the parapineal in the coelacanth appears as the major component of the pineal complex (Hafeez and Merhige, 1977).

1.3.2. Amphibians

Asymmetries at the level of the habenular nuclei have also been widely described in both Urodela (newts and salamanders) and Anura (frogs and toads). In all species asymmetries are observed only in the dorsal habenular nuclei. In the newt *Triturus cristatus* neurons in the left dorsal habenula are organized in several layers extending more laterally; conversely, few neurons in the right dorsal nucleus define a more limited region (Braitenberg and Kemali, 1970).

The habenula in the Anuran species presents both morphological and neuronal asymmetries and, in particular that of the frog *Rana esculenta*, represents one of the most widely studied example of epithalamic asymmetry in vertebrates. In anuran species, the left dorsal habenular nucleus is considerably larger than the right dorsal nucleus (Braitenberg and Kemali, 1970). This asymmetry is a feature that shows seasonal and sex-dependent variations. Furthermore, regarding the neuronal organization, while...
neurons in the right dorsal habenula are distributed around a single neuropil, a more complex organization characterizes the left dorsal habenula that can be compartmentalized into different subnuclei with unique features in their cytoarchitecture and ultrastructure (Braitenberg and Kemali, 1970; Guglielmotti and Fiorino, 1999; Kemali and Guglielmotti, 1977; Morgan et al., 1973). Studies on the frog *Rana temporaria* showed that habenular asymmetries are established early during the development, as they are already present in tadpoles and juvenile stages (Morgan et al., 1973). In this anuran species, the left habenula is composed by two nuclei with a lower physiologic activity, compared to the right habenula, formed by a unique nucleus (Vota-Pinardi and Kemali, 1990). The two nuclei of the dorsal habenula are thought to be related to sexual reproduction as it has been observed that in males and females of *Rana esculenta* they are larger during the reproductive season in spring, compared to their size in winter (Braitenberg and Kemali, 1970). This difference is probably due to the action of steroid hormones during the development (Kemali et al., 1990).

Braitenberg and Kemali (1970) also hypothesized that these asymmetries of the left habenula arose early during the evolution, suggesting that two bilaterally symmetric organs could have moved to form a single dorso-ventral complex whose original functions could have been lost or at least modified.

In anuran species the pineal complex consists of a frontal organ and a pineal organ. The frontal organ is located under the skin behind the lateral eyes, it contains photoreceptors and projects to the intracranial pineal organ (reviewed by Van De Kamer, 1965). No evidences of an asymmetrical parapineal organ have been reported (Concha and Wilson, 2001).

**1.3.3. Reptiles**

In reptiles, asymmetries of the habenular structure have been described in some species of lizards as *Uta stansburiana* (Engbretson et al., 1981) and *Lacerta sicula* (Kemali and Agrelli, 1972), while in others species of lizards (Cruce, 1974), in turtles (Ten Donkelaar, 1998), ophidians (Nagasaki, 1954) and crocodiles (Tamura et al., 1955), the habenula appears symmetric.
With regard to the habenula of the lizard *Uta stansburiana* (Engbretson et al., 1981) has been widely described, both habenular nuclei can be divided into a lateral and a medial subnucleus. Left habenula is larger that the right; moreover, neurons in the medial subnucleus of the left habenula are arranged with a unique cytoarchitectonic (Kemali and Agrelli, 1972), connectional (Engbretson et al., 1981) and immunohistochemical (Engbretson et al., 1982) features.

In reptiles the pineal complex is formed by a pineal organ and a parietal eye.

The parietal eye is a unique structure that has first been described in the sphenodon (Rhynchocephala) (Dendy, 1911) and in species of lizards living at higher temperate latitudes (Quay, 1979). The pineal organ in reptiles is a tubular or saccular intracranial structure while the parietal eye, after evaginating from the skull through a foramen, connects to the diencephalon by the parietal nerve (Gladstone and Wakeley, 1940). The parietal eye forms as an evagination of the pineal organ, thus its embryonic origin together with the pattern of its connections suggests that this peculiar formation in lizard is homologous to the parapineal organ of teleosts (Engbretson et al., 1981; Yañez and Anadón, 1996; Yañez et al., 1999). Axonal projections from the parietal eye innervate the *pars dorsomedialis* of the left medial habenula (Engbretson et al., 1981; Korf and Wagner, 1981).

The parietal eye has been demonstrated to be a fully functional photoreceptive structure able to respond to light stimuli (Solessio and Engbretson, 1993; Solessio and Engbretson, 1999). It has been thus hypothesized that, in lizard, and probably also in lampreys and teleosts, stimuli of daily light oscillations asymmetrically modulate the habenulo-interpeduncular system through photoreceptors present both in the parietal eye and in the parapineal organ (Concha and Wilson, 2001).

### 1.3.4. Birds and mammals

In birds and mammals habenular asymmetries have been poorly documented but, in general the habenula in these classes appears symmetric even considering a few subtle differences that can be detected between the right and left habenula using quantitative volumetric studies. As an example, in the
chick, asymmetry of the medial subnucleus of the habenula has shown to be sex-dependent (Concha and Wilson, 2001).

In mammals, analyses of two different species revealed some controversies. In albino rats the medial subnucleus of the habenula is remarkably larger in the left side (Wree et al., 1981), while the opposite situation is reported for albino mice that show an enlarged right habenula (Zilles et al., 1976).

With respect to the parapineal organ, this structure is absent in birds and mammals (Concha and Wilson, 2001).

1.4. Behavioral lateralization

The discovery of functional brain lateralization in the human species is associated with the classical observations by Broca in 1861 and, more recently, with the findings from the work carried out by Sperry and colleagues on split-brain patients in 1960's (Gazzaniga and Sperry, 1966).

For a very long time functional brain lateralization has been considered unique to humans until Fernando Nottebohm’s finding of differential effects on song production of sectioning the right or the left hypoglossal nerve in the canary (Nottebohm, 1971). Nowadays we know that cerebral lateralization is not a feature unique to humans and three decades of studies have reported behavioral and anatomical asymmetries in a wide range of vertebrate species, from fishes to mammals and birds (reviewed in Rogers and Andrew, 2002). Remarkable similarities have been reported even among distantly related species (Bisazza et al., 2002) and there are recent reports of behavioral asymmetries in invertebrates, for example, spiders (Ades and Ramires, 2002), insects (Pascual et al., 2004) and decapods (Byrne et al., 2004), which suggests that lateralization might be even more common than presently thought.

1.5. Development of asymmetries in the vertebrate body plan

Axes determination is a complex process that takes shape at the earliest stages of embryonic development with the establishment of an anterior-posterior and a dorsoventral axes. Subsequently the left-right axis forms,
correctly orientating itself with respect to the previous axes. Molecular and genetic mechanisms underlying the development of left-right asymmetries are found to be consistent and evolutionary conserved amongst vertebrate groups (Bisgrove and Yost, 2001). Also the direction of asymmetry is striking conserved and one of the most evident examples is the left side positioned heart in all vertebrate classes (Vandenberg and Levin, 2009).

It has been proposed that that the development of L-R asymmetries in the vertebrate body plan occurs in at least three steps during early embryonic development (reviewed by Hamada et al., 2002). In the first step the L-R axis becomes oriented with respect to the antero-posterior and dorso-ventral axes, thus defining a left and a right side of the developing embryo. This early pattern is processed during the second phase and leads to the activation of an asymmetrical gene expression cascade. During the third phase, a series of morphogenetic events control localized changes in cell migration, shape, proliferation and survival that determine the asymmetrical anatomy of organs and tissues (Mercola, 2003).

1.5.1. First step: breaking symmetry

When the anteroposterior and the dorsoventral axes define the the left and the right side, the embryo initially shows bilateral symmetry. Disruption of the determination of the left-right axis leads to the loss of asymmetry and to isomerism. Thus during the first step of L-R development, the initial bilateral symmetry of the embryo is broken and the L-R body axis becomes orientated with respect to the antero-posterior and the dorso-ventral so that all individuals are asymmetric in the same direction. Defects in the proper orientation of the L-R axis result in individuals that randomly develop either with normal or with reversed asymmetries (Vandenberg and Levin, 2009).

Currently it is thought that this first event occurs at the end of the gastrulation, when a transient embryonic structure becomes evident at the posterior end of the notochord and subsequently disappears as organogenesis begins (Blum et al., 2009). This structure is composed of a monociliated epithelium and, in all vertebrates, cilia of comparable length around 5 μm are polarized to the posterior pole of cells. These cilia posses a
rotating beating movement in a clockwise direction and it has been demonstrated that this clockwise rotation generates a leftward flow of extracellular fluid able to trigger the asymmetrical transcription of Nodal genes in the left lateral plate mesoderm during early somitogenesis (Nonaka et al., 1998; Nonaka et al., 2005). A cilia-based leftward flow of extracellular fluid has been described in almost all vertebrate groups from teleost fish zebrafish (Danio rerio) (Essner et al., 2005) and medaka (Oryzias latipes) (Okada et al., 2005), to mammals mouse (Nonaka et al., 1998) and rabbit (Okada et al., 2005), and recently also in the frog Xenopus laevis (Schweickert et al., 2007). Due to this wide evolutionary conservation it has been argued that cilia-driven vectorial flow should represent the ancestral strategy for symmetry breaking in chordates (Blum et al., 2009).

The transient ciliated structure that triggers the leftward fluid flow develops from superficial mesoderm, in a region that can be identified as the gastrocoel/archenteron and whose morphology varies in different organisms (Blum et al., 2009). In mouse and rabbit this ciliated epithelium forms in the wide posterior portion of the notochordal plate (Blum et al., 2009; Okada et al., 2005), this structure, in mice is also called the embryonic node, thus the flow that generates is known as nodal flow. The homologous structure in the amphibian embryo is called gastrocoel roof plate and localizes in a ciliated epithelium transiently embedded into the dorsal endoderm of the archenteron (Essner et al., 2002; Schweickert et al., 2007). In teleost fish medaka and zebrafish, the ciliated formation responsible for the generation of the leftward fluid flow is called Kupffer’s vesicle (Brummett and Dumont, 1978; Essner et al., 2002) and appears at the end of gastrulation in the tail bud, posterior to the notochord (Blum et al., 2009; Essner et al., 2005). It has been shown that cilia beating dynamics and the generation of the leftward fluid flow are conserved, even if the size and shape of the monociliated structure at the posterior notochord and the speed of the flow are very different in the analyzed vertebrate species (Okada et al., 2005).

Studies of targeted mutagenesis on two members of the heterotrimeric kinesin family KIF3A and KIF3B showed that these proteins are essential for both the assembly of monocilia and the normal development of L-R
asymmetries (McGrath et al., 2003; Nonaka et al., 1998; Takeda et al., 1999). Similar evidences were supported by the analysis of a mice mutation that results in the *inversus viscerum* (*iv*) phenotype, a total mirror image of normal L-R asymmetries. The *iv* mutant phenotype is due to a mutation in a gene that encodes for an axonemal dynein in monocilia of the ciliated epithelium at the posterior notochord. This particular dynein is called Left-Right dynein (*Lrd*) and mutations affecting the *Lrd* gene cause the disruption of monocilia beating movement as in homozygous *iv* mice (*iv iv*) abnormal or no leftward flow results in the randomization of normal L-R asymmetries (Okada et al., 1999; Supp et al., 1999; Supp et al., 1997).

The importance of proper ciliary formation for normal L-R development is well demonstrated considering human syndrome named primary ciliary dyskinesia (PCD). Primary cilia dyskinesia is a group of diseases characterized by recurrent infections of the respiratory tract resulting from the absence, immotility or dysmotility of the cilia responsible for airways clearance. Approximately 50% of individuals affected by PCD display *situs inversus*, and this condition is known as Kartagener syndrome. Interestingly, the first link between immotility of cilia and left–right determination had been hypothesized in the human Kartagener syndrome long before the discovery of the leftward nodal cilia in mice and the nodal flow hypothesis (Afzelius, 1976). PCD causes randomization of *situs* exactly like several mouse mutants with disrupted leftward nodal flow due to absence or immotility of nodal cilia. Because of abnormal dynein in the ciliary microtubules, affected individuals lack functional cilia, leading to a decreased mucociliary transport and sperm immotility (Eliasson et al., 1977). All these evidences suggest that also in humans the nodal flow may be conserved as an early step in L–R asymmetry (Peeters and Devriendt, 2006).

Despite the growing detailed investigations on the cilia structure and on the mechanism that generates the leftward nodal flow, the molecular and chemical nature of the fluid moving toward the left side of the embryo is still poorly understood. Many studies have investigated the composition of morphogens or parcels transported by this flow (Nonaka et al., 1998; Okada et al., 2005; Tanaka et al., 2005). Following the establishment of leftward flow,
in fact, the information provided by this event has to be interpreted by surrounding tissues in the left lateral plate mesoderm (LPM). In 2003 McGrath and colleagues proposed a model based on the findings that two populations of monocilia are present in the mouse node at the posterior notochord. A centrally located subset of dynein (Lrd)-containing monocilia are motile and are responsible for the generation of the leftward nodal flow. A second subset of non Lrd-containing monocilia are located at the periphery of the node. These cilia are immotile but the presence of the cation channel polycystin-2 has been proposed to play a mechanosensory role, by sensing the leftward flow. The proposed model argues that cilia at the periphery of the node, bent by the flow will initiate a cascade of events that confer left-sidedness to the left side of the embryo (McGrath et al., 2003; Tabin and Vogan, 2003). One of the first asymmetrical left-sided signal is an intracellular elevation of Ca^{2+} levels promoted by polycystin-2 channels (McGrath et al., 2003).

A work published in 2005 it has been demonstrated that, in mice, the fluid flow generated by monocilia at the posterior end of the notochord transports membrane-coated vesicles called ‘nodal vesicular parcels’ (NPVs). These parcels are secreted and transported to the left of the embryonic node by the leftward flow and have been shown to carry Sonic hedgehog and retinoic acid. Moreover the release of these parcels is triggered by FGF signaling and this process is supposed to create a gradient of morphogens that, in turn, induces a downstream increase of the Ca^{2+} level starting from the left margin of the node, and then propagated towards the left lateral plate mesoderm (Tanaka et al., 2005). In fact it has been widely confirmed that Ca^{2+} signaling is required, downstream the leftward nodal flow, for the proper development of L-R asymmetry (Hirokawa et al., 2006; McGrath et al., 2003; Shimeld, 2004).

In a recent review, it has been argued that cilia-driven leftward flow represent the ancestral strategy to break initial embryo symmetry in vertebrates, or even chordates, so that this flow may have been a feature present in the common ancestor of the deuterostome group (Blum et al., 2009). This hypothesis could be supported by the fact that in the amphioxus
cilia have been found to be present on the dorsal lip and on archenteron cells at late gastrula stages (Hirakow and Kajita, 1991). Moreover, in this species, ninety minutes after *Nodal* bilateral induction, its right-sided domain is downregulated until disappearance (Yu et al., 2002), and the left-sided domain should provide signals for asymmetrical morphogenesis. This is proposed to be the result of a leftward flow (Blum et al., 2009). Blum and colleagues also speculate that also in sea urchins, and echinoderms in general, a mechanism of cilia-driven vectorial flow, generated by mesodermal-derived cells of the archenteron, may be responsible for the induction of an asymmetric *Nodal* expression cascade (Duboc et al., 2005; Yu et al., 2002).

Despite the widely characterization of ciliated structures that generates a leftward flow of extracellular flow and the dynamic of this flow in all classes of vertebrates, there is an unsolved problem. The chick embryo is the model organism in which first a L-R asymmetric gene expression has been detected (Levin et al., 1995). However no ciliated structure and thus no leftward flow has been demonstrated to be present during L-R development in the chick embryo (Blum et al., 2009; Manner, 2001). Therefore, asymmetry at the posterior end of the notochord in the chick embryo arises without involvement of cilia and flow, but the molecular and cellular mechanisms responsible for symmetry breaking have not been elucidated yet. Blum and colleagues speculate that the leftward nodal flow may have been the common ancestral strategy for setting up asymmetry, as it has been reported from fish to mammals, and eventually that flow was lost secondarily in the course of chick evolution (Blum et al., 2009).

1.5.2. Second step: activation of asymmetrical gene expression cascade

Once a cilia-driven fluid flow generated an asymmetrical L-R information, this cue is subsequently transferred to left lateral plate mesoderm (LPM) where it is propagated and then transduced into cascades of asymmetric gene expression that characterize the second phase in the development of L-R asymmetries. Although the asymmetric patterns of gene expression differ among species, a conserved feature in all examined classes of vertebrates is
the production of the secreted TGFβ-like factor Nodal (or a Nodal homologue) in the left lateral plate mesoderm (reviewed by Burdine and Schier, 2000; Capdevila et al., 2000; Mercola, 2003). Morphogens moved by the leftward flow after gastrulation are known to activate an asymmetrical gene expression cascade that spread along the left side of the embryo in a posterior to anterior wave (reviewed by Burdine and Schier, 2000; Capdevila et al., 2000; Mercola, 2003). Genes involved in this process are Nodal-related genes and other members of the TGFβ superfamily of signaling molecules. Many members of the transforming growth factor-β, in fact, play important roles in different steps of body plan determination in vertebrate embryos (Schier and Shen, 2000; Whitman, 1998). TGFβ signaling pathway is mediated by ligands that bind to specific cell surface type I and type II receptors. TGFβ ligands first bind type II serine/threonine kinase activin receptors (ActRII) that subsequently phosphorylate type I receptors (ActRI) (Mehra and Wrana, 2002; Schier and Shen, 2000; Whitman, 1998). This phosphorylation step is important for proper signal propagation (Wrana et al., 1994). Then, during vertebrate development, phosphorylated type I receptors activate proteins of the Smad family which, in turns form heterodimers that bind to transcription factors to induce the activation of downstream target genes (Schier and Shen, 2000; Whitman, 1998; Wrana and Pawson, 1997).

The TGFβ ligand Nodal (or Nodal-related) associates with EGF-CFC cofactors (composed by an extracellular growth factor-like domain and a cysteine-rich domain called CFC) and this complex binds to its receptors in order to mediate roles in cell interactions in the patterning of the early embryo, especially in mesoderm induction and axes formation (Chea et al., 2005; Cheng et al., 2003; 2004).

During L-R development Nodal in the left LPM activates its own expression, the expression of other Nodal-related genes and also of its inhibitors, in a conserved regulatory loop. Moreover Nodal pathway also induce the expression of transcription factors, as Pitx2 that is important to drive asymmetrical organogenesis (Hamada et al., 2002).
It is important to notice that Nodal regulatory pathway in the left side of the embryo, involves the activation of its own inhibitors, *Lefty* genes, members of the TGFβ subfamily of Antivins. The expression of *Lefty* genes is restricted to the left LPM and starts during somitogenesis stages, in particular in the heart primordium (*Lefty2*) and in the dorsal diencephalon (*Lefty1*), where they negatively regulate *Nodal*-related genes (Bisgrove et al., 2000; Meno et al., 1997; 1998). Another important expression domain for L-R development is found along the midline, in fact, during somitogenesis the *Lefty1* gene is strongly expressed in midline tissues. This domain is important as it has been suggested that the midline acts as a molecular barrier preventing left-sided asymmetrical signals to spread over the right side of the body, and this is due to the inhibitory role of *Lefty1* on the expression of *Nodal* (Meno et al., 1998).

In fact, it has been widely demonstrated that mutations affecting proper development of midline structures, including the notochord and the floorplate, lead to a bilateral expression of *Nodal*-related genes and thus, to a randomization of L-R organs displacement (Bisgrove et al., 2000; Danos and Yost, 1996). Furthermore, even if *lefty1* mutant mice develop normal midline structures they display bilateral expression of *nodal* and *lefty2* leading to heterotaxia in organs positioning. These evidences underline the important role of the *lefty1* midline domain in L-R development, as with the inhibition of *nodal* expression it prevents left-sided signals to be expressed in the right side (Meno et al., 1998).

Further investigations demonstrated that other important signaling pathways are involved in the propagation of asymmetrical Nodal signals in the left LPM after the generation of the leftward flow. Studies performed in mice, chick and zebrafish have shown that the Notch signaling pathway plays a primary role in the establishment of L–R asymmetry by directly regulating expression of the Nodal gene. In fact it has been reported an important requirement of the Notch signaling for the activation of *Nodal* expression around the left side of the ciliated structure at the posterior end of the notochord after the onset of the leftward flow (Krebs et al., 2003; Raya et al., 2003). Notch is also a key signal for the subsequent spreading of *Nodal* expression in the left LPM (Brennan et al., 2002; Saijoh et al., 2003).
The importance of maintaining an asymmetrical left-sided gene expression cascade is well documented by studies reporting that the misexpression (bilateral, right-sided or no expression) of left-sided *Nodal*-related genes alters the normal L-R development (reviewed in Burdine and Schier, 2000; Okada et al., 1999; Supp et al., 1997). Also in humans, heterozygous mutations in patients with laterality defects have been found to involve orthologs of *Nodal*-related genes: ACVR2B (Kosaki et al., 1999b), LEFTYA (Kosaki et al., 1999a) and CFC1 (Bamford et al., 2000), suggesting that the Nodal-signaling cassette is conserved in humans (Peeters and Devriendt, 2006).

Left-sided activation of the Nodal signaling cascade has been described to be necessary for proper L-R development in all classes of vertebrates (reviewed by Burdine and Schier, 2000; Capdevila et al., 2000; Mercola, 2003). Recent evidences suggest that asymmetric Nodal-signaling is involved in L-R asymmetries in the entire chordate lineage. In fact the expression of *Nodal* and *Pitx2* has been reported to be asymmetrically restricted to the left side of embryos also in the amphioxus and in ascidians (Boorman and Shimeld, 2002; Yasui et al., 2000).

Surprisingly, latest investigations reported the first evidence for a nodal orthologue in a non-deuterostome group. In fact, *Nodal* and *Pitx* have been isolated in two in two species of snails and their expression in the left or right side of the embryo is related to body chirality. *Nodal* and *Pitx2* are both expressed on the left side of the embryo in species with leftward direction in shell coiling (e.g. *Biomphalaria glabrata*), and on the right side in species with rightward shell coiling (e.g. *Lottia gigantea*). According to their results, authors argue that the involvement of the Nodal pathway in the establishment of L-R asymmetry might have been an ancestral feature of the Bilateria (Grande and Patel, 2009).

**1.5.3. Third step: asymmetrical organogenesis**

Nodal signaling cascade in the left LPM provides cues that induce L-R asymmetric morphogenesis of visceral organs and brain structures.
Shortly after asymmetric Nodal expression is down regulated in the left LPM, situs-specific morphogenesis comes into begin. Asymmetry of internal structures is characterized by several steps starting with the loop of the cardiac tube towards the right side while the embryo turns along the antero-posterior axis in a clockwise direction. Then the asymmetric formation of the lobes starts in the lungs and with the rotation and coiling of the digestive tract, there is the unilateral regression of parts of the vascular system (Hamada et al., 2002). Asymmetric organogenesis thus occurs by the displacement of midline organs towards the left or right side or by unilateral regression of bilateral primordia or, as in the lungs, by translating asymmetric informations into the development of distinct L-R anatomies (Peeters and Devriendt, 2006).

In response to inductive Nodal activity, organ primordia will adopt left-side morphology, while in the absence of Nodal signals, organ primordia will acquire right-side morphology (Hamada et al., 2002).

In this context, the asymmetric expression of a Nodal protein is crucial as it induces the expression of the transcription factor Pitx2. Pitx2 gene encodes for a bicoid-type hoemobox transcription factor (Campione et al., 1999; Logan et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998). The expression of Pitx2 localizes in the left-LPM and appears in concomitance with Nodal and Lefty2 expression, but persists much longer. In fact, after the down regulation of Nodal and Lefty genes, Pitx2 expression is still present at the late somite stage in various left-sided organ primordia that thus will localize in the left side of the body after complete formation (Hamada et al., 2002). The asymmetric signaling pathway of Pitx2 is conserved among vertebrate species and its fundamental role in proper L-R organogenesis is documented by studies on Pitx2-null mutant mice that show laterality defects i. g. in lungs formation (Gage et al., 1999; Kitamura et al., 1999).

Current evidences suggest that a conserved signaling pathway may be involved in mesoderm formation and L-R axis determination in all deuterostomes (Chea et al., 2005). The homeobox transcription factor Pitx, has also been described in both cephalochordates and tunicates (Boorman and Shimeld, 2002; Morokuma et al., 2002; Tiozzo et al., 2005; Yasui et al.,
2000). In the amphioxus, the expression of *Pitx* is related to *nodal* expression and localizes in the anterior left endoderm, ectoderm, and mesoderm during neurulation stages (Boorman and Shimeld, 2002; Yasui et al., 2000). In ascidians, during adult stages, *Pitx* is asymmetrically expressed in two domains: in the left peribranchial chamber, which is connected to the stomach and intestine, and in the left side of the dorsal lamina (Tiozzo et al., 2005). Therefore these findings, together with the identification of *Pitx* orthologue, indicate that the evolutionary conserved mechanism in L-R development may have been an ancestral strategy in the Bilateria lineage (Grande and Patel, 2009).

### 1.6. Zebrafish as a model species

Zebrafish (*Danio rerio*) is a teleost fish of the cyprinid family (class of Actinopteriigii or ray-finned fish). It is a small tropical fish (about 3-5 cm in length see figure 1.2) and originates from the Ganges river, in the north-east region of India.

Since its establishment as a model organism in the 1970s by George Streisinger, this small teleost has been widely studied as a powerful and versatile tool for vertebrate developmental biology thanks to its important features. Zebrafish is easy to maintain and breed in laboratory conditions and females can laid more than 200 eggs per each mating event. Fertilization and embryonic development are external and mainly synchronous for large clutches of embryos. Moreover embryos are relatively larger and their development can be easily observed through the transparent chorion. Moreover, developing embryos are transparent for the first 24 hours and pigmentation can be chemically inhibited so that they can be maintained transparent for many days. This unique feature allows the *in vivo* observation...
of developing organs and tissues. Embryonic development is rapid and within two days all common features of the vertebrate body plan are established and even if they accomplish equivalent functions, zebrafish organs are like a miniaturized version of those of higher vertebrates. Zebrafish has also a relatively short generation time (2-4 months) compared to other vertebrate model, so that this fish is a versatile tool for large-scale genetic analyses and mutagenesis screenings (Nüsslein-Volhard and Dahm, 2002; Streisinger et al., 1981).

In addition, zebrafish embryos are easy to manipulate and powerful tools have been elaborated to perform microinjections and cell transplantations. In particular DNA, mRNAs and antisense oligo morpholinos are commonly injected in freshly fertilizes eggs. These microinjections techniques allow to transiently overexpress proteins during development testing the effect of overexpressing molecules in particular embryonic domains and/or at different time points. Conversely antisense oligo morpholinos are useful to test the effect of knocking down target genes during key developmental stages. Microinjection is also used to insert transgenic constructs that express reporter genes, as the green fluorescent protein (GFP), under the control of tissue-specific promoters. The creation of stable transgenic lines allows to follow the differentiation of specific organs or tissues and elucidate the genetic pathways responsible for their development (Nüsslein-Volhard and Dahm, 2002).

1.7. Asymmetries in the zebrafish

The striking properties of the zebrafish model make it a powerful tool for the study of vertebrate L-R development. Zebrafish body plan displays main evident asymmetries in visceral organs represented by pancreas position and subsequent gut coiling, and by position and morphology of the heart tube.

In adult zebrafish, the pancreas localizes in the right side of the midline, embedded by the the gut loop that, conversely, is leftward coiled. This asymmetrical organization is still present from day four post fertilization. In the zebrafish embryo at the 12 somite stage, the pancreas is first detected as
a thickening of the endoderm layer in a medial position. Cells of this primordium can be recognized because they start to express the insulin (ins) gene. Later in the development, at the 16 somite stage and at 24 hpf (hours post fertilization) respectively, cells that express glucagon and somatostatin (sst) begins to differentiate (Biemar et al., 2001). At the 18 somite stage the pancreas thickening starts to organize into a pancreatic primordium that gradually separates from the endoderm. Then, at 32 hpf a second pancreatic primordium starts to be detected in an antero-ventral position with respect to the first primordium, and at 52 hpf, subsequent to the leftward coiling of the gut, the two primordia start to fuse and move towards the right side of the dorsal midline, thus originating a fully functional organ (Field et al., 2003).

In zebrafish, as in all vertebrate lineage, the heart localizes in the left side of the body and it is the first organ to develop an evident L-R asymmetry (Stainier, 2001). Prior to the formation of the heart tube at 24 hpf, cardiac precursor cells are localized in lateral districts of the myocardial epithelium and then fuse at the midline ventral to the endoderm to form the cardiac cone (Stainier, 2001). Moreover, prior to the formation of the cardiac tube, several genes such as bmp4, lefty1, lefty2, and pitx2 display an asymmetric expression on the left LPM and in the cardiac field (Chocron et al., 2007). A model has been proposed for heart morphogenesis in which the cone undergoes a telescopic extension that gives the heart a tubular shape with central cells of the cone forming the ventricle and peripheral cells originating the atrium. In addition to this extension movement, a tilting process, named cardiac jogging, occurs. During this process the atrium becomes positioned toward the left side of the body axis, while the ventricle remains at the midline (Smith et al., 2008; Stainier, 2001). In the meanwhile, the cardiac tube also undergoes a rightward tilting (cardiac D-looping) during which the future ventricle of the heart becomes localized toward the right side of the body axis and with respect to the atrium (Ahmad et al., 2004; Long et al., 2002; Stainier, 2001). Many studies demonstrated that the disruption of the Nodal signaling pathway responsible for the proper L-R patterning of the body axis leads to
an increase in the frequency of opposite cardiac jogging and reverse cardiac looping (L-looping) (Ahmad et al., 2004; Long et al., 2003).

Moreover, important studies in zebrafish focus on neuroanatomical asymmetries. Zebrafish brain displays some main asymmetries in the epithalamus, the major subdivision of the diencephalon (Concha and Wilson, 2001). The zebrafish epithalamus (Figure 1.3), as in most teleost, is composed of the pineal complex and the bilateral habenular nuclei that are part of a highly conserved conduction system that interconnects sites in the forebrain and ventral midbrain (Bianco and Wilson, 2009; Facchin et al., 2009b).

In zebrafish both the pineal complex and the habenular nuclei display striking asymmetries (Concha and Wilson, 2001; Halpern et al., 2003). The photoreceptive pineal complex comprises the medially located pineal organ and the left-sided parapineal nucleus. During development, parapineal precursors detach from the anterior pineal anlage and migrate leftward, to reach a position adjacent to the left habenula (Concha et al., 2003). It has been shown that this migration depends on the activity of Fgf8 that is bilaterally expressed in the habenular nuclei (Regan et al., 2009).

![Figure 1.3: Dorsal representation of a 4 dpf larval zebrafish epithalamus. The pineal organ (p) is located in the midline; the parapineal (pp) is located to its left. The left (lh, red) and right (rh, black) habenulae are located on either side of the pineal complex. The left habenula primarily sends axonal projections to the dorsal interpeduncular nucleus (dIPN) while the right habenula sends projections to the ventral IPN (vIPN). From (Snelson and Gamse, 2009)](image)

The zebrafish left and right nuclei of the habenulae display asymmetries in distinct patterns of gene expression, axon terminal morphology and connectivity (Aizawa et al., 2005; Aizawa et al., 2007; Bianco et al., 2008;
In fact, the left habenular nucleus is larger and has a denser neuropil than the right nucleus. In addition, many cells of the left dorsal habenula express the leftover (lov) gene at high levels while in the right dorsal habenula lov is expressed at reduced levels and in a smaller number of cells. Conversely, cells in the dorsal right habenula express other two genes right on and dexter at high levels while their expression in the left habenula is restricted to a smaller cluster of cells (Gamse et al., 2005; Gamse et al., 2003).

Molecular L-R differences in the zebrafish habenular nuclei also extend to their efferent projections via the fasciculus retroflexus (FR) and innervations of the midbrain target, the interpeduncular nucleus (IPN). In detail, left habenular neurons project along the entire dorso-ventral extent of the IPN while right habenular neurons project predominantly to the ventral IPN (Gamse et al., 2005).

It is important to notice that the laterality of habenular asymmetry is always concordant with the sidedness of the parapineal. In fact, in mutants in which the midline is disrupted or mutants defective in asymmetrical Nodal signaling, the laterality of the lov expression pattern is L-R randomized, and most of the embryos display reversed brain asymmetries (Gamse et al., 2005; Gamse et al., 2003).

Investigations evidenced that that the establishment of parapineal and habenulae asymmetries is coordinated through a stepwise mechanism (Concha et al., 2003; Gamse et al., 2003). First, the anlage of the left habenula delivers cues that bias the direction of the Fgf8-dependent parapineal migration towards the left (Concha et al., 2003; Regan et al., 2009). Then, the parapineal promotes the elaboration of features that characterize the left habenular nucleus. When the parapineal is artificially removed by laser ablation, much of the habenular asymmetry is lost and both nuclei display predominantly a right-sided identity (Concha et al., 2003; Gamse et al., 2005; Gamse et al., 2003). A recent work better elucidated the mechanism of the establishment of L-R asymmetries in the zebrafish dorsal diencephalon (Roussigné et al., 2009). In this work it has been proposed a model in which asymmetrical Nodal signaling in the left dorsal diencephalon (Rebagliati et
al., 1998a; Rebagliati et al., 1998b) provides cues to promote differential L-R neurogenesis of prospective habenular cells. In fact Habenular neurons differentiate earlier in the left habenula than in the right. This Nodal-dependent asymmetry is supposed to provide the left bias to the parapineal migration as it is evident before the onset of parapineal detachment from the medial pineal organ (Roussigné et al., 2009). Neuroanatomical asymmetries in the zebrafish epithalamus are of particular interest because of their possible correlation with functional asymmetries.

1.8. Early steps in zebrafish L-R development

Investigations on the development L-R asymmetries in zebrafish, revealed the requirement for the three basic steps already described as a conserved mechanism in vertebrates (Hamada et al., 2002). Thus, the extreme versatility of zebrafish together with all its unique features makes this fish a good model also for the study of vertebrate L-R development.

One fundamental step in zebrafish L-R development is the formation, at the end of gastrulation, of a ciliated structure that localizes in the tail bud at the posterior end of the notochord. This structure is conserved among all teleost fish and it is called Kupffer’s vesicle (KV) from the name of the researcher who first described it in 1868 (Kupffer, 1868).

The zebrafish Kupffer’s vesicle is a transient ciliated structure, cilia in this vesicle possess a rotating beating movement that generates a counterclockwise flow of fluid directed through the left side of the developing embryo. This morphogens-containing fluid is known to accumulate on the left-side of the notochord and trigger asymmetrical nodal-dependent gene expression cascade during following somitogenesis stages (Essner et al., 2005).

Zebrafish KV is proposed to originate from a cluster of cells that do not participate to involuting epibolic movements during gastrulation. These cells are named non-involuting, endocytic marginal blastomeres (deep NEM cells) and, at late blastula stage, they localize below the surface cells of the enveloping layer that, conversely, will actively contribute to epibolic
movements (EVL). Many studies suggested that, at the beginning of gastrulation, deep NEM cells shift from the blastoderm margin to form a distinct group of cells named dorsal forerunner (DFCs) (Cooper and D’Amico, 1996; Melby et al., 1996). This striking group of DFCs migrates to the vegetal pole in close association with the EVL laying above, and subsequently DFCs differentiate into an epithelial vesicle (KV) that then produces a cilia-based leftward flow in its interior lumen (Amack et al., 2007; Essner et al., 2005; Kramer-Zucker et al., 2005). As already mentioned, Kupffer’s vesicle is a transient structure that disappears in late somitogenesis stages by a collapsing mechanism and DFCs are incorporated into notochord (Cooper and D’Amico, 1996). Recent studies have further investigated on DFCs formation and aggregation to form a functional organ (Oteíza et al., 2008). Particularly, it has been reported that DFCs originate by the ingestion of a group of surface epithelial cells at the dorsal margin of the zebrafish gastrula. These dorsal surface epithelial cells (DSE) can be observed starting from the sphere stage (4 hours post fertilization-hpf), they are in direct contact with the yolk syncytial layer and present filopodia-like protrusions at their leading edge. During subsequent developmental stages a subset of marginal and submarginal DSE cells are internalized by the overlapping movement of the EVL and displaced below the surface epithelium. Kupffer’s vesicle formation thus involves the early conversion of epithelial surface cells into deep DFCs with mesenchymal behavior; and this process is completed by 50% epiboly, prior to the formation of the zebrafish embryonic organizer (shield) (Oteíza et al., 2008). As epiboly proceeds, the cluster of DFCs increases its segregation from the dorsal margin deep cells, but remain in contact with the EVL margin moving forward (D’Amico and Cooper, 1998). Moreover, by the end of gastrulation, the number of DFCs is reported to increase from 30-40 to about 50-60 cells, and at that developmental stage, DFCs become organized in a more compact and oval shaped cluster. As epiboly comes to its final stages, DFCs undergo a phase of re-epithelialisation to be transformed into a highly organized ciliated epithelial vesicle with a rosette-like structure. Further investigations clearly demonstrated that endogenous local Ca\(^{2+}\) release in the zebrafish DFCs region during gastrulation/epiboly stages is
necessary for cells rearrangement and KV formation. In fact, brief inhibition of endogenous Ca^{2+} in DFCs does not interfere with the differentiation of this cluster of cells but severely affects DFCs rearrangement to form KV that appears as a smaller midline cluster with individual cells scattered nearby. It has been proposed that Ca^{2+} release in DFCS region regulates β-catenin activity, and inhibition of Ca^{2+} release is thus responsible for a significant increase in nuclear β-catenin and activation of its transcriptional reporter leading to the alteration of KV formation and of normal zebrafish L-R development (Schneider et al., 2008).

It has been widely been demonstrated that many important developmental signals are involved in the zebrafish Kupffer’s vesicle morphogenesis, such as the T-box transcription factor no tail (the zebrafish homolog of mouse Brachyury, (Schulte-Merker et al., 1994) that regulates KV morphogenesis by early, cell autonomous activity in the DFCs (Amack and Yost, 2004). Importantly, recent reports have shown that Nodal signaling is also involved in DFCs specification and KV morphogenesis. In fact, it has been reported that DFC number increases or decreases in response to enhanced or reduced Nodal signaling, respectively (Choi et al., 2007). According to these observations, it has been observed that overexpressing the Nodal antagonist Lefty leads to a strong reduction in the DFC number. Moreover, zebrafish maternal-zygotic one-eyed pinhead mutants completely lack differentiated DFCs at 80% epiboly stage (Oteíza et al., 2008). Zebrafish one-eyed pinhead is a EGF-CFC gene acting as an essential extracellular cofactor for Nodal signaling, so that mutants lacking of this oep cofactor are unresponsive to Nodal activation (Gritsman et al., 1999; Yan et al., 1999). These evidences strongly support a role for Nodal signaling in the early specification of DFC cluster of cells and in KV morphogenesis (Oteíza et al., 2008).

Final steps in zebrafish KV development are characterized by cellular rearrangements of DFCs that include the formation of the lumen of the Kupffer’s vesicle together with the process of ciliogenesis (Oteíza et al., 2008). KV is composed by monociliated cells expressing the ciliary gene left-right dynein related1 (lrdr1) and its expression in DFCs is required for normal L-R development in zebrafish as lrdr1 is important for cilia motility and the
generation of fluid flow into KV. In fact, antisense morpholinos knock-down of \( lrdr1 \) disrupts cilia motility and thus the leftward fluid flow into KV (Essner et al., 2005).

It has also been reported that \( polaris \) and \( polycystin-2 \) genes are expressed in several ciliated tissues during early zebrafish development including KV (Bisgrove et al., 2005). As already studied in mice, Polaris is involved in ciliary assembly, while \( Polycystin-2 (Pkd2) \) is a \( Ca^{2+} \)-permeable cation channel associated with cilia that is supposed to play a role as a ciliary mechanosensor. Defects in \( polaris \) and \( pkd2 \) functions were found to be linked to polycystic kidney disease (PKD) in mammals. In mice, disruption of these two genes also leads to L-R patterning defects (Murcia et al., 2000; Pennekamp et al., 2002; Wu et al., 1998). Studies on zebrafish demonstrated that the knockdown of Polaris or Pkd2 function results in misexpression of normally left-sided genes, including \( southpaw, lefty1 \) and \( lefty2 \), and causes randomization of L-R asymmetry in the heart and in the gut. In addition, \( polaris \) and \( pkd2 \) are shown to be required in KV cells to regulate cilia formation and subsequent L-R development (Bisgrove et al., 2005).

As already mentioned, \( pkd2 \) gene encodes for a \( Ca^{2+} \)-permeable cation channel, and recent works have investigated the role of \( Ca^{2+} \) signaling in zebrafish L-R development (Sarmah et al., 2005; Schneider et al., 2008; Shu et al., 2007). Cilia in the KV are known to express other ion pumps as the sodium pump Na,K-ATPase \( \alpha 2 \) (encoded by the \( atp1a2a \) gene) and its functional partner, the sodium-calcium exchanger Ncx4a (Shu et al., 2003). These two ion channels have been shown to play a role in modulating the intracellular calcium levels in zebrafish thus regulating very early step of L-R patterning. In fact, downregulation of \( atp1a2a \) and \( Ncx4a \) gene sin DFCs causes cilia immotility, perturbation of the leftward fluid flow, and consequently, leads to the disruption of the asymmetrical Nodal pathway in the left LPM and in the left dorsal diencephalon (Shu et al., 2007).

Generation of a leftward fluid flow by ciliary movement inside the lumen of zebrafish Kupffer’s vesicle is the cue that triggers a cascade of events leading to the asymmetrical activation of the left-sided Nodal signaling pathway (Oteíza et al., 2008). It has been shown that at the 10-12 somite stage the left-
sided asymmetric expression of the *nodal-related* gene *southpaw* (*spaw/ndr3*) starts. The gene *spaw* is considered as the earlier molecular marker of zebrafish L-R asymmetries, and further evidences demonstrated that the expression of this gene plays a crucial role in the establishment of visceral L-R asymmetries. Moreover, even if *spaw* expression is not detected within the brain, this nodal-related factor is also required for the L-R patterning of early diencephalic asymmetries. In fact the *nodal-related3 spaw* is predicted to activate the expression of another *nodal-related* gene, *cyclops* (*cyc/ndr2*) in the left dorsal diencephalon in later somitogenesis stages (Long et al., 2003). At this developmental point Spaw has also been shown to play a role in the activation of *cyclops, lefty1* and *Pitx2* expression in the left dorsal diencephalon, and of *lefty2* and *Pitx2* expression in the heart primordium. In particular, the expression of *cyc, lefty1* and the transcription factor *Pitx2* encoding components of the Nodal signaling pathway occurs in the left dorsal diencephalon region where precursors of the future pineal organ localize during somitogenesis, thus indicating the important function of this signals in directing zebrafish brain asymmetries (Bisgrove et al., 2000; Concha et al., 2000; Essner et al., 2000; Roussigné et al., 2009).

In addition *lefty1* expression is also promoted in the midline, where it is supposed to act as a molecular barrier that inhibits the expression of left-sided signals to spread over the right side of the embryo (Ahmad et al., 2004; Bisgrove et al., 1999; Bisgrove et al., 2000; Long et al., 2003).

In zebrafish the mechanisms by which counterclockwise flow in KV is translated to molecular asymmetries around KV and how this information is transmitted to the left LPM to induce asymmetric left-sided expression of *nodal, lefty* and *Pitx2* to control L-R development are still largely unknown.

### 1.9. Zebrafish functional asymmetries

The growing interest in the development of asymmetries and the availability of a model with peculiar anatomical and genetic features make the zebrafish an ideal tool for studying brain lateralization.
Many studies on functional asymmetries are carried out on animals with laterally placed eyes such as fish and birds. In these organisms each eye largely sees a different portion of the visual field. As lateral positioning of the eyes is often accompanied by an almost complete crossing of fibers at the optic chiasma, the contralateral hemisphere primarily processes visual input of each eye. To date very few studies have investigated functional asymmetries in zebrafish and despite its relevance in molecular biology, little is known on the possible association between neuroanatomical and behavioral asymmetries in this model.

Two recent studies indicate that asymmetry of the epithalamus might, indeed, be involved in the development of behavioral lateralization in zebrafish. Barth et al. (2005) have found a correlation between visceral asymmetries and certain lateralized behaviors in the frequent-situs-inversus (fsi) line of zebrafish. The mutation that causes situs inversus in the fsi line also determines high rate of reversal parapineal position and these authors have suggested that there might be a causal relationship between asymmetries of the epithalamus and behavioral laterality. Facchin et al. (2009a) have found that artificial selection for right-eye use when looking at own mirror image significantly increased the frequency of reversed asymmetry in expression of lox gene in the habenula while selection for left-eye use tended to decrease it. Together with the genetic analyses in this dissertation I will discuss the hypothesis that parapineal positioning influences behavioral lateralization of adult Danio rerio.
2. METHODS

2.1. Abbreviations

BCIP: 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt
BSA: albumin from bovine serum
DIG: digoxigenin
dpf: days post fertilization
EDTA: ethylenediaminetetraacetic acid
FLUO: fluorescein
HM: hybridization mix
hpf: hours post fertilization
LB: Luria-Bertani Broth medium
NBT: 4-Nitro blue tetrazolium chloride
NTP: ribonucleotides triphosphate
O/N: over night
PBS: phosphate buffered saline
PBT: phosphate buffered tween
PFA: paraformaldehyde
PTU: 1-phenyl-2-thiourea
RT: room temperature
SSC: saline standard citrate
ssDNA: single stranded DNA
TAE: Tris-acetate buffer-EDTA
Tris: Tris base (2-amino-2-hydroxymethyl-propane-1,3-diol)
TE: Tris-ETDA;
WT: wild type.
2.2. Methods for keeping and raising zebrafish

Zebrafish are kept and raised, with the suitable adaptation to laboratory conditions, according to methods described in two main texts, the Zebrafish Book (Westerfield, 2000) and Zebrafish: a Practical Approach (Nüsslein-Volhard and Dahm, 2002).

Zebrafish laboratory strains are maintained in large-scale aquaria systems (“Aquarienbau Schwarz, ZebTech, TECNIPLAST and Müller-Pfleger). Recirculation systems with biological filters and a reverse osmosis supply are used to provide high-quality water and a regular water exchange rate. These large-scale recirculating systems allow raising and maintaining very large numbers of fish located in hundreds of tanks serviced by a common filter unit. High-quality water is sterilized by UV radiation before distribution to the tanks to reduce the risk of disease spreading.

Zebrafish are maintained in overflow containers according to the number of individuals, 1-liter plastic boxes for keeping single individuals, 5 or 10-litre glass or plastic boxes for 2 up to 30 fish. Fish tanks are placed in rows on to rimmed plastic or glass shelves in several suitable aquaria racks provided with constantly recirculating water from reservoirs located above the racks. Water enters into each container through a little outlet and exits through an overflow on to the shelf, from where it is drained to the filter. In racks where mouse cages are used as tanks, these are equipped with an outflow at the top margin where a hole of 3,5 cm diameter is covered by grids of different mesh size, depending on the age of the fish, in order to prevent fish to escape through the overflow. For 1-liter tanks a 2 cm long slit of a 2 mm width is cut into the top edge in the front side of the box. The water enters into the tanks via a series of outlets tubes made of silicone equipped, at their end, with a cut-off Eppendorf pipette tip so that tanks are provided with overflow water.

Healthy and fecund fish need to be maintained in constant saline, temperature and pH conditions. Fish recirculating water is provided with a suitable saline salt supply and maintained at a pH of about 7 and a conductivity between 200 and 400 μS at a temperature of 28.5°C.
Adult fish are fed with a dietary suitable to keep them in good breeding conditions. Fish are fed once with dry food flakes (TetraMin) and twice with live food composed of *Artemia salina* nauplia. *Artemia*, a species of small shrimps, is bought as cysts from suppliers and then raised to nauplia stage into appropriate hatcheries. These hatcheries are made of inverted plastic cones of about 15 liters with cut-off bottoms provided with an outlet and lighted with a neon lamp. Each cone contains 8 liters of tap water added with 192 gr of sea salt (NaCl) and 80 ml of shrimp cysts. Air pump with a bubbling stone has to be inserted into the cone to ensure vigorous mixing and to keep *Artemia* live. After two days the shrimps are completely hatched and reach the nauplia stage, stage at which they have the suitable nutritional value, and they can be harvested. To harvest *Artemia* nauplia aeration has to be stopped so that shrimps can sink to the bottom of the plastic cone, while the cysts’ shells are floating on the water. Then, the nauplia are collected through the outlet at the bottom of the cone, filtered and washed to remove excess of salt. *Artemia* nauplia are resuspended in fresh tap water and fed with a laboratory squirt bottle.

Fry zebrafish are fed twice a day from 6 dpf with powder made of dry *Artemia* (Novo Tom, JBL).

Zebrafish are usually kept on a 14-hour light - 10-hour dark cycle regulated with appropriate timers.

Fish are bred according to the Zebrafish Book (Westerfield, 2000), and mating crosses are set up in late afternoon using suitable mating boxes. These boxes consist essentially of plastic tanks with a removable inner container that fit tightly with the external one. The inner container holds the paired fish and its base in made of a grid through which the eggs fall into the outer container so that they are protected from being eaten by their parents. Sometimes a small bundle of green plastic grass is added in the inner containers as a way to reduce fish aggression.

Zebrafish mate and spawn when the light turns on in the morning. Fish eggs held in the outer box of the mating tanks are collected by pouring the water from this outer container through a fine tea net. Eggs are then transferred from the tea net to a Petri dish in embryo medium (Fish Water or Ringer’s
solution). Laid eggs are then observed under a dissection microscope and unfertilized eggs or dead embryos have to be regularly removed because they are a substrate for growth of bacteria and moulds. Embryos are incubated at a temperature range between 25°C to 33°C and then fixed at suitable stages for in situ hybridization or immunohistochemistry protocols. Alternatively embryos can be raised to adulthood: at 6 dpf larvae are transferred in 1-liter tanks (about 50-80 larvae per tank) and maintained in an incubator at 28.5°C until they reach 1 month of age when they are moved to the ZebTech, TECNIPLAST aquarium. Tanks of this system are equipped with mesh that does not allow young fish to escape so that they can grow to adulthood. Females zebrafish used in genetic screening for the maternal effect mutation described in this study were stocked and named separately in 1-liter containers.

2.3. Recipes

FISH WATER 1X:
100 ml SALINE STOCK SOLUTION 50X
50 μl Methylene Blue 1000X
dH₂O up to 5 lt.

SALINE STOCK SOLUTION 50X:
25 mM NaH₂PO₄ (3.43 g)
25 mM Na₂HPO₄ (4.45 g)
75 g ISTANT OCEAN
ddH₂O up to 5 lt.

RINGER’S SOLUTION NORMAL:
116 mM NaCl
2.9 mM KCl
1.8 mM CaCl₂
5 mM HEPES, pH 7.2.
PTU:
0.003% PTU (1-phenyl-2-thiourea) in fish water.

TRICAIN:
(3-amino benzoic acid ethyl ester also called ethyl 3-aminobenzoate) comes in a powdered form from Sigma. Make tricaine solution for anesthetizing fish by combining the following in a glass bottle with a screw cap.

Stock solution 25X:

400 mg tricaine powder
97.9 ml DD water
~2.1 ml 1 M Tris (pH 9).

Adjust pH to ~7. Store this solution in the fridge.
To use tricaine as an anesthetic combine the following in a 50 ml beaker: 2 ml tricaine solution
~48 ml clean fish water.

2.3.1. Whole-Mount in situ Hybridization Solutions

HYBRIDIZATION MIX (HM):
60% formamide
4.6 μM citric acid pH 6
SSC 5X
0.1% Tween-20
50 μg/ml heparin
500 μg/ml torula yeast total RNA (tRNA)
dH₂O up to 100 ml

WASHING MIX (HM WASH):
HM without tRNA and heparin
PBS 1X:
150 mM NaCl
10 mM Na₂HPO₄
ddH₂O up to volume

PBT 1X:
1X PBS
0.1% Tween-20

PFA:
4% paraformaldehyde in PBS 1X

ANTIBODIES ANTI DIGOXIGENIN/FLUORESCEIN:
Antibodies used for whole mount in situ hybridization are provided by Roche, they are diluted 1:1000 in a PBT 1X/2% sheep serum/200 mg:ml BSA solution and pre-adsorbed using 50 fixed embryos/ml of various developmental stages. After 2 h at RT, the antibody solution is diluted to 1:3000 and filtered. Then NaN₃ is added for better storage at 4°C.

NBT/BCIP STAINING BUFFER:
100 mM Tris-HCl pH 9.5
50 mM MgCl₂
100 mM NaCl
0.1% Tween20
ddH₂O

NBT/BCIP STAINING SOLUTION:
NBT/BCIP (La Roche) 20 µl/ml in staining buffer.
Alternatively use separate components
- 7.5 µl/ml NBT (Nitro Blue Tetrazolium provided by Sigma) 50 mg/ml (50 mg NBT dissolved in 0.7 ml anhydrous dimethylformamide and 0.3 ml H₂O). Store in the dark at ~20°C.
• 3.5 µl/ml BCIP (5-Bromo 4-Chloro 3-Indolyl Phosphate, provided by Sigma) 50 mg/ml (50 mg dissolved in 1 ml anhydrous dimethylformamide). Store in the dark at –20°C.

BENZYL BENZOATE-BENZYL ALCOHOL


2.3.2. Solutions for bacteria transformation and RNA probe synthesis

LB MEDIUM:
5 g tryptone
2.5 g yeast extract
5 g NaCl
ddH₂O up to 500 ml and autoclave to sterilize.

LB AGAR:
2 g tryptone
1 g yeast extract
2 g NaCl
3 g agar
ddH₂O up to 200 ml and autoclave to sterilize.

DEPC WATER (Nuclease-free water):
1 ml DEPC (Diethyl pyrocarbonate) in 1lt ddH₂O. Shake for 1h to O/N. DEPC must then be completely destroyed by autoclaving.

TAE (50X) ELECTROPHORESIS RUNNING BUFFER

Tris base 240 g
Glacial acetic acid 57.1 ml
0.5M EDTA 100 ml
ddH₂O to 1 lt.
2.4. Transformation of *E. coli*

Bacterial transformation allows to obtain large plasmids copy number. Vectors containing part of the cDNA of interest were introduced in bacterial *E. coli* cells (One Shot® TOP10 Chemically Competent *E. coli* provided by Invitrogen™). Vectors confer to the bacterial cells antibiotic resistance as a selectable marker for plasmid-containing cells.

Chemically competent *E. coli* stored at -80°C were placed on ice, a small amount of plasmid DNA (10-50 ng) is added to the bacteria cells that were left on ice for 15 minutes before preforming heat shock. A 30-second long heat shock at a temperature of 42°C allow the *E. coli* cells to take up vector DNA. Transformed cells are replaced on ice for 2 min adding 200 μl of nutrient S.O.C medium (Invitrogen™) and then incubated for about 45 min in a shaker at a temperature of 37°C. This incubation allows antibiotic resistance to be expressed so that cells can be plated on agar containing ampicillin or kanamycin. Plates are incubated over night and plasmid-containing colonies can grow on solid medium. Positive colonies are then selected and singles clones are cultured in Luria-Bertani medium where the suitable antibiotics has been added to improve selection of vector-containing clones. Liquid cultures provide massive bacterial growth in order to obtain a large plasmid copy number after extraction and purification.

2.5. Purification of plasmid DNA

The extraction and purification of plasmid DNA was performed using commercial kits provided by Qiagen (QIAprep®) based on the alkaline lysis of bacterial cells in order to release plasmid DNA. These kits are equipped with special silica matrix columns that binds plasmid DNA with high affinity in the presence of high salt (Vogelstein and Gillespie, 1979). Then two wash with suitable buffers are performed to remove excess of salts and improve DNA recovery.

High-quality plasmid DNA is then eluted from the column with 50–100 μl of nuclease-free water pre-heated at 65-70°C in order to increase elution efficiency.
2.6. Anti-sense probe synthesis

An improving possibility to study the expression of genes of interest is to generate an epitope-tagged antisense RNA probe directed against the targeted gene/mRNA. To construct digoxigenin or fluorescein-labelled antisense RNA probes the partial cDNA of the gene of interest is cloned in a plasmid vector such it is under the control of a phage promoter. These phage promoters are recognized by the RNA polymerases encoded by phages as T3, T7 or SP6 and they allow large amounts of RNA to be produced in vitro. After extraction and purification, the plasmid is linearized, using a suitable restriction enzyme, at the 5’ end of the inserted gene of interest (Table 3.1). This step is necessary as polymerases will fall off the end of the linearized plasmid after transcription and the enzyme can start a new transcription, in a process called run off synthesis.

<table>
<thead>
<tr>
<th>Vector</th>
<th>linearization</th>
<th>transcription</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBK-CMV_lov (Gamse et al., 2003)</td>
<td>EcoRI</td>
<td>T7</td>
</tr>
<tr>
<td>pGEM-T-Easy_spaw (Long et al., 2003)</td>
<td>SpeI</td>
<td>T7</td>
</tr>
<tr>
<td>pBSSKII_lefty1 (Thisse and Thisse, 1999)</td>
<td>NotI</td>
<td>T7</td>
</tr>
<tr>
<td>cyclops (cyc) (Rebagliati et al., 1998b)</td>
<td>NotI</td>
<td>T7</td>
</tr>
<tr>
<td>sox17 (Amack et al., 2007)</td>
<td>EcoRI</td>
<td>T7</td>
</tr>
</tbody>
</table>

Table 3.1: Vectors containing part of the genes of interest to synthesize anti-sense RNA probes, each one with the suitable restriction enzyme and RNA polymerase.

Linearization reaction is performed in a 50 μl total volume with approximately 5 μg of plasmid to be digested, and using 1.2 μl of the suitable restriction enzyme coupled with its buffer and BSA when requested to enhance endonuclease performance:

- ddH2O up to 50 μl
- plasmid DNA 5 μg
• 10X buffer 2 μl
• BSA 100x (if needed) 0.5 μl
• restriction enzyme 1.2 μl

Linearization reaction goes 2 h at 37°C or at a temperature suitable to improve enzyme performance.

The linearized plasmid is then purified and precipitated over night at -80°C.

The purification of the vector after linearization is carried out using Phase Lock gel tubes (Eppendorf):
  • centrifuge column tubes 1 minute max speed to pack the gel at the bottom of the tube;
  • add nuclease free water to 100 μl to the sample of linearized DNA, and transfer the volume on the top of the gel of Phase Lock column;
  • add 50 μl phenol and 50 μl chloroform;
  • centrifuge 5 min max speed;
  • the Phase Lock gel separates solvents with saline buffers and enzymes of the linearization reaction from the aqueous phase containing the DNA;
  • add 100 μl chloroform and centrifuge 5 min max speed;
  • collect the aqueous phase containing purified linearized DNA (approx. 100 μl) that is on the top of the gel, and transfer it in a new 1,5 ml tube.

Then add to the purified DNA 0,1 volumes (10 μl) NaCl and 2,5 volumes of Ethanol absolute, mix well and precipitate at least 2 h or over night at -80°C.

After precipitating add 2 μl of glycogen to the linearized DNA mixture and centrifuge 15 min max speed;
  • remove the surnatant being careful not to touch the pellet;
  • wash the pellet 3 times with 200 μl 70% ethanol centrifuging 2 min max speed each time and removing surnatant;
  • dry the pellet from ethanol residuals and the re-suspend it in 14 μl of nuclease-free water.

Then run an electrophoresis on 1,5% agarose gel loading 1 μl of purified linearized plasmid DNA in order to control the quality of the digestion.
If the linearization was successfully carried out the antisense RNA probe can be transcribed starting from a phage promoter (T7, T3 or SP6) flanking the 3’ end of the inserted gene of interest. Nucleotides added by RNA polymerases are UTP digoxigenin- or fluorescein-labelled. Synthesis of mRNA probes were performed in a 20 μl reaction volumes at a temperature of 37°C from 2 hours to 6 hours depending on probe length.

- linearized plasmid DNA 13 μl
- 10X transcription buffer (Roche) 2 μl
- DIG- or FLUO-RNA Labeling Mix (Roche) 2 μl
- RNAsin® Ribonuclease Inhibitor (Promega) 1 μl
- (T7, T3 or SP6) RNA Polymerase (Roche) 2 μl

After the transcription, a step is required to digest template plasmid DNA with 2 μl of DNase (RNAse-Free DNase, Promega) at 37°C for 20 min. This allow to purify a high quality RNA probe.

The purification of antisense RNA probes is performed using a commercial kit (MEGAclear™ provided by Ambion®). Once purified, the RNA probe can be stored at -80°C. Then an electrophoresis has been run on 1,5% agarose gel loading 1 μl of purified probe in order to control and quantify the transcription. Prior to being loaded on gel, the probe has to be denatured at 65°C for at least 5 min.

2.7. Single-probe Whole-Mount In Situ Hybridization (WISH)

*In situ* hybridization is a method useful in studying the expression of genes of interest. This method is based on the generation of epitope-tagged RNA probes directed against the gene/mRNA of interest. Zebrafish embryos and larvae allow to perform whole-mount in situ hybridization, thus permitting three dimensional analysis on tissues and organs.

The most common protocol for *in situ* hybridization implies fixing zebrafish embryo in 4% paraformaldehyde (PFA) in PBS 1X for 2 h at room temperature (RT) or overnight at 4°C in 2 ml eppendorf tubes. Embryos are selected at different developmental stages and, if necessary, removed from their chorion using dissection needles. Before late somitogenesis stage
embryos have to be dechorionated after fixation, while in later and larval stages it is more suitable to carefully dechorionate embryos alive to avoid they remain curved as if they were fixed inside the chorion.

After fixation PFA is removed, embryos are washed in PBS and then transferred in methanol 100% for at least 2 h at -20°C before starting the *in situ* hybridization protocol. Embryos can be stored in methanol for months but this step is necessary for permeabilization of embryos (Nüsslein-Volhard and Dahm, 2002).

**Day 1**

1. Rehydration of samples stored in Methanol 100% at -20°C.
   - 75% MetOH - 25% PBS 1X 5 min RT
   - 50% MetOH - 50% PBS 1X 5 min RT
   - 25% MetOH - 75% PBS 1X 5 min RT
   - 100% PBT 4 x 5 min RT

2. After PBT washes, digest with proteinase K (10 µg/ml). This step permeabilizes the embryos permitting access of the RNA probe. The digestion time is dependent on the developmental stage. No digestion is needed for embryos at blastula, gastrula and somitogenesis stages (up to the 18 somite stage). For 24 hpf old embryos digest for 5 min and for older embryos (from 36 h to 5 days old embryos) digest for 40-60 min. Proteinase K digestion works at RT and it is stopped by incubation in 4% paraformaldehyde in 1 x PBS.
   - Post-fixation of digested embryos with PFA 4% in PBS for 20 min at RT. Also embryos that do not need proteinase K digestion are usually post-fixed to strengthen the samples.
   - Washes with PBT 5 x 5 min RT

3. Pre-hybridization step is performed by incubation in 500-800 µl of Hybridization Mix (HM) for 2 to 5 h in a waterbath set at 60-70°C, depending on the melting temperature of the probe.
4. Hybridization step: remove and discard the pre-hybridization mix. Replace with 200 µl of hybridization mix containing about 100-200 ng of antisense DIG (or FLUO) labelled RNA probe. Hybridize overnight in a waterbath at 60-70°C.

Day 2
Remove the hybridization mix with the RNA probe and recover it: probe-containing solutions can be used several times.

1. Wash briefly with HM for washes (HM without tRNA and heparin) at 65°C
   - 75% HM Wash - 25% SSC 2X 15 min 65°C
   - 50% HM Wash - 50% SSC 2X 15 min 65°C
   - 25% HM Wash - 75% SSC 15 min 65°C
   - 100% SSC 2X 15 min 65°C
   - 100% SSC 0,2X 2 x 15 min 65°C
   - 75% SSC 0,2X - 25% 10 min RT
   - 50% SSC 0,2X - 50% PBT 10 min RT
   - 25% SSC 0,2X - 75% PBT 10 min RT
   - 100% PBT 10 min RT

2. Incubation with blocking buffer of PBT/2% sheep serum/2 mg:ml BSA, at RT for 2 to 4 hours.

3. Incubation in 400 µl of antibody solution diluted at 1/3000 in blocking buffer overnight at +4°C under slow agitation on a horizontal orbital shaker.

Day 3
Remove the antibody solution diluted at 1/3000 and recover it, antibody solutions diluted can be used up to three times.
1. Wash briefly with PBT at RT, then wash extensively 6 x 15 min at RT under slow agitation on a horizontal orbital shaker. Before starting the staining step, embryos are washed 3 x 5 min in NBT/BCIP staining buffer.

2. Staining step: embryos are transferred from eppendorf tubes into wells of a 24-well plate, the staining buffer is then removed and NBT/BCIP staining solution is added. Embryos are incubated in staining solution at room temperature under slow agitation on a horizontal orbital shaker and protected from day light by a layer of aluminium foil. Staining reaction is regularly monitored under a dissection microscope.

3. The staining reaction is topped by removing the staining solution, washing 3 x 5 min with PBT and then placing back the embryos in the eppendorf tubes in PFA 4%.

Stained samples are usually stored in PFA 4% in the dark at 4°C.

2.8. Mounting and imaging

Labelled embryos are mounted in 100% glycerol. Embryos at early developmental stages are observed in glycerol on depression slides. Whole mount pictures were taken with a Nikon SMZ 1500 Macroscope provided with a digital camera. Embryos starting from 20 somite stage were manually de-yolked using dissection needles and then flat mounted on slides. Slides were provided with small chambers cut into several layers of adhesive tape (1 layer until 24 hpf, 2 layers for 48 hpf and so on), the embryos is placed, in the desirable position, in glycerol into the chamber and then flattened using a coverslip. Flat mount pictures were taken with a Leica DMR Microscope. Digitalized pictures are saved as TIFF files, then adjusted for contrast, brightness and color balance using a Photoshop CS2 software and stored as such or after conversion to the .jpeg format to reduce the files size.
2.9. Fluorescent beads injections

This procedure has been performed according to protocols described in previous works of other authors (Essner et al., 2005; Neugebauer et al., 2009).

Fluorescent beads of 0.5 µm diameter (Polysciences) were diluted 1:100 in dd water and 0.1% of Phenol Red (Sigma) and then injected into KV of live embryos at 8-10 somite stage. Embryos were manually dechorionated, placed into 3x3 mm wells of injection plates made in 0.8% agarose in Ringer’s solution on coverslips and mounted in 1% low melting agarose (Sigma) with the KV facing upwards, as shown in figure 3.1.

Microinjection needles need to be thick enough to penetrate the embryo but not so to damage Kupffer’s vesicle and compromise its function.

Microinjection capillaries are prepared in a needle puller (DMZ Universal; Zeitz Instruments) to obtain narrow and fine needles. The needle is then filled with 2.5 µl of beads solution, using a micropipette fitted with tips used to load sequencing gels (Eppendorf Microloader Tips). The closed end of the needle is removed using fine forceps to give an opening suitable for KV diameter (adapted from Nüsslein-Volhard and Dahm, 2002).

Microinjection set-up was composed by a stereomicroscope and a micromanipulator connected to a magnetic clam stand and a pneumatic microinjector (Pneumatic Picopump) with air compressor.

Beads movement was visualized using a 40x/0.80 water immersion lens and recorded on a Nikon fluorescence microscope (Nikon ECLIPSE 80i) using ATI Multimediacenter software.
2.10. Bright field screening of Kupffer’s vesicles

Embryos at the 10 somite stage were mounted as previously described for microinjections and vesicles were visualized using a 40x/0.80 water immersion lens on a Nicon ECLIPSE 80i microscope. Using the software Image Pro Plus 6.0 we could measure the AP diameter, LR diameter and the area of vesicles.

2.11. Behavioral tests

2.11.1. Test subjects

The first phase of behavioral analyses involved fish derived from crosses between females of the fifth generation of TLRE zebrafish line of behavioral selection described in (Facchin et al., 2009a) and males from the Tg(foxD3::GFP)zf15. In this transgenic line the GFP, under the promoter of the foxD3 is expressed in the pineal complex (Gilmour et al., 2002) and this allows the in vivo discrimination of the left- or right-sided position of the parapineal organ at 3 dpf. Embryos derived from these crosses were were anesthetized in Tricaine and analyzed using a stereomicroscope (LeicaMZFLIII) equipped with a UV-lamp. The whole sample of larvae was then divided into two groups according to the position of the parapineal organ: L-PPO (subjects with the parapineal organ on the left side) and R-PPO (subjects with the parapineal organ on the right side). Embryos were then raised to adulthood and they were subjected to the tests at 6-10 months of age. All the tests were performed at approximately 1-month interval and, when possible, the same subjects underwent all of these tests.

2.12. Laterality tests

2.12.1. Mirror test

In the first laterality test 62 subject have been tested: twenty-nine zebrafish with left-sided parapineal organ (L-PPO; 17 females and 12 males) and thirty tree zebrafish with the parapineal organ on the right side of the diencephalon (R-PPO; 18 females and 15 males).
The apparatus used for testing zebrafish in eye preference for viewing their mirror image (Fig. 3.2) was similar to that already described in a previous study (Dadda et al., 2007). It consisted of a glass tank (60 cm x 60 cm x 45 cm; width/length/height) where eight mirrors (27 cm × 37 cm; length/height) have been placed around the aquarium’s wall to create an octagonal shape. The bottom of the aquarium was white and with no gravel in order to improve the visibility of the subject. At the center of the experimental tank an octagonal shaped starting-box made of white plastic (each side 13 cm x 16 cm; length/height). This starting-box housed the experimental fish for acclimation before the beginning of the test: the subject was dip-netted into a corridor (30x13 cm) provided with a trapdoor (3.5 cm × 16 cm; width/height) that could be lifted via a nylon thread connected on a pulley system allowing the fish to leave the starting-box and explore the new environment. The apparatus was filled with 9 cm of system water at a temperature of 28.5 °C and lit by four neon lights (18 W) placed along the aquarium’s wall. A video camera was positioned above the experimental tank at a distance of 2 m and the whole apparatus was surrounded by black curtains. Each subject was dip-netted from the home tank and placed in a small plastic cylinder (diameter 7 cm, height 8 cm) to be transported to the apparatus where the fish was gently released into the starting box. After a 2-min period the trapdoor was lifted and, once outside the starting box, the subject’s swimming behavior was immediately recorded for a period of 8 min. At the end of the experiment the subject was captured and released back to his home tank. Each subject was observed once.
The original video recordings were edited using Adobe Premiere® Pro 2.0 in order to obtain 2 frame per second (fps) clips. With the help of a suitable computer program (written in Delphi4 – Borland®) fish positions were scored per each frame. Subject positions have been calculated as the distance (expressed in millimeters) and the degree of alignment between the test fish and the mirror. The degree of alignment refers to the angle (expressed in degrees) formed by the prolongation of the anteroposterior body axis of the fish with respect to the closest mirror (Fig. 3.3). To calculate the laterality index we considered the observations in which the fish was swimming along a mirror (±30° from a parallel line) within 9 cm from the mirror. We excluded from the analyses all cases in which the experimental fish was orthogonal to the mirror and when the distance between the subject and the mirror was wider than 9 cm because the fish could not be seen as reflection in the mirror. The index of eye use was calculated as: [(frequency of right-eye use):(frequency of right-eye use + frequency of left-eye use)]. From the video recordings we also scored the latency to reach the mirror, that is the time interval between the emergence of the experimental fish from the corridor of the starting box and the frame in which they reach 2 cm from the mirror.

2.12.2. Eye use in predator inspection

Forty-nine zebrafish were analyzed in this experiment 24 L-PPO (15 females and 9 males) and 25 R-PPO (14 females and 11 males). Data on eye preference were measured during the personality test called “distance from the predator” (see below for more details). The behavior of the subject was observed in a glass tank (the subject tank, 40 cm x 60 cm x 35 cm; width/length/height). During the test fish could observe a live predator, *Amatitlania nigrofasciata*, housed in an adjacent tank (Fig. 3.4). The position of the subject was sampled every 2 s during a 10-minutes test period. We
measured the number how many time the subject was observing the predator with his right or left eye excluding those cases in which the fish was closer than 6 cm (corresponding approximately to one body length) to one of the walls. In these areas the subject’s eye use could be constrained by proximity to the glass. An index of eye preference was calculated as: \([(\text{frequency of right-eye use}) : (\text{frequency of right-eye use} + \text{frequency of left-eye use})]\). In addition, we recorded the zone and the sector occupied by the test fish in each valid measurement. A few subjects spent most of the time attempting to escape from the compartment and scored less than eight valid observations. These subjects (15 fish; 6 R-PPO and 9 L-PPO) were discarded from the analyses.

The test fish was dip-netted from his home tank, transported in a plastic container (diameter cm, height 8 cm) and gently released to the centre of the apparatus where he spent 10 min of acclimation. Then a green plastic panel that obscured the subject tank from the predator tank was lifted up ad the zebrafish behavior was video recorded and observed for 10 minutes.

2.12.3. Rotational preference in a familiar environment

In this behavioral test, fifty-seven fish were tested, 26 L-PPO (16 females and 10 males) and 31 R-PPO (16 males and 15 males).

The apparatus was a modification from that described in a previous study in which it has been observed the swimming direction in a familiar environment in *Girardinus falcatus* (Bisazza et al., 2005). The apparatus consisted of a squared glass tank (60 cm x 60 cm x 45 cm) containing four smaller tanks made of white plastic (upper Ø 28 cm, lower Ø 20 cm, 27 cm height). A ring-shaped swimway was created into each plastic tank (inner radius 15 cm, outer radius 20 cm). Each swim way was filled with 12 cm of system water at a temperature of 28.5 °C. The whole apparatus was lit by four neon lights (18

Figure 3.4: Picture showing an adult male of *Amatitlania nigrofasciata*. 
W) connected on a timer (8:30 a.m. to 10:30 p.m. L–D cycle) and covered with a green plastic net acting like a one-way screen. Each subject was housed singly in the tank and the presence of four identical tanks in the apparatus allowed us to record simultaneously two adjacent swimways with a R-PPO and two with a L-PPO fish using a video camera mounted above the apparatus at a distance of 2 m. Experimental fish were placed into the tanks at 6:00 p.m. and left undisturbed until 3:00 p.m. of next day. The subject’s behavior was then recorded for 2 h consecutively. We measured the number of complete rotations and calculated an index of rotational preference as: 

\[
\frac{\text{frequency of clockwise rotation}}{\text{frequency of clockwise rotation} + \text{frequency of counterclockwise rotation}}.
\]

2.12.4. Turning direction in the dark

In this experiment sixty subjects were used, 31 L-PPO (18 females and 13 males) and 29 R-PPO (15 females and 14 males). The apparatus was similar to that used in a previous work concerning the study of swimming direction in the dark in *Girardinus falcatus* (Bisazza et al., 2005) and consisted of a plastic circular tank (upper diameter 102 cm, lower diameter 98 cm, height 36 cm). A hollow cylinder (diameter 11 cm) was placed in the center of the experimental tank and connected on a pulley system so that it could be lifted via a nylon thread from a remote location (Fig. 3.5). The tank was filled with 11 cm of system water at a temperature of 28.5°C and placed in a completely darkened room. The apparatus was lit with two 20 W infrared lamps placed 300 cm above the tank. Infrared lamps have been used according to the characteristics of zebrafish visual system Wavelength emission of infrared light ranged 800–950 nm with a peak at 850 nm, therefore well outside the sensitivity of both zebrafish rods and cones (Bilotta and Saszik, 2001). Each
subject was dip-netted from his home tank, transported to the apparatus and introduced into the cylinder that was gently raised up after 10 min of acclimation and video recording started. Every trial was video recorded for 10 min while the experimenter could see the session on a monitor outside the darkened room. Video recordings were analyzed frame by frame and we considered those turnings that occurred at least 10 cm from the wall.

Valid measurements were taken when changes of direction with respect to the previous swimming direction were of at least 20° within a period of 400 ms (Fig. 3.6). An index of turning direction was calculated as: \[ \text{index of turning direction} = \frac{\text{frequency of right turn}}{\text{frequency of right turn} + \text{frequency of left turn}} \].

In addition we measured the total distance covered by fish in the first minute after being released.

**2.13. Measures of personality**

In these experiments sixty-four subjects were tested: 31 L-PPO (19 females and 14 males) and 31 R-PPO (18 females and 13 males). Each fish underwent in sequence three test measuring called (1) social distance, (2) activity in open field under social isolation, (3) distance from the predator during which we also measured the eye use in predator inspection.

The apparatus used for the tests was similar to that previously described for zebrafish by Moretz et al (2007) and consisted of a central glass tank (40 cm x 60 cm x 35 cm; width/length/height) flanked on both sides by two smaller glass tanks (40 cm x 40 cm x 35 cm; width/length/height). The central tank housed the experimental fish and was divided into 10 identical sectors (6 cm x 40 cm; width/height). This tank was filled with 30 cm of water at a temperature of 30±2 °C and lit by two neon lights (18 W); there was no gravel in order to improve visibility of the experimental subject. Two green plastic panels acting as one-way screens covered the longer walls of the tank. One of
the lateral tanks housed a social group of wild-type zebrafish (three females and three males) who was confined in the fore portion of this tank by means of plastic partitions that concealed a filter. The tank was lit by two neon lights (18W) and the floor was covered with 3 cm of gravel. The second lateral tank housed a live predator, an adult male of *Amatitlania nigrofasciata*, a cichlid fish whose diet includes small fish (Fig. 3.7). Two glass partitions (15 cm x 34 cm; width/height) placed at 30° served to confine the predator in the central part of his tank when it approached the subject’s tank.

![Figure 3.7: Schematic representation of the apparatus used for the measure of personality traits: (a) subject tank, (b) tank holding social companions and, (c) predator tank.](image)

The predator tank was also provided with a shelter, a plastic jar with a diameter of 16 cm. The subject central tank was separated from the two lateral tanks by two green plastic removable panels (35 cm x 40 cm; width/height) connected on a pulley so that they could be lifted at distance. A video camera was fixed 2 m above the apparatus. The social companions were introduced into their tank the day before the test while the predator was housed in his tank for the whole duration of the experiment.

### 2.13.1. Social distance

At the beginning of this test the experimental subject was dip-netted in the tank, the panel between the subject and the companions tank was lifted up so that the test fish could interact with the stimulus shoal. Conversely the predator compartment was obscured by the green partition. The subject was video recorded for a 5 min period and recordings were then examined
scoring every 2 seconds (150 total observations) the sector of the tank that the test fish occupied during the experiment. This scores represent a measure of the distance from the stimulus shoal.

2.13.2. Activity in open field under social isolation
After the social distance test, the green opaque partition between the shoal and the subject tank was lowered and the behavior of the test fish in the open field was observed for another 5 min. In order to score the activity, the tank was divided on video recordings into 15 rectangular squares (width/length 12 cm x 13.3 cm each). So the total number of squares crossed by the subject in each of the 5 min of the test was scored. Then the subject tank, on the screen, was divided into an outer portion formed by an area of 5 cm (approx. one body length) from the walls and an inner portion of 30 cm x 50 cm. The amount of time spent in the center of an open field is commonly considered as a measure of a low-anxiety behavior (Bale et al., 2002; Carola et al., 2002). The proportion of time spent in the two portions was calculated in each of the 5 min of the test.

2.13.3. Distance from the predator
At the end of the previous test, at the moment when the test fish was at least 48 cm from the predator tank, the green opaque panel was lifted so that the subject could see the predator. The test lasted 10 min and was video recorded. The distance of the predator was measured scoring every 2 seconds the sector occupied by the test fish excluding the sector closest to the predator since in a great portion of this sector the predator was not visible.

2.14. Statistical methods
Statistics were performed using SPSS 14.0. We checked for homogeneity of variance and where necessary the appropriate transformations have been applied. Means are expressed ± standard deviation. In the four laterality experiments the number of observations differed among subjects; cases were therefore weighted by the logarithm of the number of observations.
3. RESULTS

3.1 PART I: genetic analysis

3.1.1. Isolation of the maternal effect gene

In a recent work, Facchin and colleagues observed that two lines of zebrafish selected for opposite behavioral lateralization, also showed differences in anatomical left-right asymmetries. This research aimed at verifying whether wild type zebrafish (the GT and TL strains) could be artificially selected for left- or right-preference in eye use during monocular inspection of their mirror image. The results of this artificial selection suggested that behavioral laterality has a genetic basis in zebrafish as it led to a rapid change in phenotypic frequencies. In both selected lines (GTLE with a bias in left-eye use and TLRE with a preference for right eye use), Facchin and colleagues observed that selection increased the frequency of individuals significantly lateralized in a specific direction, conversely selection decreased the frequency of individuals significantly lateralized with the opposite preference in eye use. In addition, authors also investigated if there was a correlation between visceral positioning, brain asymmetries and selection for behavioral lateralization. As a result, they evidenced that selection for a preference in right-eye use for monocular inspection of a social stimulus also increased in the population the frequency of individual with reversed epithalamic asymmetries and pancreas position. In particular, data reported in this work indicate that, after five generation of artificial selection in the TLRE the frequency of embryos with reversed asymmetry in the position of the parapineal organ increased from 12.5% in the base wild type stock (TL), to 35.8% in the fifth generation of selection (TLRE). Results suggest that selection for right-eye use significantly increased the frequency of reversed asymmetry in the epithalamus, starting from a base wild type stock with a frequency individuals with reversed brain asymmetries that was significantly higher compared to other wild type stocks (Facchin et al., 2009a).

Considering these preliminary data we decided to perform selective crosses in order to assess the genetics underlying this selected trait. The first step
was to analyze the offspring from reciprocal crosses. In these reciprocal mating crosses fish used were from the 5th generation of selected lines. In the first cross females of the GTLE line (with a preference for left-eye use) were paired to males of the TLRE line with the opposite preference, and in the second cross, females of the TLRE line were paired to males of the GTLE line. For each reciprocal cross, embryos were collected and raised until 3 dpf, then they were fixed and in situ hybridization protocol was performed using an antisense probe for the leftover gene (Gamse et al., 2003). This gene is a marker of the habenular nuclei and its expression has been reported to be stronger in the left dorsal habenula than in the right (Fig. 3.1), and this is related to the neural connection of the left-sided parapineal organ with the left habenular nuclei. When diencephalic asymmetries are reversed and the parapineal organ is on the right side of the epithalamus, the expression of leftover is stronger in the right habenular nucleus (Gamse et al., 2005; Gamse et al., 2003).

According to Facchin's data (2009a), results presented in this thesis show that when crossing females from the of GTLE line with a preference for left eye-use with males from the TLRE line with the opposite preference, more than 97.2% (n=273/281 embryos) of offspring showed left-sided parapineal organ, as demonstrated by detection of leftover expression in the habenular nuclei in 3 dpf larvae. This frequency is similar to those found for wild type strains reported in Gamse and collaborators (Chi-square=0.011; p=0.915, Gamse et al., 2003) and for GTLE selected line reported in Facchin and

Figure 3.1: In situ hybridization on 4 dpf larvae showing the expression of the leftover (lov) gene, a marker of habenular L-R asymmetries. Normal lov expression is stronger in the left habenular nucleus (a), while in larvae with reversed asymmetries lov expression is stronger in the right habenula (b).
collaborators (Chi-square=1.11; p=0.292, Facchin et al., 2009a). This result was unexpected because, as already mentioned, incross of subject from the TLRE selected line generated 35.8% of embryos with reversed brain asymmetries (Facchin et al., 2009a). This result was unexpected because, as already mentioned, incross of subject from the TLRE selected line generated 35.8% of embryos with reversed brain asymmetries (Facchin et al., 2009a).

Another surprising result was found analyzing progenies from the second reciprocal cross. Mating pairs between females from TLRE line and males from GTLE produced offspring in which approximately 25% of larvae showed reversed epithalamic asymmetries. This results are strongly persistent and similar considering offspring from different TLRE females (mean=23.9%±5.6). Moreover, the same frequencies of reversed parapineal were reported when TLRE females were crossed to males of wild type strains present in the laboratory facility (mean=23.3%±5.2). In fact no significant difference has been found between the two series of crosses (two-sample t-test, t[5]=0.32, p=0.76).

These results led to the hypothesis that the behavioral selection promoted the isolation of a spontaneous mutation in a maternal effect gene and thus experiments have been programmed to validate this hypothesis.

Maternal effect occurs when a specific phenotype in the zygote is controlled by the mother's genotype, that is already present in the egg cells prior to the fertilization, and does not involve extranuclear genes. Maternal effect genes are mostly required in early developmental stages, before the onset of the transcription of the zygotic genome, that in the zebrafish starts at the midblastula transition stage (about 1000 cells) (Kane and Kimmel, 1993; Kimmel et al., 1995). Maternal transcripts are provided and stocked in the eggs cell during oogenesis and thus maternal effect means that during the development the embryo does not express products from its gene but it inherits the gene product from the mother’s transcript. If the transcript originates from a mutated gene, the gene product will be altered even if the zygotic gene is normal. For this reason, in order to determine the mother's genotype for the considered trait, the screening of the progeny phenotypes is necessary.
The discovering that a higher frequency of the reversed phenotype could be detected only when selected TLRE females were crossed with any male allowed us to cross those females to transgenic males of the Tg(foxD3::GFP)zf15 line (Gilmour et al., 2002), this creating the F₀ generation. The Tg(foxD3::GFP)zf15 transgenic line under the control of the foxD3 promoter, in which GFP expression is localized also in the pineal complex thus allowing the in vivo discrimination of the position of the parapineal organ (Fig. 3.2). Offspring derived from F₀ mating compose generation F₁, fish were screened for the position of the parapineal organ at 3 dpf and fish with left (72%) and right (28%) parapineal organ were raised separately.

Once raised to adulthood, F₁ males and females with right or left parapineal were incrossed to obtain the F₂ generation (Fig. 3.3). According to the maternal effect gene hypothesis F₀ TLRE females, have been considered as recessive homozygous for the putative mutation (-/-), and foxD3::GFP males as homozygous wild type (+/+), the resulting F₁ is genetically heterozygous (+/-) but its phenotype is the expression of maternal mutated genotype. As a consequence, F₂ generation should be composed by 1/4 homozygous wild type (+/+); 1/2 heterozygous (+/-) and 1/4 recessive homozygous for the putative mutation (-/-). The hypothesis of this genetic transmission could be supported even if the position of the parapineal organ in the parental F₀ is unknown. To validate this supposition
F₂ females, once raised to adulthood, were mated to different males, wild type for the considered trait and their progeny have been screened for the position of the parapineal organ, in order to identify 1/4 females recessive homozygous for the putative mutation (-/-).

A total of 102 females have been raised to adulthood but only 83 have been included in the analysis as we considered data derived from females that underwent at least two distinct mating events using different males. The remaining 19 females died before all analyses could be performed. We plotted all data collected from the females’ progenies (Fig. 3.4) and we should classify our group of individuals into three classes based on the frequency of embryos with reversed parapineal organ produced by each female. Considering our data, females that produced less that 5% of embryos with right parapineal were called left-parapineal generating females (LGF), as the main part of their progeny have normal brain asymmetries. Conversely, females that produced over 16% of embryos with reversed brain asymmetries were called right-parapineal generating females (RGF). Then remaining females fell into the intermediate group (IGF) as they produced 5 to 16% of embryos with reversed parapineal.

Figure 3.3: Pedigree that shows the inheritance predicted for a maternal effect gene.
Results

**Figure 3.4:** Plots of data collected from 83 females based on the percentage of embryos with reversed brain asymmetries produced. Based on this distribution an inferior (5%) and a superior (16%) limit have been established to divide the females’ population into three groups.

We choose 5% as a minimum value according to literature data (Facchin et al., 2009a; Gamse et al., 2003) and refers to a normal condition in a wt population. The 16% value has been chosen as it is the point at which our distribution changes its growth rate after it seems to reach a “plateau” (see figure 3.4).

All following analyses have been performed according to the subdivision of the population of females into these three groups.

Twenty-four LGF produced a mean of 2.9%±1.3 of reversed parapineal organ over 16226 embryos. Thirty-nine IGF produced a mean of 9.5%±3.3 of reversed brain asymmetries over 27095 embryos. And finally twenty RGF produced a mean of 22.2%±4.8 of reversed parapineal over 12742 embryos.

The three groups of females significantly differed in the percentage of reversed parapineal offspring ($F_{(2,83)}=184.812$, $p<0.001$, see Fig. 3.5).
The main goal of the project is to try to map the mutation, but the positional cloning technique (Talbot and Schier, 1999) requires a large number of samples to be collected and, unfortunately, managing a spontaneous maternal effect allele to perform the mapping is quite a long and complex work. For this reason the present work is aimed at providing a wide phenotypic characterization of the mutated gene.

3.1.2. Putative mutation in a maternal effect gene randomizes the expression of genes of the Nodal pathway during somitogenesis

During the first step in the characterization of the putative mutation isolated after Facchin’s behavioral selection (2009a) it has been decided to investigate if the affected gene influenced the asymmetric expression of left-sided genes of the Nodal pathway responsible for the L-R establishment of epithalamic structures. We decided to perform in situ hybridization protocol using the antisense probe for lefty1 that localizes in the left dorsal diencephalon at the 20-22 somite stage.

Embryos generated by wild type females from our laboratory strains, Giotto Leo and Umbria were used as control. As widely explained in the

![Figure 3.5: Percentage of reversed parapineal position for the three groups of females classified after the genetic analyses. Mean±SE are expressed.](image-url)
Results

Introduction, in normal developmental conditions, genes of the Nodal pathway are expressed in the left side of the embryo. As resumed in figure 3.6, 93.2% of embryos (n=41/44) derived from wild type control females showed normal left-sided expression of lefty1, while 6.8% of embryos (n=3/44) showed bilateral expression.

![Figure 3.6: Percentage of lefty1 expression in wt control embryos and in embryos derived from females of the three analyzed groups.](image)

Then we analyzed the expression of lefty1 in the offspring from females of the three considered groups. As a result, 91% of embryos (n=30/33) derived from LGF presented left-sided expression, the remaining 9% (n=3/30) presented randomized expression of lefty1 and more precisely, 6% had bilateral expression and 3% right-sided expression (Fig. 3.7).

Analysis of IGF revealed that 83% of their progeny (n=361/435 embryos) had normal left-sided expression of lefty1, while 12.9% (n=56/435 embryos) showed bilateral expression and 4.1% (n=18/435 embryos) evidenced lefty1 expression on the right-side of the dorsal diencephalon. Finally, a strikingly lower percentage of embryos derived from RGF (60.5%, n=244/403) had normal expression of lefty1 in the left dorsal diencephalon while we could observe an increased in the number of embryos with of altered expression (39.5%), compared to progenies from IGF, LGF and to wild type-derived
embryos. In particular, 28.5% of embryos from RGF (n=115/403) showed bilateral *lefty1* expression and 11% right-sided expression (n=44/403).

Moreover, in embryos from RGF females we also checked for the expression of *cyclops* (*nodal-related2*) that overlaps with the expression of *lefty1*. The expression of *cyclops* in the left dorsal diencephalon is important as it provides cues for the subsequent development of L-R asymmetries in the zebrafish diencephalon (Bisgrove et al., 2000; Concha et al., 2000; Essner et al., 2000; Roussigné et al., 2009). The results reflect those already observed for the expression of *lefty1* with a strong reduction in RGF of the percentage of embryos from RGF displaying normal *cyclops* expression (55%, n=311/566), while 30.9% (n=175/566) and 14.1% (n=80/566) of embryos had respectively bilateral and right-sided *cyc* expression (Fig. 3.7).

As previously described, it has been reported that the left sided expression of *cyclops* and *lefty1* in the dorsal diencephalon at the 20-22 somite stage is
dependent upon the upstream expression of the zebrafish \textit{nodal-related3} gene \textit{southpaw} in the left lateral plate mesoderm (Long et al., 2003). According to this evidences we decided to analyze also \textit{spaw} expression in the LPM at the 15-18 somite stage. Embryos generated by wild type females from our laboratory strains, Giotto Leo and Umbria were used as control. Results are presented in figure 3.8, where it can be observed that 98.2\% of embryos (n=166/169) derived from wild type control females showed normal left-sided expression of \textit{southpaw}, while 1.8\% of embryos (n=3/169) showed bilateral expression.

As for \textit{lefty1} we analyzed the expression of \textit{southpaw} in 18-somites embryos generated from females classified in the three groups. LGF produced 95.2\% of embryos (n=120/126) displaying left-sided \textit{spaw} expression, while the remaining 4.8\% (n=6/126) of embryos presented bilateral expression. Analysis of progeny derived from IGF revealed that 82.3\% of embryos (n=167/203) had normal left-sided expression of \textit{spaw}, while 13.8\% (n=28/203 embryos) showed bilateral expression and 3.9\% (n=8/203 embryos) evidenced \textit{spaw} expression in the right lateral plate mesoderm.
Finally, only 48.6% of embryos derived from RGF (n=366/753) had normal expression of \textit{spaw} in the left LPM while an increase in the percentage of embryos with bilateral expression (42.5%, n=320/753) has been observed, compared to progenies from females of the other two groups and to wild type-derived embryos. Moreover, 8.9% of embryos from RGF (n=67/753) showed right-sided \textit{spaw} expression (Fig.3.9).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3_9.png}
\caption{Dorsal view of \textit{southpaw} (\textit{spaw}) expression in the left dorsal diencephalon (a), bilateral expression (b) and right-sided expression detected in 15-18 somite-stage embryos (c).}
\end{figure}

Results found for the expression of \textit{southpaw} in the LPM in 15-18-somite stage embryos are similar to those of the expression of \textit{lefty1} in the left dorsal diencephalon at later somitogenesis stages.

As discussed in the introduction, the \textit{southpaw (ndr3)} gene has been shown to be the earlier marker of L-R asymmetries expressed in the left LPM of the zebrafish embryo starting from 10-12 somite stage (Long et al., 2003). The activation of \textit{spaw} expression is supposed to occur after the onset of the leftward fluid flow generated by the Kupffer’s vesicle (Essner et al., 2005). Evidences provided by above described results on \textit{spaw} expression in embryos generated by RGF suggested that the characterization of our putative mutation may extend to earlier developmental stages.

3.1.3. The mutated gene does not affect the generation of the leftward fluid flow in the Kupffer’s vesicle

Cilia-driver flow of extracellular fluid generated by the zebrafish Kupffer’s vesicle is one of the main event upstream the activation of the Nodal-
dependent asymmetrical expression cascade (Essner et al., 2005). According to evidences that demonstrated how disrupted leftward flow affect the normal left sided expression of *Nodal* in the left LPM (Essner et al., 2005; Kramer-Zucker et al., 2005; Shu et al., 2007), we decided to analyze this event in embryos derived from RGF. The hypothesis was thus that this spontaneous mutation isolated in our laboratory should affect (blocking or at least reducing) the directional flow in the KV, leading to a randomization in the expression of *nodal* and nodal-dependent downstream genes during somitogenesis.

Beads microinjection procedure (Essner et al., 2005; Neugebauer et al., 2009) was performed on embryos derived from wild type crosses for control, and on embryos derived from RGF. The analysis were restricted only to these two groups because of technical constraints due to difficulty in keeping the embryos still and correctly orientated during the injection phase of the procedure. Furthermore, due to the small size of zebrafish embryos and the need for high-precision tools, it was difficult to strictly inject in to the vesicle. However our trials reflect those already documented in previous works (Essner et al., 2005).

Twenty-five wild type control embryos have been successfully injected (over a total of 50 selected) and all of them showed a counterclockwise movement of fluorescent beads inside the lumen of KV. When considering embryos derived from RGF, only 15/50 embryos have been successfully injected, but again all positive trials presented a counterclockwise beads movement the lumen of KV, similar to wild type embryos.

### 3.1.4. The size of Kupffer’s vesicle is reduced in embryos derived from putative mutant females

When performing beads microinjection procedure the main constraint was that in most embryos derived from RGF, KVs were difficult to detect under a standard stereomicroscope. For this reason a reduced number of embryos have been successfully injected compared to control.

Starting from this observation, we decided to *in vivo* analyze the morphology of Kupffer’s vesicles in embryos at the 10 somite stage.
Morphological analysis has been carried out on embryos derived from females grouped within the three groups. Embryos generated by wild type females from our laboratory strains, Giotto Leo and Umbria were used as control.

According to a previous study on the morphological characterization of the zebrafish Kupffer’s vesicle (Kreiling et al., 2007). We focused on the major cross-section to measure the antero-posterior (AP) and left-right (LR) diameters and the total area of Kupffer’s vesicle.

Results are showed in figures 3.10 and 3.11. Considering the AP diameter, 110 embryos in wt control showed a mean 68.3 μm and 25 embryos from LGF showed a mean of 65.5 μm. Smaller AP diameters were found for 206 embryos from IGF (mean 57 μm) and for 39 embryos from RGF (mean 48.6 μm). The difference between the four groups was statistically significant (F(3,376)=34.08, p<0.001). Post hoc analyses (LSD method) revealed no significant difference between control and embryos from LGF (p=0.291).

Considering the LR diameter, 104 embryos in wt control showed a mean 69.4 μm and 25 embryos from LGF showed a mean of 66.7 μm. Again smaller LR diameters were found for embryos from IGF (mean 58.5 μm; n=206) and for 39 embryos from RGF (mean 50.3 μm). The difference between the four groups was statistically significant (F(3,369)=34.65, p<0.001). Post hoc analyses (LSD method) revealed no significant difference between control and embryos from LGF (p=0.287).

And finally, when measuring the area of Kupffer’s vesicle, 104 embryos in wt control showed a mean 3544.6 μm² and 25 embryos from LGF showed a mean of 3156.7 μm² (Fig. 3.12a). As a consequence of previous results on diameters measures, smaller KVs (Fig. 3.12b) have been observed for 205 embryos from IGF (mean area of 2565.3 μm²) and for 38 embryos from RGF (mean area of 1903.3 μm²). The difference between the four groups was statistically significant (F(3,368)=36.10, p<0.001, see Figure 3.13 for a frequency distribution). Again post hoc analyses (LSD method) revealed no significant difference between control and embryos from LGF (p=0.074).
In addition, a percentage of 12.5% of embryos from RGF completely lacked Kupffer’s vesicle (Fig. 3.12c), while no embryos lacking KV have been reported for wt control and for females classified in the other two groups (IGF and LGF).
A correlation analysis has also been performed to verify if there was a correspondence between the percentage of embryos with reversed parapineal produced by females of the three classified genotypic classes and the size of KV of embryos. As reported in the graph (Fig. 3.14) a significant
negative correlation has been found, as the area of the Kupffer’s vesicle increases the percentage of embryos with reversed parapineal decrease (Pearson correlation r=0.565, p<0.001, n=83, Fig. 3.14).

Figure 3.14: The relationship between KV area and percentage of right parapineal in the three groups of fish. Fit line for total is reported.

3.2. PART II behavioral analysis

Here we tested the hypotheses that parapineal positioning influences behavioral lateralization and personality of adult Danio rerio. RGF were crossed to foxD3::GFP transgenic males larvae were examined at 3days post fertilization and sorted according to the left or right parapineal position; once raised to adulthood, they were compared in four standard tests of behavioral lateralization and in three measures of personality traits.
3.3. Laterality tests

3.3.1. Mirror test

To compare fish with left (L-PPO) and with right parapineal (R-PPO) we performed a repeated measure analysis of variance considering the parapineal position as between-subjects factor and the 8 minutes of the test as within-subjects factor (ANOVA): $F(1,58) = 5.308, p = 0.025$; Fig. 3.15). This means that the Mirror test revealed a significant difference in eye preference between subjects with L-PPO and with R-PPO. Analyses revealed no effect of sex ($F(1,58) = 0.798, p = 0.375$) or minute of test ($F(7,406) = 1.170, p = 0.319$) and no interaction was significant (all, $p > 0.05$). This means that the eye preference is not significantly influenced by the sex nor by the time spent in the apparatus. Moreover the interaction of these two factors do not direct eye preference.

When considering the whole test statistical analysis that compared the means of the two groups revealed that L-PPO subjects showed a significant right eye preference (one-sample t-test, $t(28) = 2.648, p = 0.013$) whereas R-PPO did not show eye biases in mirror viewing ($t(32) = 0.543, p = 0.591$).

We also measured the latency to reach the mirror image after the emergence of the experimental fish from the corridor of the starting box. The latency did not differ between L-PPO and R-PPO ($F(1,58) = 0.025, p = 0.876$) and was shorter for males than for females (sex: $F(1,58) = 4.264, p = 0.043$). Latency to reach mirror was not correlated with the laterality index (Pearson correlation $r = 0.164, n = 62, p = 0.203$).

In general, L-PPO and R-PPO did not differ in the average distance kept from the mirror during the test (ANOVA: parapineal $F(1,58) = 0.0915, p = 0.904$); this distance significantly decreased during the test (minutes: $F(7,406) = 36.520, p < 0.001$) and was shorter for males than for females (sex: $F(1,58) = 5.719, p = 0.020$ and minutes by sex: $F(7,406) = 4.296, p < 0.001$). No other interaction was significant.

There was no correlation between distance from the mirror and laterality index ($r = -0.198, n = 62, p = 0.124$).
Results

3.3.2. Eye use in predator inspection

To analyze zebrafish lateralized responses when facing a real predator, we performed a repeated measure ANOVA (between-subjects factor is the PPO position and within-subjects factor is represented by the minutes of the test). As a result of this L-PPO and R-PPO zebrafish significantly differed in eye preference to inspect a live predator (ANOVA: $F_{(1,45)} = 6.916, p = 0.012$; Fig. 3.16A). There was no significant effect due to sex ($F_{(1,45)} = 1.170, p = 0.285$) nor to the interaction between sex and parapineal position ($F_{(1,45)} = 0.646, p = 0.426$).

Comparing the means of the whole performance we found that there was a statistically significant preference for right-eye use in R-PPO zebrafish (one-sample t-test, $t_{(24)} = 2.260, p = 0.033$) while L-PPO failed to reveal any significant preference ($t_{(23)} = 1.256, p = 0.222$).
3.3.3. Rotational preference

In this test fish became acclimatized to the apparatus environment and then we measured their direction of swimming in familiar conditions. We found that L-PPO and R-PPO fish significantly differed in the direction of rotational preference (ANOVA: $F_{(1,53)} = 6.735$, $p = 0.012$; Fig. 3.16B) and there was no significant effect of sex ($F_{(1,53)} = 0.001$, $p = 0.974$) nor interaction between sex and position of parapineal organ ($F_{(1,53)} = 0.175$, $p = 0.678$). Comparing the means of the performances of the two groups we revealed that L-PPO subjects swam preferentially in a clockwise direction (one-sample t-test, $t_{(25)} = 5.143$, $p < 0.001$) whereas R-PPO revealed no significant preference ($t_{(30)} = 0.762$, $p = 0.452$).

3.3.4. Turning direction in the dark

In this test we observed zebrafish swimming behavior in the absence of visible light and our analysis revealed that L-PPO and R-PPO subject showed a significant difference in turning direction (ANOVA: $F_{(1,58)} = 7.944$, $p = 0.007$; Fig. 3.16C). This difference was not influenced by sex, in fact the effect of sex and also the interaction between sex and parapineal position were not
Results

significant \((F_{(1,58)} = 0.466, \ p = 0.497\) and \(F_{(1,58)} = 0.056, \ p = 0.814,\) respectively). The difference between the two groups is due to the fact that L-PPO fish showed a significant bias to turn to the right (one-sample t-test, \(t_{(32)} = 2.227, \ p = 0.033\)) while R-PPO showed a marginally non-significant tendency to turn to the left \((t_{(28)} = 1.740, \ p = 0.093\). L-PPO and R-PPO did not differ in the total number of turns performed during the whole test (two-sample t-test, \(t_{(58)} = 0.987, \ p = 0.328\).

In this test we also measured the total distance covered by fish in the first minute after being released and we observed that L-PPO subjects covered a greater distance than R-PPO (ANOVA: parapineal \(F_{(1,58)} = 5.426, \ p = 0.023\). Our analysis revealed that this difference is mainly due to the higher activity of L-PPO males compared with R-PPO males. This means that the effect of sex and the interaction between sex and parapineal position were statistically significant \((F_{(1,58)} = 6.724, \ p = 0.012,\) and \(F_{(1,58)} = 6.996, \ p = 0.011\) respectively). However there is no correlation between laterality index and distance covered \((r = -0.138, n = 60, \ p = 0.295\).

3.4. Personality tests

3.4.1. Social distance

This test allowed us to measure zebrafish social behavior. We observed that fish significantly tend to decrease the distance from conspecifics along with the 5 min of the test (repeated measure ANOVA: \(F_{(4,244)} = 12.387, \ p < 0.001;\) Fig. 3.17). Social tendency was not affected by the position of the parapineal \((F_{(1,61)} = 0.103, \ p = 0.749),\) by sex \((F_{(1,61)} = 0.039, \ p = 0.844\) nor by the interaction between these two factors \((F_{(1,61)} = 2.399, \ p = 0.127\). The interaction between minutes of the test and position of the parapineal was not significant \((F_{(4,244)} = 1.007, \ p = 0.404),\) as for the remaining interactions (all, \(p > 0.05\)). Further analyses revealed that social tendency was not significantly correlated with the laterality of eye use measured during predator inspection \((r = 0.044, n = 49, \ p = 0.770).\)
3.4.2. Activity in open field under social isolation

The total activity in an open environment, as inferred by the number of squares crossed, did not differ significantly between L-PPO and R-PPO fish (251.6±72.7 and 238.2±79.1, respectively; ANOVA: F(1,60) = 0.084, p = 0.773; Fig. 3.18A). Fish significantly tend to decrease their activity through the 5 min of the test as they acclimate to the environment (F(4,240) = 12.571, p < 0.001) and males were more active compared with females (290.8±74.9 and 211.4±57.1 crosses, respectively, F(1,60) = 30.793, p < 0.001); Fig. 7A suggests a different pattern of activity of L-PPO and R-PPO fish, but the interaction between parapineal and minutes fell just short of significance (F(4,240) = 2.101, p = 0.081). No other interaction was statistically significant.

We also observed that R-PPO subjects showed a greater tendency to stay in the inner portion of the open field compared with L-PPO (Fig. 3.18B). The difference in the proportion of time spent in the inner portion is significant in the first and in the second minutes (two-sample t-test, t(62) = 2.053, p = 0.044 and t(62) = 2.241, p = 0.029, respectively) but not in the remaining 3 min or in the whole period (all, p > 0.05). Laterality of predator inspection did not correlate with time spent in the inner portion (r = 0.114, n = 49, p = 0.43)
while it correlated negatively with activity \( r = -0.32, n = 49, p = 0.027 \), meaning that fish using predominantly the right eye tend to move less after being separated from social companions.

![Figure 3.18: Activity of L-PPO and R-PPO zebrafish after separation from companions: (A) total number of square crossings, (B) proportion of time spent in the inner portion of the open field.](image)

### 3.4.3. Distance from the predator

While observing zebrafish behavior when facing a real predator we reported that L-PPO and R-PPO subjects did not differ in their tendency to move close to a predator (repeated measure ANOVA: \( F_{(1,54)} = 3.321, p = 0.074 \); Fig. 3.19). As the time increases, the subjects tend to move away from the predator \( (F_{(9,486)} = 2.620, p = 0.006) \). There was no significant effect of sex \( (F_{(1,54)} = 1.053, p = 0.309) \) nor interaction between sex and the position of parapineal organ \( (F_{(1,54)} = 0.720, p = 0.400) \). As shown by Fig. 3.19, the behavior of L-PPO and R-PPO fish tends to diverge with time and R-PPO fish tend to maintain a shorter distance from the live predator in the second half of the test. If we analyze separately the first and second parts of the test, we find a significant difference between L-PPO and R-PPO fish in the last but not in the first 5 min of the test (two-sample t-test, \( t_{(56)} = 0.918, p = 0.363 \) and \( t_{(56)} = 2.120, p = \))
0.038, respectively). Laterality index (eye preference for inspecting the predator) was correlated with the distance in the second 5 min but not in the first 5 min ($r = 0.321$, $n = 49$, $p = 0.024$ and $r = 0.024$, $n = 49$, $p = 0.872$, respectively), meaning that fish using predominantly the right eye tend to swim closer to the predator.

Figure 3.19: Mean distance (±S.E.M.) from a live predator over the 10 min of observation.
4. DISCUSSION

4.1. Genetic and molecular analysis

Axes determination, in all organisms, is a complex process whose foundations are laid in many cases even before fertilization of the egg cells. The vertebrate body plan begins to take shape at the earliest stages of embryonic development with the formation of the dorso-ventral and anterior-posterior axes. Subsequently the left-right axis forms, correctly orientating itself in relation to the previous axes. The vertebrate embryo develops left and right halves that are mostly externally symmetric but internally asymmetric. The heart and spleen lie on the left hand side and the liver on the right with the large intestine coiling from right to left (Bisgrove et al., 2000).

In the vertebrate embryo the event responsible for breaking of the initial body symmetry occurs during late gastrulation at the posterior end of the notochord (the node of mammalian embryos) where a transient ciliated structure becomes evident. In zebrafish, this structure is identified as the Kupffer's vesicle (Essner et al., 2005; Essner et al., 2002; Okada et al., 2005). Cilia in this structure posses a rotating beating movement that generates a leftward flow of extracellular fluid that triggers the asymmetrical transcription of Nodal and Lefty genes in the left lateral plate mesoderm, and the transcription factor Pitx2 during early somitogenesis (Blum et al., 2009). Disturbance or absence of nodal leftward flow results in laterality defects and randomization of left-right asymmetries (Essner et al., 2005). Recently it has been reported first evidence of Nodal and Pitx orthologues isolated in two species of snails with opposite body handedness and direction of shell coiling (Grande and Patel, 2009). Authors found that nodal and Pitx are both expressed in the right side of the embryo in the dextral species and in the left side in the sinistral species. These results suggest that the asymmetrical expression of nodal and Pitx may represent an ancestral conserved feature in the evolution of Bilateria.

Together with visceral asymmetries, the comprehension of neuroanatomical asymmetries is notably important. The brain is a complex, asymmetric structure, in lower vertebrates the epithalamus is the major subdivision of
the diencephalon, which consists of the habenular nuclei and the pineal complex and is located in the vertebrate forebrain. Structural asymmetries in this region are common and highly conserved amongst lower vertebrates. There are however differences in size, neuronal organization, connectivity and neurochemistry between species (Concha and Wilson, 2001). In most extant vertebrate species epithalamic asymmetries are established early on in development, which suggests a genetic regulation of brain laterality during development. As mentioned before the laterality of asymmetries varies between vertebrate species and the evolutionary framework is not clear, however it has been proposed that the consistency of lateralization within a particular species and especially within a population may play a role in the variability of social behavior between individuals of the species (Concha and Wilson, 2001).

In this dissertation we report the characterization of a spontaneous mutation in a maternal effect allele involved in the development of left-right asymmetries in the zebrafish. This mutation has been isolated, in a previous study, after five generations of selection based on behavioral lateralization. In fact, starting from a wild type base stock, the TL strain, that initially showed a slight preference for right-eye use (RE) for inspecting their own mirror image, the selection significantly increased the number of individuals lateralized for right-eye use. Conversely the GT strain has been selected for the opposite preference, as behavioral selection significantly increased the frequency of individuals with a preference for left-eye use (LE). Furthermore it has been reported that five generations selection for a preference in right-eye use in monocular inspection of a social stimulus also increased in the TLRE line the frequency of individual with reversed epithalamic asymmetries, suggesting a strong genetic basis for behavioral laterality in zebrafish and thus a possible correlation between neuroanatomical and functional asymmetries (Facchin et al., 2009a).

The study described in the present work argues that the increased frequency of reversed brain asymmetries in the TLRE selected line is due to alterations in a maternal effect allele involved in early stages of left-right development in zebrafish. Based on a preliminary mendelian analysis of this trait, we
performed selective crosses to show that the transmission of the allele reflected those of a maternal effect inheritance, and affected females could be identified with the screening of their progeny for the position of the parapineal organ at the 3 dpf stage. Analyses revealed that analyzed females could be classified into three groups to the percentage of reversed brain asymmetries in their offspring: females generating a frequency of 0-5% (LGF), between 5 and 16% (IGF) and females generating more than 16% (RGF) of embryos with reversed asymmetries. We grouped 24 females in the (LGF) group, 39 females were grouped as IGF and 20 females fit into the RGF group.

According to this subdivision of the females’ population I decided to investigate early steps in the development of zebrafish L-R asymmetries.

With respect to the expression of genes of the asymmetrical Nodal signaling cascade, \textit{lefty1} and \textit{southpaw} genes were considered. As reported in literature (Bisgrove et al., 2000; Concha et al., 2000; Essner et al., 2000) \textit{lefty1} is expressed on the left dorsal diencephalon, where precursors of the future pineal organ differentiate during late somitogenesis. We therefore decided to verify if the isolated mutation influenced the expression of \textit{lefty1} in the dorsal diencephalon at the 22-25 somite stage. Results demonstrated that a reduced percentage of embryos derived from RGF showed normal left-sided \textit{lefty1} expression (60.5%) compared to females of the other two groups. LGF females do not significantly differed from wild type females (Chi-square=0.018; p= 0.894) while class IGF females showed an intermediate value in the percentage of embryos generated with normal left-sided \textit{lefty1} expression (83%).

These data were consistent with the hypothesis of a maternal effect gene controlling the generation of L-R asymmetries and thus it has been supposed that this gene should influence early developmental stages. According to this we decided to perform a backward stepwise analysis of key stages of L-R development. First, considering the expression of \textit{lefty1} and the corresponding expression of \textit{cyclops} (\textit{ndr2}) in embryos from class RGF, we decided to analyze the step upstream \textit{lefty1} and \textit{cyclops} activation. It has been widely demonstrated that during L-R development \textit{nodal} activate its own
expression and also the expression of its inhibitors of the Lefty/Antivin family (Bisgrove et al., 2000; Hamada et al., 2002; Meno et al., 1997; 1998). Three nodal-related genes have been isolated in the zebrafish genome: *squint* (*ndr1*), *cyclops* (*ndr2*) (Rebagliati et al., 1998a) and *southpaw* (*ndr3*) (Long et al., 2003). The *nodal-related3* gene is the earlier gene with asymmetrical expression along the left lateral plate mesoderm described in the zebrafish and has been predicted to activate the expression of *cyclops* (*cyc/ndr2*) and of *lefty1* in the left dorsal diencephalon in later somitogenesis stages (Long et al., 2003). We therefore decided to compare the expression of this upstream signal in embryos generated by previously analyzed females. Results clearly reflect those found for the *lefty1* gene with LGF that do not significantly differ from control wt females (Chi-square=2.177; p= 0.140). Conversely, RGF females generated a significantly lower percentage of embryos with normal left-sided expression of *southpaw*, and IGF are again characterized by intermediate frequency values.

These observations revealed that our mutated allele will probably act upstream the activation of *southpaw* and the subsequent asymmetrical Nodal signaling cascade. Focusing on the "nodal flow" generated by the ciliated cells inside the lumen of the Kupffer’s vesicle, as reported by recent investigations, disruption on the leftward counterclockwise flow leads to the randomization of *nodal* expression and of organs positioning (Bisgrove et al., 2005; Bisgrove and Yost, 2006; Essner et al., 2005; Kramer-Zucker et al., 2005). We hypothesized that the randomization of *spaw*, *cyclops* and *lefty1* expression in embryos from RGF should arise as a consequence of a disrupted nodal flow. Microinjection of fluorescent beads into the KV lumen did not support this hypothesis as both in embryos from wt control females and from RGF the flow was normal. Despite this results, some experimental constraints have been encountered due to the difficulty in visualizing KV of embryos from RGF under a dissection microscope. In those embryos KV appeared to be smaller. Results strongly support the hypothesis that KV size was significantly reduced in embryos generated by RGF, while KV size in embryos from LGF did not significantly differ from wild type control and again embryos from IGF showed an intermediate phenotype.
Considering the observed distribution based on percentage of progeny with reversed brain asymmetries generated by each females, the minimum (5%) and the maximum (16%) values that have been fixed divide our population into a Mendelian distribution. Thus we could hypothesize that LGF are homozygous wt (-/-) while RGF females are recessive homozygous (-/-) for the putative mutant allele. Females grouped as IGF can therefore be considered as heterozygous and thus the genetic of our allele can be described by an incomplete dominance inheritance where the heterozygous group display an intermediate phenotype. However, the development of asymmetries is a complex multiple-step process, and further genetic analyses are necessary to establish if our trait is controlled by more that one locus.

Most important in the present work we report the first evidence for a maternal effect gene involved in L-R development in a vertebrate species. In fact maternal effect inheritance in a gene controlling L-R development has only been reported for the direction (also called handedness or body chirality) of shell coiling in snails (Limnaea sp.) (Boycott and Diver, 1923; Sturtevant, 1923). In this species, in fact body chirality (dextral or sinistral) is hereditary determined by a single locus that act maternally (Freeman and Lundelius, 1982; Hosoiri et al., 2003) but the handedness-controlling gene(s) and the developmental mechanisms in the determination of body chirality are not determined yet. A recent work shows that nodal and Pitx orthologues have been isolated in two species of snails with opposite direction in shell coiling (Grande and Patel, 2009). Authors found that the side of the embryo that expresses nodal and Pitx is related to body chirality: both genes are expressed on the right side of the embryo species with dextral shell coiling (Lottia gigantea) and on the left side in the species with sinistral coiling (Biomphalaria glabrata). It is also known that the direction of shell coiling in dextral and sinistral snails correlates with the spiral blastomere arrangement (Crampton, 1894). Further experiments in two gastropods species of the Limnaea species that demonstrated the clockwise or counterclockwise chiral blastomere arrangement at the third cleavage (eight-cell stage) specifically direct the activation of the Nodal pathway in the right or left side of the embryo at the late trophophore stage. Moreover, the cytoskeletal dynamics of
the chiral blastomere arrangement at the eight cell stage is directly controlled by maternal handedness-controlling gene(s) (Kuroda et al., 2009). Our results are further supported by previous findings demonstrating that a maternal effect gene called hecate controls the establishment of the dorso-ventral axis in the zebrafish. Females homozygous for the hecate mutation produce a high proportion of embryos displaying defects in dorsal–anterior structures, and similarly to what we observed in our experiments those females display a variable expressivity of the phenotype (Lyman Gingerich et al., 2005). This mutation influences intracellular Ca\(^{2+}\) release affecting Wnt/β-catenin signaling pathway during D-V axis development: embryos from mutant females display an increase in Ca\(^{2+}\) levels at blastula stages that subsequently affects the expression of dorsal genes and inhibits the Wnt/β-catenin pathway (Lyman Gingerich et al., 2005).

Other than the hecate gene, three more maternal effect genes have been reported to control the induction of the dorsal axis, ichabod (Kelly et al., 2000), tokkaebi (Nojima et al., 2004), and brombones (Wagner et al., 2004), and thus it is also reasonable that maternal effect genes are involved in the development of the two other body axes, as the L-R axis and acting with similar mechanisms. To further support this hypothesis, a recent work demonstrated that Ca\(^{2+}\) and Wnt/β-catenin signaling is also involved in early stages of L-R development. In fact artificial inhibition of endogenous Ca\(^{2+}\) release after the onset of gastrulation causes an increase in Wnt/β-catenin signals resulting in altered coalescence/cohesion of dorsal forerunner cells (DFC). As DFCs migrate to form the Kupffer's vesicle, this inhibition of endogenous Ca\(^{2+}\) also results in defective KV formation, and randomized expression of asymmetrical L-R genes of the nodal pathway as well as subsequent alteration of organ laterality (Schneider et al., 2008).

In this context the present study aims at providing new insights on the genetic regulation of laterality in a vertebrate species. Our results indicate that the mutated allele alters Kupffer's vesicle morphogenesis, without disrupting the generation of counterclockwise leftward flow inside KV. Thus this allele appears not to influence ciliogenesis as the flow, visualized with fluorescent beads injected into KV, is normal in embryos from RGF, compared
to control. Despite these observations, RGF generate embryos with striking smaller KV and our hypothesis is that smaller KV still work properly generating the directional fluid flow but the amount of morphogens accumulated on the left side of the posterior notochord is probably not sufficient to direct a L-R information and thus to activate unambiguously the left-sided Nodal expression cascade, and consequent asymmetrical organ positioning. It is predicted that left-sided expression of genes of the Nodal pathway will result in normal organ positioning with parapineal on the left of the diencephalon; conversely right-sided activation of the Nodal pathway will result in reversed parapineal positioning. Finally embryos with bilateral expression of Nodal-related genes are predicted to have parapineal organs on the left or right side of the diencephalon at random and with equal frequency.

Our hypothesis is that smaller KV depends upon a smaller group of dorsal forerunner cells (DFCs) that differentiate and migrate to form the ciliated structure at the zebrafish tail bud. This hypothesis is supported by preliminary results showing that at the 50% epiboly-shield stage (Kimmel et al., 1995; Oteíza et al., 2008) a smaller number of DFCs are visible in embryos from RGF putative mutant females compared to wild type embryos, as evidenced by in situ hybridization with sox17 probe (Fig. 4.1).

**Figure 4.1:** *In situ* hybridization showing sox17 expression in DFC in the zebrafish embryo at the 50% epiboly stage. In embryos from wt control (a) the mass cell is notably larger (n=15) than in embryos (n=25) from putative mutant females (b).

It has been show that in zebrafish, the *no tail* gene (*ntl*, homologous to mouse brachyury) encoding for Ntl transcription factor functions cell autonomously in DFCs to regulate KV morphogenesis and LR determination (Amack and
Yost, 2004). Authors selectively knocked down ntl only in DFCs at midblastula stages thus not affecting the expression of this transcription factor in other cells. Blocking the expression of Ntl in DFCs led to failure in the KV morphogenesis. According to these observations our next efforts might be directed at analyzing molecular pathways acting early in the differentiation and specification of DFCs, between midblastula transition stages and the beginning of epiboly movement when DFCs start to migrate.

4.2. Behavioral analysis

In close association with genetic and molecular analyses, we also investigated for a possible correlation between brain asymmetries and behavioral lateralization. The use of GFP reporter under the control of foxD3 promoter as an in vivo marker of parapineal position in zebrafish larvae allowed us to investigate the influence of early neuroanatomical asymmetries on behavior of adult fish. Fish with typical left parapineal position and fish with reversed asymmetry differed in all measures of behavioral lateralization. Moreover, different reactivity to the same environmental stimulation evidenced that opposed epithalamic asymmetries are associated with variation of some temperamental dimensions. In fact, as humans show differences in personality, a wide range of other animal species exhibit stable individual differences in behavior that are correlated across different contexts and have been also termed as animal temperaments, coping styles or behavioral syndromes (Dall et al., 2004; Sih et al., 2004). Some authors (Andrew, 2005; Andrew, 2006) have recently showed that disruption of the genetic mechanisms leading to reversal of anatomical asymmetries also determines reversal in some but not all lateralized functions. This partial reversal would be evolutionarily important since it could generate new behavioral phenotypes and in particular it could enable the production of individuals with new combinations of personality traits.

The results of our study provide strong support for the hypothesis that establishment of early diencephalic asymmetries, as the left–right positioning of the parapineal driven by the asymmetrical Nodal signaling in the first
hours post-fertilization, influences development of functional brain lateralization. Fish with left and right parapineal were found to differ significantly in all four laterality tests in which they were compared. Since we measured different lateralized functions, social recognition, anti-predator response and motor asymmetries, there appear to be a broad effect of parapineal position on the organization of lateralization in the central nervous system. In a previous work with the zebrafish frequent situs-inversus (fsi) mutant line, Barth et al. (2005), reported that adult zebrafish with normal heart position preferred to bite targets on their right, while fish with reversed heart position did the reverse. Also, at 8 dpf, fry with right and left heart were found to have an opposite pattern of eye use in the mirror test, although they did not differ in two other lateralized measures (in the emergence into a novel environment and in the startle response). As the mutant fsi line shows a 90% concordance between heart and parapineal positioning, it is very likely that Barth and colleagues studied the same phenomenon we are evidencing in the present study.

In contrast, in a recent study on the possible function of epithalamic asymmetries, Facchin et al. (2009b) found no correlation between parapineal position and several measures of behavioral lateralization including the mirror test. However it must be considered that the main difference of Facchin and colleagues’ work is that they obtained a large frequency of reversed-parapineal fish by disrupting the asymmetrical Nodal signaling pathway through the injection of an antisense morpholino against the southpaw gene. Conversely, we compared fish with normal and reversed parapineal position originated from a spontaneous mutation in a maternal effect gene influencing early stages in zebrafish L-R development. To date we have little information about other possible effects that morpholino knock down of Nodal-related signals can have on the development of the central nervous system in zebrafish, but it is possible that both the uncommon absence of laterality bias in all tests and the lack of an association between asymmetry of the habenulae and behavioral lateralization were determined by disruption of signals necessary for the normal development of cerebral lateralization. Another difference is that this dissertation presents results
from testing adult fish while both Barth et al. (2005) and Facchin et al. (2009b) observed 6-8 dpf larvae.

Although in our experiments parapineal position influenced all laterality tests, the relationship between anatomical and behavioral asymmetries is complex to elucidate. In three tests (mirror test, rotational preference and turning direction in the dark), fish with normal left-sided parapineal organ (L-PPO fish) showed a significant population bias, while fish with reversed parapineal position, R-PPO, did not. Conversely, in another test, eye preference for predator inspection, we found a significant bias among R-PPO fish but no bias in fish with normal parapineal position. In general laterality biases were not particularly strong and a large overlap in laterality scores was observed between L- and R-PPO fish.

Interestingly while L-PPO fish showed a preference for left eye use in the mirror test (and no preference in predator inspection), R-PPO used preferentially the right eye during predator inspection but did not revealed any significant preference in the mirror test. This is consistent with findings from previous research where one eye was used preferentially to monitor dangerous stimuli while the other eye was preferred in viewing neutral or socially relevant stimuli (Bisazza et al., 2005; Facchin et al., 1999; Rogers et al., 2004). However the fact that in each case the other parapineal group did not show any significant bias could be strictly related to our data set.

Therefore the picture emerging from this study is that the parapineal position is not an all-or-nothing determinant of cerebral lateralization in zebrafish. Clearly factors other than the parapineal position must influence the development of functional asymmetries in zebrafish and determine inter-individual differences in laterality scores.

Recently Andrew and co-workers (Andrew et al., 2009; Budaev and Andrew, 2009) found that exposure to the light during embryonic stages greatly influences the development of lateralized eye use in zebrafish. This phenomenon is well studied in birds where differential exposure to light during the incubation period influences development of lateralized neural circuits and has long-lasting effects on feeding, social and anti-predator behavior (Rogers and Workman, 1989; Rogers et al., 2004). Neurons in the
parapineal of fish contain retina-specific proteins that make them photosensitive and Andrew (Andrew, 2009) has suggested that light may act by modulating the influence of the parapineal on asymmetric development of the habenula.

Regarding the influence of unilateral parapineal positioning on other asymmetries of the epithalamus until very recently, different lines of evidence concurred on its crucial role in determining habenular L–R differences in gene expression and connectivity (Concha et al., 2000; Gamse et al., 2003; Kuan et al., 2007). A new study by Bianco et al. (Bianco et al., 2008) suggests, however, that the situation is far more complex. These authors analyzed in great detail the consequences of ablation of parapineal precursor cells at 24–28 hpf, prior to their migration towards the left epithalamus, including examination at the level of individual projection neuron morphology. While it was confirmed that asymmetric innervations by the parapineal are essential for the development of both left and right habenular axon terminals with appropriate morphology and connectivity, they found that, even in the absence of signaling from the unilateral parapineal, neurons of left and right habenulae continued to elaborate distinct lateralized axonal arbors while subtle L–R differences remain for leftover gene expression and neuropil organization.

A second issue is whether there are brain asymmetries other than habenular, which affect lateralization. Several studies on mammals – especially primates, revealed associations between neuroanatomical and behavioral asymmetries that involves different areas of the brain (Cantalupo and Hopkins, 2001; Hopkins et al., 2007b; Phillips and Hopkins, 2007) while all the above mentioned studies on zebrafish are related to the epithalamus and leaves open the question whether the habenulae are the sole source of functional asymmetry in this species.

In conclusion, our data indicate that parapineal positioning is not the single initial symmetry-breaking event specifying the unique identity of the left and right side of the brain; rather, it works in concert with other developmental and environmental signals in determining asymmetric circuitry and lateralized behaviors.
The habenulae are part of a highly conserved pathway, the dorsal diencephalic conduction system, that is found in all vertebrates and connects the limbic forebrain areas with midbrain and hindbrain structures (Sutherland, 1982). Evidence has been provided of the implication of this system, and of the habenular complex in particular, in several important cognitive functions and emotional behaviors, including spatial learning, visual attention, response to aversive stimuli and exploratory behavior (reviewed in Klemm, 2004; Sutherland, 1982). In the present study, we found significant differences between L- and R-PPO fish in response to a live predator and in activity in the open field. R-PPO fish maintained a shorter distance from the predator, spent more time in the inner portion of the open field and covered a shorter distance when released in the dark. The behavior of L- and R-PPO did not, however, differ in other measures such as the latency to exit from their compartment and reach their mirror image, the distance kept from social companions or the total activity in an open field test. Two studies have previously attempted to correlate anatomical asymmetries with differences in behavioral response. In one (Facchin et al., 2009b), during the mirror test, zebrafish with reversed parapineal position showed a delay in the onset of swimming and covered a shorter distance. We do not have a comparable measure for the mirror test, but we observed that R-PPO covered a shorter distance compared to L-PPO in the first minute after they were released in the dark. In the second study (Barth et al., 2005), fish with reverse heart position (and probably with reversed parapineal position, too) showed a greater boldness when confronted with a novel object, a result that seems in agreement with our finding that R-PPO fish swim to a closer distance from a predator than L-PPO fish.

Rapidly accumulating evidence indicates that animals show consistent individual differences in behavior that are correlated across different contexts and have been variously termed as animal personalities, coping styles or behavioral syndromes (Brown et al., 2007; Wilson et al., 1994). The shy–bold continuum (i.e. the likelihood that an individual is inclined to risk taking), is probably the best studied of these characteristic (Wilson et al., 1994). Differences along the shy–bold axis are thought to reflect a trade-off
between growth and mortality since bold individuals grow faster but suffer higher mortality rates compared with individuals that take less risk (Biro and Stamps, 2008; Smith and Blumstein, 2008). Since the two hemispheres of the brain play different roles in emotion, attention, perception and control of motor responses (reviewed in Demaree et al., 2005; Rogers and Andrew, 2002) some authors have suggested that differences in hemispheric dominance can lead to individuals that differ in cognitive styles and personality (Andrew, 2006; Barth et al., 2005; Reddon and Hurd, 2009). Andrew (2006; 2009) in particular has suggested that reversal of neuroanatomical asymmetries in the habenulae causes reversal of some, though not all, lateralized functions and that this partial reversal of lateralized abilities is an important adaptive mechanism in vertebrates, since it contributes to the generation of individuals with new behavioral phenotypes. We found some evidence of this effect, as zebrafish that used predominantly the right eye to inspect a predator tended to swim to a shorter distance from it and be less active in the open field test. The influence of lateralization was not, however, evident in other behavioral measures. What emerges from this study is that the relationship among anatomical asymmetries, lateralization of cognitive functions and differences in personality is complex. It appears that in zebrafish the parapineal position largely influences the pattern of cerebral lateralization. It also directly affects some behavioral responses to environmental stimulation, while others are influenced indirectly through the mediation of lateralization.

Interestingly a recent work argue that pond snails of the species Limnaes stagnalis display a lateralized mating behavior set up early in the development as a consequence of the asymmetry (dextral or sinistral) of the entire body. As already mentioned, snails body chirality is controlled by a maternal effect gene and thus authors suggest that also the lateralized mating behavior in snails is maternally inherited (Davison et al., 2009). We can therefore argue that the maternal effect allele studied in the present dissertation is important in setting up early stages of L-R development, influences brain asymmetries and behavioral lateralization. From an ecological point of view this means that mothers may control early
development of their offspring and also direct complex phenotypic traits as behavioral responses to environmental stimuli.
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Alice