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Heterotaxy in *Caenorhabditis elegans*

Defects in embryo gut organogenesis underlie natural variation in hermaphrodite left/right organ arrangement

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Biological Sciences.

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Abstract

The internal organ arrangement of most metazoans is markedly left-right asymmetric, with the predominant handedness arbitrarily termed dextral. Occasionally, some individuals present a complete reversal of all organs or a partial reversal, known as heterotaxy. For years, the nematode *Caenorhabditis elegans* was assumed to have an invariant dextral organ arrangement. However, recently we found that both males and hermaphrodite *C. elegans* show substantial variation in the left/right (L/R) arrangement of the two major organs: gut and gonad, affecting the relative position of the gonad arms within the animal body cavity. Gut/gonad heterotaxy appears to affect the development of hermaphrodites’ anterior and posterior gonad arms independently, resulting in three morphologically distinguishable phenotypes. This thesis focuses on the investigation of gut/gonad heterotaxy in hermaphrodites, describing the phenotype variation among wild isolates and recombinant inbred lines (RILs) generated from two strains at the extreme ends of the distribution of phenotypes. The study of more than 40,000 individual animals revealed phenotype sensitivity to temperature, and heterotaxy rates up to ~20% in some RILs, which showed evidence of transgressive segregation, suggesting a complex and multifaceted underlying cause. Genome-wide analyses studies (GWAS) and quantitative trait loci (QTL) mapping revealed that the phenotype is polygenic. However, these analyses did not detect any loci significantly associated with the phenotype. Evidence suggests that the underlying cause of reversals may in part be traced back to an early embryonic event, during which the developing intestinal primordium undergoes a L/R asymmetric twist in comma-stage embryos. Thus, variations in the anatomical handedness among wild isolates and RILs may reflect varying fidelity in the mechanism controlling the rotation of gut cells during mid-embryogenesis.
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CHAPTER ONE

General Introduction

Developmental biology is the process by which complex organisms (plants and animals) grow, with the outcome of producing a fully-formed adult composed of specialized sets of cells that function together as tissues, organs, and organ systems. The first step in the development of every animal starts with a single cell known as the zygote. This cell contains all the necessary information and “instructions” to progressively specialise into a complex, multi-cellular individual. Developmental processes are widely variable among animal species, but as all animals share a common ancestor in the metazoan kingdom, there are common processes used and mechanisms used by all animals. For example, embryogenesis involves cell division followed by differentiation into a large diversity of cell types by conserved regulatory mechanisms. However, the strategies used by embryos can differ greatly even within a single phylum.

Early development and cell differentiation must occur at the right pace, with precise timing and coordination through a very complex process. Errors or mis-coordination can lead to disease, malformation or death of the embryo. The study of human development is challenging because of its complexity and the ethical considerations of studying human zygotes and embryos. For this reason, animal models are used that can help us to understand the mechanisms of human development.

1.1 C. ELEGANS AS A MODEL ORGANISM FOR DEVELOPMENT STUDIES

Among the many animal models that have been used for developmental studies, the simplicity and ease of studying Caenorhabditis elegans (C. elegans) makes it particularly attractive. This animal has been described more completely than any other
animal (Sulston and Horvitz, 1977). It is a free-living soil nematode whose experimental attributes include its small (~1mm) size, the ease of culturing in laboratory conditions, its transparency (such that the internal organs can be easily observed), and its amenability to genetic manipulation. In addition, the animal is anatomically simple, with a tubular body composed of precisely 959 somatic cells in the hermaphrodite and 1031 somatic cells in the male. The number of germline cells (sperm and eggs) is indeterminate. The rapid life cycle is also an advantage for developmental studies. The N2 laboratory strain can develop from fertilized egg to a fully formed adult in ~three days at 20°C. In addition, the hermaphroditic nature of the worm allows the rapid creation of inbred lines. That is, hundreds or thousands of *C. elegans* individuals can be generated that are genetically identical. In addition, males and hermaphrodites can be crossed, generating new combinations of alleles. Despite its relatively few cells (compared to other animals) around 200 distinct cell types are present in the animal and most major organ functions found in more complex animals are also used in the animal, albeit with far fewer cells. For example, the *C. elegans* equivalent of the kidney is a 3-cell organ. Thus, many *C. elegans* organs have similar functions to those in humans, but with fewer cells.

The development of *C. elegans* has been completely described from the very first cell to a completely developed adult (Sulston and Horvitz, 1977). This cell lineage describes the ancestry and fate of every single cell. This is of major importance because the identity and history of every cell can be known, where a single cell can be continually followed through development as well as its position in the animal. This information was first described by John Sulston, who directly observed the *C. elegans* embryo and represented the division of each cell in a tree diagram (Sulston et al., 1983). The result of this is that now any cell in a *C. elegans* adult can be chosen and its development traced
back to the 1-cell embryo stage. Sulston also reported that some cells are programmed to
die: 131 somatic cells undergo a stereotyped pattern programmed cell death during the
normal development of the hermaphrodite over its lifetime.

In terms of variation, there exist >100 haplotypes of *C. elegans* (Zwaal *et al.*, 1996).
(Cook *et al.*, 2017). Each wild isolate (WI) or isotype is genetically unique, thus offering
very substantial genetic variability, which makes it possible to dissect the genetic basis of
a range of characteristics, including quantitative – or continuous - traits. However, the
majority of *C. elegans* studies have been performed on a single isolate: N2. First isolated
by L.N. Staniland in Bristol, UK, N2 was established as the reference strain in 1970 by
Sydney Brenner, (EL *et al.*, 1959). Although studies on N2 have given considerable insight
into the genetic basis of many aspects of *C. elegans* development and biology, more recent
studies on N2 have concluded that the N2 laboratory strain does not represent a “natural”
population of *C. elegans*; rather it has adapted to a laboratory environment (Zwaal *et al*.,

### 1.2 ESTABLISHMENT OF ANTERIOR-POSTERIOR POLARITY IN THE *C. ELEGANS* EMBRYO

How are the first two daughter cells of *C. elegans* embryo made to be different from
one another? How is polarity established in the embryo? Prior to fertilisation, there is no
anteri/or/posterior polarity in the spheroid oocyte. The oocyte must break its polarity,
which is initiated by the point of sperm entry (Marston and Goldstein, 2006). During
development of the gonad at the L4 larval stage of *C. elegans* hermaphrodites, each of the
two growing gonad arms produces ~150 amoeboid sperm (Hubbard and Greenstein,
2000). After that, the germline switches to oocyte production. Considering a normal
situation in which each sperm fertilizes one oocyte, each hermaphrodite can produce
~300 individuals by self-fertilization before they become sperm depleted. The unfertilized oocytes enter in an arrested state in which they remain in meiosis I prophase until fertilisation. As oocytes move forward through the proximal gonad, they grow in size as well as maturing. As they approach the spermatheca (where the sperm is stored) the first event in polarization can be observed (McCarter et al., 1999). The nucleus of the oocyte, roughly central, moves distally so it distances itself from the spermatheca and sperm entry point (Figure 1a). After sperm entry, two important events occur: oocyte meiosis is completed, and the embryo now exhibits a clear anterior-posterior (AP) polarity with the hermaphrodite pronuclei (HP) indicating the anterior cortex, and the sperm pronuclei (SP) indicating the posterior (Albertson, 1984). An eggshell is then produced by the zygote, which maintains its shape and constrains the growing cells influencing their future divisions (Wharton, 1980). The pronuclei then move toward each other. However, the HP moves a greater distance than the SP, suggesting that the pulling forces are stronger with the SP. During this event, cell membrane contraction is observed, which culminates in a deep invagination similar to the later cytokinesis furrow. This is termed the pseudo-cleavage furrow (Figure 1b). At this point, the embryo exhibits a marked polarity, resulting in the asymmetric division of the zygote, or P0 cell, into two daughter cells: AB and P1 (Figure 1b). These two cells are different in size, cytoplasm content, and cellular fate.

The PAR proteins (partitioning defective) determine the asymmetric division in P0 (one cell zygote). PAR-3 and PAR-6 form a complex associated with the actin-myosin network and therefore are spatially restricted to the cell cortex (Schneider and Bowerman, 2003). On the other hand, PAR-2 and PAR-1 are uniformly distributed through the cytoplasm. When the sperm enters the oocyte, it locally disassembles the actin-
myosin network which contracts promoting a cortical contraction towards the anterior pole (Jenkins, 2006). PAR-2 and PAR-1 then occupy the posterior cortical region previously occupied by PAR-3 and PAR-6. Importantly, this cortical contraction promotes a compensatory cytoplasmic flux towards the posterior pole. Therefore, the two daughter cells have different cytoplasmic determinants. During anaphase, the spindle elongates in the AP axis asymmetrically. This displaces the mitotic spindle to the posterior cortex resulting on a displacement of the cytokinesis furrow. This will establish the asymmetrical division where the anterior cell is larger than the posterior.

In summary, the PAR proteins direct a molecular mechanism for cell polarity establishment and are responsible for the polarity of the first asymmetric division. This division includes different cell size and cytoplasm determinants, allowing differential assignment of cell fates between the two daughters.
Figure 1. Cell orientation from fertilization to 6-cell *C. elegans* embryo. Left lateral view and dorsal (top) view (left and right column respectively). a) *C. elegans* embryo at the moment of fertilization. No polarity whatsoever can be observed. Yellow arrows mark sperm entry point, marking the future posterior embryo pole. Please note position of male and hermaphrodite pronuclei. b) 2-cell stage embryo. The spindle in AB and P1 cells is formed following the Anterior-Posterior (AP) axis. c) 4-cell stage embryo. The spindles in ABa and ABp form perpendicular to the AP axis but skew as cleavage proceeds. The spindles in P2 and EMS follow the asymmetry established by ABa and ABp. At this point the dorsal-ventral (DV) axis of the embryo is established. d) 6-cell stage embryo. ABa and ABp spindles form perpendicular to both AP and DV axes. However this new LR axis proceeds asymmetrically clockwise resulting in ABal & ABpl (red) slightly anterior to ABar & ABpr (orange). e) Alternative 6-cell stage embryo obtained by gpa-16 mutation or micromanipulation. LR axis proceeds asymmetrically anti-clockwise resulting in ABal & ABpl (red) slightly posterior to ABar & ABpr (orange).
1.3 ESTABLISHMENT OF DORSAL-VENTRAL POLARITY IN THE *C. ELEGANS* EMBRYO

In early *C. elegans* development, the division direction is determined by the spindle orientation (Hyman and White, 1987). The centrosome pair in P0 is provided by the sperm (Hyman and White, 1987; Hyman, 1989; Schneider and Bowerman, 2003). This centrosome duplicates and each copy positions between the pronucleus and distal cell cortex. After the first division is completed, the AB and P1 nuclei form between the centrosome pair and the cleavage furrow. Then each centrosome splits and moves 90° along the transversal axis. In AB the centrosomes remain in that position, while in P1, both centrosomes rotate 90° to lie on the longitudinal axis but on opposite and equidistant sides of the nucleus. This centrosome positioning will define the orientation of the next divisions. By irradiation of centrosomes and cytoplasm regions, we know that when the P1 centrosomes are aligned along the transverse axis, they both initiate a competitive rotation toward the anterior cortex but one centrosome eventually “wins”. These pulling forces between the centrosomes and the anterior cortex result in a small invagination that can be observed during this centrosome re-positioning.

The division of AB and P1 defines the next axis: dorsal-ventral (DV). AB divides first, but owing to the eggshell constraints, the spindle orientation skews from an original orthogonal position into an oblique angle, resulting in one of the daughter cells positioned anterior and ventral to the other (ABa and ABp respectively). P1 then divides into P2 and EMS daughter cells, with P2 positioned posterior and dorsal to EMS (Figure 1c)
1.4 ESTABLISHMENT OF LEFT-RIGHT POLARITY - EVOLUTIONARY APPROACH

After the establishment of the AP and DV axis, the next step in *C. elegans* embryogenesis is the creation of a left-right (LR) axis, i.e. the establishment of the handedness of the animal. This is the process by which animals differentiate their left and right sides.

Most metazoans, including humans and *C. elegans*, show a pseudo-bilateral symmetry. That is, they are roughly symmetrical externally, but this symmetry is broken in the internal organization of the organs. For example, in humans there is asymmetry in the placement of our heart, liver and spleen. The LR axis is the most challenging one to establish, as it cannot be determined by exogenous inputs such as the sperm entry point or gravity. Rather, it needs to be set by internal determinants (Levin, 2005). The nature of the handedness itself has been amply discussed (Cooke, 2004). Why does LR asymmetry exist? Would not be easier just to program a perfectly symmetrical development? It has been argued that in evolution, vertebrates have developed a pseudo-bilateral symmetrical body plan to improve the efficiency of our organ function in the limited space available and to avoid unnecessary organ duplications.

Molecular details establishing LR asymmetry in vertebrates began with the identification of the left-sided expression of the *nodal* gene in the chick gastrula (Levin *et al.*, 1995). As with many bilateral animals, chicks have an overall symmetric body plan, with a number of internal components that are asymmetric with respect to the LR axis. Levin *et al.* (Levin *et al.*, 1995) demonstrated that 4 genes are asymmetrically expressed in the chick embryo, with 3 of them forming part of the initial regulatory network that will influence the asymmetric structure of the heart. The researchers focused on the
dextral bending of the heart hook as it is the earliest gross anatomical feature than can be observed. The LR asymmetric fate of the chick embryo is established long before the first morphological asymmetry appears, with evidence of the existence of molecular signalling molecules expressed asymmetrically before gastrulation (Levin et al., 1995).

Handedness is apparent across the animal kingdom, for example several molluscs develop an exoskeleton with the shape of a conical spiral with two alternatives: left-handed or right-handed (Murray and Clarke, 1966). The handedness of this exoskeleton varies among the different species of mollusc. Some species show invariant right-handed handedness, others show invariant left-handed, and a few show variation in handedness. It has been suggested that three main processes exist to establish LR asymmetry and can be classified according to the protostome-deuterostome classification (Spéder et al., 2007). Nodal flow and ion flows can be found in deuterostomes, while actin/microtubule cytoskeleton directed processes appear to be restricted to protostomes, including C. elegans.

The signals that initiate the final asymmetry of the gut in humans are well known (Burn and Hill, 2009) and can be divided into 4 phases, the first one of which is the break of polarity and establishment of a LR axis based on the previously formed AP and DV axes. This mechanism was discovered in mouse models, and is defined as a nodal flow. In a universally conserved manner, cilia rotate in a clock-wise handedness dependent on the invariant arrangement of microtubule structure. At this point, and in contrast to C. elegans and other protostomes, this rotation creates an extraembryonic flow that appears to organize many determinants of handedness. The rotation is followed by the transmission of LR positional signals which lead to asymmetric gene expression and finally an asymmetric organ morphogenesis. This cilia-based mechanism has been shown
in other vertebrates including zebrafish (Essner, 2005), medaka (Okada et al., 2005), rabbit (Okada et al., 2005) and Xenopus (Schweickert et al., 2007). Thus, the cilia clockwise rotation for establishment of LR asymmetry is a well-conserved mechanism among vertebrates.

The second mechanism in the establishment of the LR axis in deuterostomes, ion flows, was based on the observation that gene products originated from ion pumps and channels become asymmetrically expressed in Xenopus embryos. This asymmetric expression was hypothesized (Levin et al., 2002) to form an asymmetric ion flux resulting in an asymmetric pH gradient, and thus establishing a controlled LR asymmetry. Interestingly, Levin (Levin et al., 2002) observed that treatment with ion-pump inhibiting drugs led to heterotaxy – the abnormal distribution and arrangement of one or more of the internal organs - in Xenopus and chick embryos. This suggests that ion flows have an important role in LR asymmetry establishment.

In contrast to the mechanisms known in vertebrates, it is believed that cilia may not be involved in LR asymmetry establishment in protostomes. Rather, LR asymmetry in protostomes is established by modulation of actin and myosin contraction. This mechanism can be observed in Drosophila, some snails and C. elegans (Spéder et al., 2007).

An example of handedness establishment controlled by actin/microtubule can be found in the protostome Drosophila melanogaster. It has been suggested that the gut left-right asymmetric development in Drosophila is influenced by the ventral midline structures adjacent to the gut (Maeda et al., 2007)(Hozumi et al., 2008). In Drosophila, mutations in a type ID myosin, Myo31DF, causes reversals at both embryo and adult
stages. This fact is reflected in reversals in the LR position of the testis, hindgut and spermiduct. This result suggests that normal activity of *Myo31DF* is required for normal dextral *Drosophila* development. In addition, it provides evidence that actin-based functions (in this case type I Myosin) play a critical role in the left-right asymmetry generation.

The study of echinoderms, within the deuterostome phyla, has added additional information on the mechanisms of left-right symmetry breakage that help us to understand the evolutionary conservation of this process in deuterostomes (Takemoto *et al.*, 2016). Expression of *nodal*, a member of the transforming growth factor-beta (TGF-β), is the first detectable event of a bilateral symmetry break in the sea urchin *Hemicentrotus pulcherrimus* (Takemoto *et al.*, 2016)(Duboc *et al.*, 2005). The asymmetric expression of *nodal* leads to the establishment of a left-right organizer, located on the right side of the archenteron (digestive tube). This organizer will lead the unique asymmetric cell differentiation on the left side of the animal body. (Takemoto *et al.*, 2016) also reported that the establishment of left-right asymmetry of the sea urchin was partly controlled by ciliary motion on the small micromeres, small cells resulting from unequal segmentation of the zygote. This structure, which is involved in germline proliferation later in development, is located adjacent to the left-right organizer at gastrula stage. Takemoto *et al*, concluded that the ciliary motion during early embryo development is an ancestral and well-conserved mechanism for left-right asymmetry establishment in deuterostomes.

A key point in the subject of handedness is that in most species, this handedness can be reversed. That is some individuals may show a perfect mirror-image of the normal handedness. The most common handedness structure in a species is arbitrarily termed
dextral, while the mirror image of the dextral handedness is termed sinistral. A very interesting approach in the study of handedness reversal was performed in the gastropod *Lymnaea peregra* (Freeman and Lundelius, 1982). Around 1-2% of individuals show a naturally sinistral pattern (opposite exoskeleton coil) when cultured in normal conditions. (Freeman and Lundelius, 1982) hypothesised that there may be a dextral gene product that is responsible for the dextral coiling, which was absent in the sinistral individuals. The researchers attempted to characterize it by transferring cytoplasm from one egg to another and observing any changes in the expected coiling pattern. At the competent stage of the egg (before first polar body formation) the injection of cytoplasm from a dextral homozygote egg (both parents dextral) into a sinistral homozygote egg (both parents sinistral) changed the fate of the sinistral embryo resulting in dextral development. Cytoplasm injection had no effect after the competent stage; hence the establishment of the LR handedness is time dependent and cannot be reversed once it has started. Similarly, cytoplasm was injected from sinistral eggs into dextral eggs and it was observed that the dextral fate was not changed. The researchers concluded that the sinistral cleavage pattern reflects a default state that is generated in the absence of a dextral gene product.

1.5 **ESTABLISHMENT OF LEFT-RIGHT POLARITY IN THE *C. ELEGANS* EMBRYO**

Further insights in the mechanisms that establish LR handedness asymmetry have come from studies in *C. elegans* (Sulston and Horvitz, 1977)(Wood and Kershaw, 1991; Wood, Bergmann and Florance, 1996; Wood, 1998). As in humans, this animal is bilaterally symmetric externally, but shows profound LR asymmetry in the placement of many cells and the major organs. In *C. elegans*, this handedness is seen at several levels: first, the positioning of the gonad to one side of the intestine in the anterior and the
opposite side in the posterior of the pseudocoelom, second, the asymmetric placement of a number of cells, including neurons and the coelomocytes, and third, the path of the central nerve chord around the hermaphrodite vulva (Wood and Kershaw, 1991). This asymmetry can be first observed very early in embryonic development, specifically, at the 6 cell stage (Figure 1d), after the anteroposterior and dorsoventral polarity are established at the 2 and 3 cell stage respectively (Figure 1b, c). At that point, ABa and ABp cells divide along the LR axis (Figure 1d). The two spindles are originally located orthogonal to both the AP and DV axis, but as they elongate, they skew resulting in one pole of the spindle posterior to the opposite. From a dorsal view, the skew follows a clockwise direction and the final product is that the cells on the right side (ABar and ABpr) lie slightly posterior to their sisters ABal and ABpl (Figure 1d). Handedness is established at this point. Following the asymmetrical left-right division of ABa and ABp, anteroposterior cleavages of EMS and P2 cells occur, but these divisions are also slightly asymmetrical, resulting in an asymmetrical 8 cell stage embryo. This asymmetry is reflected in a consistent positioning of ABal and ABpl descendants slightly anterior to their homologues on the right. Later in development, this asymmetry in the 8 cell stage embryo will cause distinct and particular cell lineages for each AB left-right progeny set.

1.6 HANDEDNESS REVERSAL IN C. ELEGANS

Handedness reversal in C. elegans can be accomplished by micromanipulation during the cleavage of ABa and ABp. As ABa and ABp elongate on the LR axis during anaphase, Wood and Kershaw manually applied pressure on the left side pushing posteriorly (Wood, 1991; Wood and Kershaw, 1991). These manipulated embryos developed into adults with complete handedness reversal for all the organs and cells – i.e., they were mirror image individuals. These worms were healthy, moved normally and
produced normal numbers of self-progeny, all of which showed dextral handedness. This process of reversal was termed \textit{situs inversus totalis} (i.e., total reversal). Therefore, the authors concluded that first, the handedness of the embryo at 4-6 cell stage determines the final handedness of the adult, second, that ABal and ABar are developmentally equivalent, as are ABpl and ABpr, and third, the differences between cell lineage in cell homologues is determined by cell-cell interactions, which differ on the left and right side because of the asymmetric positioning of AB daughters. This dominant handedness is seen in much of the animal kingdom, but there are some examples of randomization of handedness. In the case of the dung beetle parasite \textit{Brynema rigidum}, 50% of their individuals are dextral and 50% sinistral, with no apparent control of handedness specification (Wood, 1998).

Wood observed more than 10,000 adult N2 hermaphrodites and found that all of them showing the normal dextral organization of internal organs, with the anterior-right and posterior-left gonad structure (Wood, Bergmann and Florance, 1996). The authors concluded that the handedness of the asymmetries in normal development is invariant for \textit{C. elegans}. However, there are some treatments that can lead to the development of sinistral individuals. The F3 progeny of adults treated with the mutagen ethyl methanesulfonate showed a sinistrality rate of 0.2% (Wood, Bergmann and Florance, 1996). These reversed animals produced 100% normal dextral progeny. It was also argued that the eggshell constraints may be responsible for the asymmetric placement of AB descendants. To test this hypothesis, embryos were subjected to a chitinase-chymotrypsin treatment to produce embryos without an eggshell. The result of this was a spherical cellular configuration of the blastomeres; without the eggshell constraints, the 4-cell stage embryo is T shaped instead of rhomboidal (Wood and Kershaw, 1991). If the
eggshell constraint were the only factor responsible for the asymmetric placement of the AB descendants, 50% sinistrality would be observed. However, survivors of chitinase treated embryos (~70%) developed into adults of which only 5.5% were sinistral totalis. This observation suggests that relaxation of the eggshell constraints occasionally permits early asymmetry reversal, but it is not the definitive factor in handedness establishment (Wood and Kershaw, 1991). Another factor that has been found to alter the invariant dextral handedness in C. elegans is low temperature. It has been observed that the N2 strain cultured at 10°C showed a frequency of situs inversus totalis of ~0.5% (Wood, Bergmann and Florance, 1996). All sinistral animals showed handedness reversal in coelomocytes, ventral nerve chord and gut/gonad. It has also been observed that the temperature-sensitive period for this event is before the 6-cell stage, and appears to occur during gametogenesis in the parents. A 10°C treatment on the oocytes before fertilization was enough to elicit ~0.5% sinistrality. However, the effect on sperm has not been assessed, which would be informative, as it is the gamete providing the centrosome and it is the key factor in spindle formation (Wood, Bergmann and Florance, 1996).

Given this background, three important questions arise. First, how is one handedness chosen over the other? Second, why there is a bias toward dextral or sinistral configuration in most species? And third, what are the downstream factors that regulate the asymmetrical development of the two sides of the embryo given only the cell position?

Further studies that aimed to understand the genetic bases of handedness reversal have been performed (Bergmann et al., 2003). The initial hypothesis was that the handedness determination was resolved by autonomous factors and independent of external cues. Jansen et al., demonstrated that control of spindle orientation by G proteins
during the first two cleavages determined the asymmetric divisions (Jansen et al., 1999). There is evidence that Gα, Gβ and Gγ proteins control centrosome behaviour (Zwaal et al., 1996). One of the G protein genes, gpa-16 has been studied. It codes for a G protein α subunit and is essential for spindle positioning. In gpa-16 (loss-of-function) mutants the spindle organization randomizes and the embryo aborts (Bergmann et al., 2003). Therefore, the loss-of-function gpa-16 mutation was combined with an it143 mutation, which lead to a partial loss of gpa-16 gene function (termed hypomorphic mutation). gpa-16(it143) is a maternal effect hypomorphic mutation with a temperature-sensitive penetrant lethality which causes aberrant behaviour of mitotic spindles during the first three cleavages. When homozygous individuals are grown at 25°C, only 30% of the embryos survive, but among the survivors, 40% show sinistral organization of internal organs, i.e., situs inversus totalis. However, the lethality and sinistrality were lower when cultured at 20°C (30% and 19% respectively) and even lower when cultured at 16°C (2% and 6% respectively) (Bergmann et al., 2003).

Focusing on the effects of situs inversus totalis, it has been assessed whether internal organ and brain asymmetry correlates with asymmetric behaviour (Downes et al., 2012). In humans, for example, there is a preferential use of the right hand in 90% of the population. Interestingly, however, this is not correlated with anatomical asymmetry. Human individuals with situs inversus totalis show the same preferential use of their right hand as dextral people (Hardyck and Petrinovich, 1977). Researchers tested whether sinistral C. elegans animals showed a reversal of behaviour (Downes et al., 2012). The feature used to assess the behaviour asymmetry was the turning behaviour of C. elegans males during mating. As a population, a clear handedness bias towards right-handed turning was observed in males. This bias was not changed after manipulation of
handedness and was maintained throughout the lifetime of the male worm. When \textit{gpa-16(it143)} dextral and sinistral males were observed, no significant differences in behaviour were observed: both dextral and sinistral worms showed the same right-handed preference in their mating behaviour. Therefore, it was concluded that the behavioural handedness is independent of the internal organ arrangement (Downes \textit{et al.}, 2012).

\section*{1.7 \textbf{LEFT-RIGHT ORGAN REVERSAL}}

In addition to the complete reversal of internal organs, \textit{situs inversus totalis}, in which reversed individuals are perfectly healthy and show no organ dysfunction (Hardyck and Petrinovich, 1977), there is another situation in which a partial reversal of internal organs can be found: \textit{situs inversus incompletes} (i.e., incomplete reversal). This event can be found in humans as well as \textit{situs inversus totalis}, but in contrast, \textit{situs inversus incompletus} is often associated with organ dysfunction and disease (Levin, 2005). The medical term for this condition is heterotaxia or \textit{situs ambiguous}, and refers to an abnormal distribution and arrangement of one or more of the visceral organs, with defects in the left-right laterality (Shiraishi and Ichikawa, 2012). For example, Primary Ciliary Dyskinesia is an immotile ciliary syndrome that causes ciliary dysfunction (Shapiro \textit{et al.}, 2016). Patients often also present with partial lateralization of certain organs, in which case it is called Kartagener syndrome.

In \textit{C. elegans}, several studies (Wood, 1991; Wood, Bergmann and Florance, 1996; Bergmann \textit{et al.}, 2003; Callander \textit{et al.}, 2014) involving more than 25,000 animals have led to the following conclusion: under normal conditions, left-right reversals in the arrangement of the two major organs (gut & gonad) in the laboratory strain N2 do not
occur naturally. This suggests that *C. elegans* has adopted an invariant dextral body plan. In other words, *C. elegans* development is believed to be virtually invariant.

### 1.8 RELEVANCE OF NORMAL ORGAN ASYMMETRY IN BILATERIANS

Most bilaterian animals exhibit a stereotypic LR asymmetry in the position and shape of internal organs. It has been hypothesized (Blum *et al.*, 2014) that the first organ system to undergo an asymmetric organogenesis was the digestive tube. In snails, the total length of the digestive system exceeds the main body length. This inevitably implies an asymmetric packaging of the digestive tube. Although this packaging may not be functionally relevant, it may be explained by space optimization. An asymmetric compartmentalization of the digestive system appears to be universally seen in animals, including protozoans (Fok and Allen, 1990; Plattner and Kissmehl, 2003). Additionally, through evolution, asymmetric compartmentalization of the digestive system has evolved to be organised in a reliable manner, rather than stochastic placement. It appears that most animals have developed a digestive system organogenesis that results not only in the most efficient intestine packaging in the body cavity, but also the most functionally advantageous. Evidence of human syndromes associated with intestinal malrotations support this claim (Strouse, 2004; Burn and Hill, 2009; Sutherland and Ware, 2009). Similarly, the primitive heart was a symmetric contractile muscle pumping haemolymph throughout the animal body (van Helden, 2014). Such simplicity did not require asymmetric morphogenesis. However, the human heart is now asymmetric, not only in the relative body position, but also in the very organ structure, with atriums and ventricles functionally and structurally different, resulting in a highly asymmetric organ (Laflamme and Murry, 2011). It is therefore hypothesized that this asymmetry has been achieved through evolution to obtain the most efficient structure to perform its vital
function. Interestingly, the lungs are also asymmetric, but their asymmetry appears to reflect the space constraints in the body cavity as a result of the asymmetric heart (Blum et al., 2014). Therefore, it appears that organ asymmetry in animals can be acquired by asymmetric organogenesis (developmentally controlled) or by space constraints forced by other organs previously developed in the same body cavity.

1.9 HETEROTAXY IN HUMANS

How normal healthy asymmetry (situs solitus) is achieved in animal organogenesis is not fully understood. Similarly, the mirror-image organogenesis known as situs inversus totalis (also called situs transversus or oppositus) is also poorly understood. Situs inversus is found in humans with a prevalence of 1:10,000 (0.01%). Situs inversus individuals present no medical symptoms or complications associated (Spoon, 2001; Levin, 2005).

Additionally, a syndrome affecting the normal asymmetric organogenesis, known as heterotaxy or situs ambiguous occurs. This abnormal arrangement of the body cavity viscera is seen in humans in the form of asplenia, polysplenia, Ivemark syndrome, primary ciliary dyskinesia, etc (Sutherland and Ware, 2009). They are all characterized by congenital malformations, with those affecting the heart carrying the highest morbidity and mortality (Lin et al., 2000). It is estimated that heterotaxy affects humans with a prevalence of 1:10,000 (0.01%) (Lin et al., 2000). Most of these “classic” heterotaxy syndromes generally involve cardiac defects plus other visceral anomalies. Most likely, the heterotaxy prevalence is much higher in human population as heterotaxy defects are usually incompatible with embryo development and generally result in the abortion of the embryo before it can be included in the heterotaxy statistics. In terms of heterotaxy prevalence, a study including 200 patients who underwent a Fontan operation concluded that Asians show a higher prevalence of any form of heterotaxy syndrome compared to
Westerns (Kim et al., 2008). Chromosomal anomalies are rarely associated with human heterotaxy (Kim, 2011).

1.10 CAERNORHABDITIS ELEGANS GENETICS

*Caenorhabditis elegans* is the best-characterized species in the *Caenorhabditis* genus. The complete genome of *C. elegans* was sequenced and published in 1998 (The *C. elegans* Sequencing Consortium, 1998) being the first multicellular organism to have its whole genome sequenced. However, it contained tens of gaps in form of unfinished yeast artificial chromosome (YACs), cosmids and fosmids. They were all progressively completed over the years (McCombie et al., 1992; Waterston et al., 1992; Collins et al., 2004), culminating in all remaining gaps being filled, including the mitochondrial genome, in October 2002 (Hillier et al., 2005).

The *C. elegans* genome is composed of at least 20,470 protein-coding genes organised into six chromosomes I, II, III, IV, V, and X. The sex-determination system in *C. elegans* is determined by chromosome X, with hermaphrodites diploid and males haploid. Autosomes are diploid for both sexes. Gene density is estimated in *C. elegans* to be one gene per five kilo-base pairs, or 200 genes/Mb. Compared to humans, who have 12-15 genes/Mb, the *C. elegans* genome is highly compacted (Brenner, 1974). Interestingly, *C. elegans* is peculiar in having polycistronic gene clusters (mRNA carrying several open reading frames (ORFs), each of which is translated into an independent polypeptide), termed operons, containing between two and eight genes (Blumenthal et al., 2002). Operons contain ~15% of *C. elegans* genes, which usually are gene expression machinery, and rarely contain tissue-specific genes.
In 2005, *C. elegans* was estimated to have ~1,300 RNA genes (Stricklin, Griffiths-Jones and Eddy, 2005). However this number was sharply increased in 2006 with the discovery of 21U-RNA and additional microRNAs genes (Ruby *et al.*, 2006). Ongoing research is being conducted in this area, adding, modifying and removing genes to the list, but it is estimated that *C. elegans* has ~16,000 RNA genes.

*C. elegans* developmental studies have proven to be of great importance for human developmental studies because of conservation of core genes and genetic pathways across animals (Sulston and Brenner, 1974). Around 38% of *C. elegans* genes have human homologues (Shaye and Greenwald, 2011). Human genes have been proven to successfully replace nematode homologues (Lodish *et al.*, 2000). In addition, gene function has been seen to be highly similar to humans and other mammals for large number of genes. These observations suggest many conserved genetic pathways in metazoans (Marsh and May, 2012).

In contrast to discrete traits, which can be categorised into a small number of categories, such as left/right handed, a quantitative trait is a continuous phenotype which is typically measured on a scale, such as height or weight. Although there are exceptions, quantitative traits tend to be influenced by many factors, both genetic and environmental, in contrast to discrete traits, where it is more common that variation in a small number of genes determines the phenotype (Falconer and Mackay, 1996). Quantitative genetics is based on identifying the genetic contribution to phenotypic differences among individuals. A number of approaches can be taken to understand the contribution of individual genes, or quantitative trait loci (QTL) to these differences, including linkage mapping (often termed QTL mapping), or and genome wide association studies (GWAS). Both these approaches rely on correlating genetic differences between individuals with
differences in their phenotype, in order to identify regions of the genome that contribute significantly to the trait (Falconer and Mackay, 1996).

1.10.1 Domestication of *C. elegans*

*C. elegans* is an ideal model organism for understanding the genetic basis of quantitative traits. Large clonal populations can be easily obtained, and large numbers of individuals can easily be housed in the laboratory. To date, the majority of quantitative genetic studies in *C. elegans* have been performed on the laboratory strain N2 (Kammenga et al., 2008). However, the process by which a wild strain is isolated from nature and domesticated into a laboratory strain is a complex process. Evidence of several pleiotropic effects in *Saccharomyces cerevisae* (Yvert et al., 2003) and *Arabidopsis thaliana* (van Zanten et al., 2009) suggest that *C. elegans* domestication may also have led to unusual phenotypes that are not commonly seen in the wild.

In nature, *C. elegans* is found in rotting hogweed, fruits and compost. The strategy this nematode follows is establishing a population that grows exponentially competing for resources and quickly feeding on bacterial food. When food is limiting, *C. elegans* enters a resistance larval stage called dauer, capable of surviving long periods of time without food. These circumstances are harshly different compared to normal laboratory conditions, where it is common that food is available continuously (Félix and Braendle, 2010).

Historically, laboratory domestication of *C. elegans* began in 1951 when L.N. Staniland collected nematodes from mushroom compost in Bristol, UK, for nematology courses. Then, in 1957, nematodes were transferred to the Dougherty Lab in California (USA). At this point, in 1963, Sydney Brenner became interested in the nematode and
isolated nematodes from his garden, which he called N1. He then requested the Bristol strain from the Dougherty Lab, which he called N2. Brenner then isolated a single N2 hermaphrodite initiating a monoclonal population, which was frozen in 1969 (Sterken et al., 2015).

The ability to freeze worms in 1969 enabled the globalization of the *C. elegans* N2 laboratory strain. Before freezing, however, the N2 strain propagated for an estimated ~300-2000 generations from 1951. This leads to a predicted number of approximately 1,000 natural mutations that could have accumulated in this time, due to random germline mutation rate and selection of random mutants (Sterken et al., 2015). Interestingly, many *C. elegans* distinct wild isolates appear to have been contaminated by the N2 strain (McGrath et al., 2009), perhaps as a consequence of a recently discovered phenomenon whereby *C. elegans* animals “escape” from their culturing plates and migrate short distances (unpublished results, Rothman Lab, UCSB, USA). However, this migrating behaviour does not explain the high genome similarity between ~two thirds of other (i.e., non-N2) strains. Rather, this genetic similarity seems to be the result of evolutionary advantageous allele selection as these strains have adapted to a laboratory environment (Andersen et al., 2012).

How is the N2 laboratory strain different from the rest of the *C. elegans* natural population? Many features provide evidence of the “uniqueness” of N2 – aggregation behaviour, maturation time, fecundity, fertility, etc. All differ markedly from more recently collected natural isolates (Sterken et al., 2015). Specifically, the N2 laboratory strain presents differences in three genes that differ substantially from the rest of the wild isolates: *npr-1, glb-5*, and *nath-10* (McGrath et al., 2009; Lee et al., 2012; Andersen et al.
al., 2014). This is reflected in N2 aerotaxis behaviour absence, different behavioural response to O\textsubscript{2} and CO\textsubscript{2} concentration, and an increased fitness advantage.

In summary, N2 laboratory strain does not represent the *C. elegans* natural population, and hence those studies including behaviour or phenotype expression should not be based solely on N2 and mutant derivates, but instead will benefit from experimental approached that incorporate the global diversity of *C. elegans*.

### 1.10.2 Population biology and evolution

*Caenorhabditis elegans* isolate N2 (Bristol, UK) is the first animal of which the complete genome sequence was available (The C. elegans Sequencing Consortium, 1998; Koch *et al.*, 2000). By comparing to the Bristol N2 laboratory strain, variation among different *C. elegans* wild isotypes from around the world was determined using whole-genome sequence reads. The pattern obtained suggests that differences among isotypes raised from an interbreeding process elicited by isolation and facilitated by hermaphroditic inbreeding reproduction occurring naturally in *C. elegans* populations in the wild (Koch *et al.*, 2000). Interestingly, recombination in *C. elegans* autosomes is observed to be the highest on the terminal thirds and the lowest on the autosome centre. Following this, essential genes tend to be located in the autosome centres, displacing gene families to the arms. Also, SNP differences among isotypes are shown to be highest on the arms (Barnes *et al.*, 1995; Koch *et al.*, 2000).

Recently, the thousands of sequenced genome fragments of 200 wild isolates has been made public by Erik Andersen (Princeton University, USA) (Andersen *et al.*, 2012). Sampling of wild *C. elegans* covered all continents (except Antarctica) providing the best and most comprehensive population study of *C. elegans* strains to date. Genomic
characterization was performed using an approach termed restriction-site-associated DNA (RAD), identifying 41,188 SNPs in 8 Mb of sequence. The RAD-seq method involves cutting the genome with a restriction enzyme, and then sequencing ~100 bases neighbouring each cut site, producing high coverage sequencing of a subset of the genome, known as a reduced representation approach (Davey et al., 2011). The hermaphroditic nature of *C. elegans* implies that most reproduction is via selfing, leading to high levels of inbreeding, hence strains collected from nearby locations were expected to be genetically identical. Of the total panel of 200 strains sampled, Andersen found 47 unique haplotypes plus 50 sets of near-identical strains. In total, Andersen described the 97 distinct and unique *C. elegans* strains, which are now known as wild isotypes, isolates, or haplotypes (Andersen et al., 2012). Isotypes exhibited high levels of linkage disequilibrium across the genome, likely as a consequence of the high levels of inbreeding as a result of hermaphroditic selfing.

The authors also tested population subdivision, and using STRUCTURE (Falush, Stephens and Pritchard, 2003) and found evidence of one worldwide population. They also found a correlation between distance and genetic similarities, although the correlation was weak and only applied to isotypes 700km or closer, suggesting that genome-wide diversity was not well predicted by geographic distances between populations. Genome-wide coverage studies showed a low genetic variation in *C. elegans* haplotypes, and genetically similar haplotypes were distributed worldwide geographically (Andersen et al., 2012). This fact could be explained by the movement of *C. elegans* globally as a consequence of internationalization of human travels and carelessness in transport of agricultural products.
1.10.3 Comparative biology of \textit{C. elegans} with \textit{C. briggsae}

In order to obtain a better understanding of \textit{C. elegans} evolution, \textit{C. elegans} has been compared with another member of the \textit{Caenorhabditis} genus – \textit{C. briggsae} (Hillier et al., 2007). These two distinct species share many similarities – they are almost morphologically identical, and both share the same ecology. Their genome size is similar, with around $\sim$100Mb organised in 6 chromosomes. Also their genes and gene pathways are comparable, as genes from one species rescue mutants from the other (Sarov et al., 2006). By the construction of a genetic map based on DNA variations between these two species, the authors concluded that, as reported previously, intrachromosomal rearrangements are more frequent on chromosome arms and less frequent in the centre (Barnes et al., 1995; Koch et al., 2000). Also, an analogous variation in gene density and distribution across the genome was found. This evidence can be endorsed with a common misunderstanding often made in undergraduate levels: \textit{C. elegans} is not more primitive than humans. They evolved in a different way than humans, towards simplification and optimization of the genome.

1.11 DISSECTING QUANTITATIVE TRAITS

A Single Nucleotide Polymorphism (SNP) is the difference in a single nucleotide at a given site in the genome between individuals or populations. When a gene presents two or more possible SNP variants, they are called allelic forms.

Through RAD-seq genotyping, Andersen \textit{et al}, (Andersen et al., 2012), created a SNP map for the 97 natural wild isolates. Using polymorphism data including 4,690 SNPs, Andersen group created a toolkit for genome-wide association studies. These studies are based on SNP variation among natural \textit{C. elegans} populations.
1.11.1 QTL mapping

Variation in quantitative traits is usually influenced by multiple genetic loci and environmental factors. Thereby, the study of those loci (Quantitative Trait Loci – QTL) can lead to the dissection of quantitative traits, in particular, determining whether there are loci of major effect influencing differences between individuals, or whether many loci of small effect explain variation in a population (Falconer and Mackay, 1996). The general idea of a QTL mapping is to associate genotypes and phenotypes in a population showing genetic variation, and relies on the co-inheritance of genetic markers with phenotypes through family groups. QTL mapping is particularly useful with model organisms, as we can control the environment and life conditions, therefore reducing noise (Lambot et al., 2004). The QTL mapping approach on which this thesis is based (Lambot et al., 2004), focuses on the experimental crosses between inbred lines (Rockman and Kruglyak, 2008), which, in addition to detecting regions of the genome influencing differences between individuals, can allow the estimation of the degree of dominance at a detected QTL.

Phenotype and genotype data within family groups, and the marker map are the three pillars upon which QTL mapping is structured. The marker map is defined by the location of genetic markers along the chromosomes, measured in genetic distance (usually centimorgans) (Lambot et al., 2004).

In general, QTL mapping offers low resolution but provides high statistical power for detecting a QTL. The main disadvantage is that studies are limited to the genetic diversity present in the parents of the segregating population. Because of this, QTL mapping provides a finer mapping when inbred lines are used, because there are many fixed differences between the lines across the genome (Lambot et al., 2004; Rockman and
Kruglyak, 2008). The idea behind QTL mapping and GWAS is very similar. However, QTL mapping tests the influence of genomic regions on differences between phenotypes, typically within family groups (e.g., a cross of inbred lines), while GWAS tests association between a phenotype at each marker position, and has historically relied on samples of unrelated individuals from a population (although methods to correct for related individuals are now available).

1.11.2 GWAS

A genome-wide association study (GWAS) is a statistical approach that aims to test whether differences in genotype are associated with differences in a given phenotype (Nuzhdin, Friesen and McIntyre, 2012). GWAS compares allele frequencies between populations looking for statistical support for variants in region or regions of the genome—termed casual regions—contributing to differences in a quantifiable phenotype. In order for a region of the genome to be significantly associated with a phenotype, the underlying genetic cause must be shared by a large number of isotypes. Typically, GWAS treats each SNP individually, meaning GWAS disregards the genome-wide similarities and differences between isotypes (Mitchell-Olds, 2010).

GWAS has proved to be a useful approach in the study of genetic natural variation. As a general rule, GWAS is particularly useful when inbred lines are available, as once they are genotyped they can be phenotyped multiple times. This allows the study of different traits under different conditions, reducing environmental noise (Cheng et al., 2010).

GWAS is prone to false positives as a result of population structure. Differences in phenotype are correlated with global non-causal genetic differences, leading to false
signals of association to genetic variants that are unrelated to the phenotype. As a consequence, when testing for association it is important to first control for confounding population structure (Lander and Schork, 1994). An example of this is in the self-fertilizing plant *Arabidopsis thaliana*. A total of 96 lines were genotyped using a custom Affymetric SNP chip, obtaining 250,000 SNPs (Kim et al., 2007). Also, 107 different phenotypes were recorded, including flowering under different environmental conditions, defence, and developmental traits (Atwell et al., 2010). Their results showed that most phenotypes gave rise to a distribution of $p$ values that was strongly skewed towards zero, as expected by the presence of confounding population structure. They also concluded that the population structure was highly complex, involving patterns of relatedness on all scales.

### 1.11.3 Missing heritability

Heritability is used in statistics to measure the variation of a given trait being it entirely dependent on genetic variation, not associated with environment. When facing complex genotypes, for example complex diseases such as diabetes, cancer, or neurological diseases, GWAS has identified hundreds of variants and significant loci associated. Unfortunately, these variants only explain a small fraction of the observed heritability of the disease. This phenomenon is known as missing heritability (Manolio et al., 2009; Slatkin, 2009; Lee et al., 2011; Zuk et al., 2012). The missing heritability problem implies that the susceptibility of an organism to inherit a given trait or phenotype depends more on "the combined effect of all the genes in the background than on the phenotype genes in the foreground" (Arango, 2017). A good example is human height or intelligence, for which genetics can explain no more than 80% of the heritability.
It has been proposed that missing heritability is a combination of many factors including: epigenetics (Manolio et al., 2009) and variability in the underlying cause of the same effect (i.e., the same disease caused by two different mutations) (Slatkin, 2009).

Phenotypic robustness is the ability of a given genotype to produce a constant phenotype, even when under different environmental perturbations (Rutherford, Hirate and Swalla, 2007). A good example of phenotypic robustness can be found in section 2.8.2 (Figure 9a). The gut/gonad heterotaxy rate in the isolate MY16 remains constant regardless of the environmental perturbation. Probably, this is due to feedback loops, gene redundancy and microRNAs. Phenotypic robustness is often associated with an increased mutation rate. In C. elegans, decreased function of hub genes has been observed to enhance the phenotypic consequences of decreased function in other genes (Lehner et al., 2006).

It appears that decreased robustness correlates with, and may produce genome instability at several levels (Queitsch, Carlson and Girirajan, 2012). The authors also hypothesize that differences in phenotypic robustness among individuals is another factor that contributes to the missing heritability of a trait.

1.12 AIMS AND PRESENTATION OF THIS THESIS

The main objective of the work described in this thesis is to study natural left-right gut/gonad heterotaxy in C. elegans from a variety of wild isolates, to identify the cellular and molecular basis of heterotaxy, and to investigate the genetic background responsible for such partial handedness reversal.

This study provides new evidence that may not support the generally accepted conclusion of an invariant C. elegans development towards dextrality. Taking advantage
of the large collection of *C. elegans* natural isotypes available, I have characterized the variation in cell/organ heterotaxy. I will have analysed variation in sets of laboratory-generated recombinant inbred lines (RILs) in order to extend the exploration of left-right development in *C. elegans*. Chapter 2 describes biological features associated with gut/gonad heterotaxy including parent-of-origin effects, variation in biological stress and reproducibility of the trait. Chapter 3 explores candidate developmental events downstream from the observable heterotaxy in adult worms. Finally, these sections of trait characterization and “observation” of the thesis are followed in Chapter 4 by available quantitative trait loci (QTL) mapping methods aimed to dissect the genetic basis contributing to the observed traits, with the goal of identifying major loci contributing to differences between wild isolates and between RILs. Each chapter will be structured with a brief introduction, setting up the basis for a complete understanding of the experiments and results to follow. Each chapter will also include relevant conclusions and a brief discussion that will be explained and extended thoroughly in Chapter 5. Specific materials and methods will be incorporated into each chapter when relevant.
CHAPTER TWO

Variation in LR organ arrangement across the C. elegans population

As concluded by several studies (Wood, 1991; Wood, Bergmann and Florance, 1996; Bergmann et al., 2003; Callander et al., 2014), as described in the previous section, the general consensus is that C. elegans has adopted, through evolution, an invariant development by which the LR organ arrangement adopts a dextral body plan. However, the Rothman laboratory recently reported that this stereotyped gut/gonad arrangement is not invariant, but rather shows substantial variation among the C. elegans natural isotypes.

2.1 VARIATION IN LR GUT/GONAD ASYMMETRY IN C. ELEGANS MALES

Situs inversus incompletus has been found in C. elegans (Callander et al., 2014). Males from the N2 strain were examined at 20°C and occasional cases (0.38%; 2 in 522) of males with reversed position of gut and gonad (gut/gonad reversal) were found (Figure 2b). However, this frequency was sharply increased when the same strain was cultured at 25°C (5.36%; 25 in 466). However, no cases of natural reversal in hermaphrodites had been described at either 20°C or 25°C. In order to assess the nature of the gut/gonad reversal observed, C. elegans males with gut/gonad reversal were isolated and the gene expression of the ASE gustatory neuron pair was established as a marker of the handedness of the individual. The ASER neuron (on the right side of the animal) expresses gcy-5. However, ASEL (on the left side) is silent for this gene. In contrast, in sinistral individuals, gcy-5 is expressed on the neuron on the left and not in its right pair (Poole and Hobert, 2006). When gut/gonad reversal males were assessed in this aspect, it was found that the gcy-5 expression was the same as that of a dextral worm.
Variation in LR organ arrangement across the C. elegans population

(Callander et al., 2014). Therefore, LR gut/gonad reversal occurs independently of the handedness of the animal established at embryonic stage. The handedness of CB4856 strain (Hw) individuals, which showed 5.47% of reversals at 20°C, was assessed (Callander et al., 2014). The overall handedness of the animal was observed in a significant number of individuals through the ASE neuron pair and it was concluded by the authors that gut/gonad reversal was independent of the overall handedness of the animal. In other words, all animals showed a dextral configuration of all internal organs except for the gut and the gonad, which were on opposite sides (situs inversus incompletus). One hypothesis is that this gut/gonad reversal is produced by a dysfunction in gonad migration as it elongates during somatic development, which often happens between the L2 and L4 stage (Hedgecock et al., 1987). One possible main driver could be the linker cell, which leads the growing gonad during morphogenesis and initiates the union with the cloaca at the tail of the worm.

Callander et al., (Callander et al., 2014) also suggested the possibility that the heterotaxy observed in males could be the result of aberrant gonad migration events occurring at C. elegans early stages of development. 40 wild isolates were selected and the frequency of errors in gonad migration during the L1 linker cell migration was assessed, and compared with the frequency of gut/gonad reversals. The results showed no significant correlation between the frequencies of these two events. In summary, LR gut/gonad reversals in males are not directly explained by gonad migration errors during the L1 stage (Callander et al., 2014).

It has been argued that the gut/gonad reversal observed in N2 males could be a simple result of mistakes in the LR-determining system resulting from the long-term culturing of the N2 laboratory strain in the laboratory environment. However, it was also
observed that LR gut/gonad asymmetry is not restricted to sporadic cases in N2 males. Fully developed adult males with fully developed gonad arms occupying the space in the pseudocoelom normally occupied by the intestine in dextral animals were found in the Hawaiian isolate (Hw, CB4856) and in Hw/N2 derived Recombinant Inbred Advanced Intercross Lines (RIAILs) (Rockman and Kruglyak, 2008, 2009). These results suggest that *C. elegans* male LR gut/gonad reversals may be occurring naturally in the inbred wild isolates.

We reported (Alcorn *et al.*, 2016) a large variation across the wild isolates in the LR gut/gonad reversal of *C. elegans* males. For each strain, the gut/gonad handedness was scored in a significant number of *C. elegans* male individuals (~141) at 20°C. Results showed a total of 12 strains showing 0% frequency of reversal animals, i.e., *C. elegans* males with the gonad on the left side of the body (Figure 2b). The other 88 strains showed frequency of reversals varying from 0.78% to 11.15%.
Variation in LR organ arrangement across the C. elegans population

Figure 2. Overview of different gut/gonad heterotaxy phenotypes found in C. elegans males and hermaphrodites. Ventral representation. Black-texture represents the intestine while the gonad is in white. Anterior pair of coelomocytes ccAR and ccPR are marked on red and always found on the right body side as marker of dextral embryo chirality. 

- **a)** Dextral (normal) male. Unique gonad arm and coelomocyte pair is on the right.
- **b)** Reversed male. Gonad arm is on the left.
- **c)** Dextral (normal) hermaphrodite. Anterior gonad arm is on the right and the posterior on the left side of the animal.
- **d)** Complete Reversal. Both gonad arms are reversed in a mirror-image representation of the dextral hermaphrodite. Note coelomocyte pair remains on the right (dextral) side.
- **e)** Posterior heterotaxy. Reversal restricted to the posterior gonad arm. Both gonad arms are aligned on the right side.
- **f)** Anterior heterotaxy. Reversal restricted to the anterior gonad arm. Both gonad arms are aligned on the left side.
2.2 HETEROTAXY IN *C. ELEGANS* HERMAPHRODITES

Given the observation of heterotaxy (i.e., LR gut/gonad reversal) in males, it is important to address whether the same phenomenon occurs in hermaphrodites. The internal organ organisation of *C. elegans* hermaphrodites entails an additional level of complexity compared to males. Hermaphrodites have two gonad arms (Figure 2c), which start ventrally at mid-body, close to the vulva, and elongate in opposite anterior-posterior directions. Importantly, the anterior gonad arm elongates along the right side of the pseudocoelom, in contrast to the posterior gonad arm, which elongates along the left (Sulston and Horvitz, 1977).

2.2.1 Gonad organogenesis in post-embryonic *C. elegans* development

The gonad primordium in *C. elegans* is morphologically identical in both males and hermaphrodites during the first larval stage L1 (Kimble and Hirsh, 1979). It is located mid-ventrally, with its 4 cells (Z1, Z2, Z3, and Z4) organized along the AP axis. Z1 and Z2 are slightly shifted to the right side, whereas Z3 and Z4 take the opposite worm’s midsagittal plane. Hence, the gonad primordium in *C. elegans* is LR asymmetric from its very beginning. Z1 and Z4 cells start dividing during the L1 larval stage, and the somatic gonad arms arising from these cells remain on the side in which the mother cell was originally placed, resulting in the asymmetric gonad arm handedness described previously (Figure 2c). During later L3 and L4 larval stages, all cells of the somatic primordium (excepting the anchor cell) divide, generating the somatic structures of the adult gonad. These structures are the sheaths (germline components), the spermathecae, (sperm storage) and the unique central uterus (Kimble and Hirsh, 1979). Each spermatheca will then undergo a single left-handed twist following the AP axis. In addition, they will be constantly contorted by the muscular contractions of nearby
somatic gonad structures (Sulston and Horvitz, 1977; Kimble and Hirsh, 1979; Sulston et al., 1983). In summary, hermaphrodites have two gonad arms, elongated on opposite sides of the body (anterior–right/posterior–left) (Figure 2c). Each gonad arm is connected mid-ventrally, with a constant oocyte production at the gonadal tips and a limited storage of sperm next to the connecting uterus.

In order to determine whether natural reversal of the LR orientation of the gonad occurs in hermaphrodites as well as males, an initial experiment was performed on a small number of isolates (data not shown). Although several isolates never showed reversals for hermaphrodites, I initially observed the aberrant positioning of the gonad relative to the intestine in isolates JU778 and MY16. This example of hermaphrodite heterotaxy is observed in three different classes: heterotaxy involving only the anterior gonad arm (“anterior heterotaxy” Figure 2f); heterotaxy involving only the posterior gonad arm (“posterior heterotaxy” Figure 2e); and heterotaxy involving both gonad arms (“complete reversal” Figure 2d).

2.2.2 Heterotaxy in C. elegans hermaphrodite population

What is the prevalence of heterotaxy in C. elegans hermaphrodite population? As described afterward, a small experiment involving ten C. elegans isotypes (Alcorn et al., 2016) detected heterotaxy in a number of strains. In order to assess the variation in heterotaxy more broadly across C. elegans strains, and to begin to dissect the possible genetic and environmental effects on heterotaxy, a much larger study was required. We have at our disposal a collection of isolated natural C. elegans population from around the world known as isotypes or wild isolates (Félix and Braendle, 2010), all of which are genetically sequenced or genotypes (Andersen et al., 2012).
The specific heterotaxy phenotype and the focus of this chapter will be the arrangement of the gonad arms relative to the intestine. As described above, three different hermaphrodite phenotypes can result from gut/gonad heterotaxy: anterior heterotaxy, posterior heterotaxy and complete reversal (Alcorn et al., 2016) (Figure 2f, e, d). The objective of this experiment is to perform a quantitative study of a sample of 91 wild isolates to investigate the prevalence of gut/gonad heterotaxy. In other words; what is the variation of hermaphrodite LR gut/gonad heterotaxy across the C. elegans wild populations?

2.2.3 Results - Wild isolates hermaphrodite scoring

I found that, among the 91 wild isolates scored, there is widespread variation in the propensity of gut/gonad heterotaxy in hermaphrodites (Figure 3). 17 wild isolates never showed heterotaxy individuals - 100% of the animals scored (sample size per strain 171-737) showed normal dextral gut/gonad configuration. These include the N2 laboratory strain, which has been previously reported to present high-fidelity development (Wood and Kershaw, 1991; Wood, Bergmann and Florance, 1996; Bergmann et al., 2003). Most wild isolates showed some variation of gut/gonad heterotaxy ranging up to 10.5% (MY16 strain; n=53/504) (Table 1).

Importantly, we observed that the three classes of gut/gonad heterotaxy in hermaphrodites had significantly different prevalence within each wild isolate and within the C. elegans population. Those classes involving only one gonad arm (anterior and posterior heterotaxy) were significantly more prevalent than those involving both (Complete Reversal) (**p<0.0001, based on T-test). Additionally, posterior heterotaxy is significantly more frequent than anterior heterotaxy (**p<0.0001, based on T-test). This
result suggests that the event leading to these reversals may be occurring independently on each gonad arm.
Variation in LR organ arrangement across the C. elegans population

**Figure 3.** Percentage of LR gut/gonad heterotaxy in L4 hermaphrodites in the different natural wild isolates grown at 20°C. Each wild isolate is labelled below and sorted on total heterotaxy frequency observed. The sample size per wild isolate ranges from 171 to 737 (Average 236). The frequency of each class of heterotaxy represented within each column by colour code. The sum of the three classes of heterotaxy represent the total frequency of gut/gonad abnormalities in L4 hermaphrodites.
Variation in LR organ arrangement across the *C. elegans* population

Table 1. Complementary data for Figure 3. The propensity for L/R gut/gonad reversals varies widely in hermaphrodites of *C. elegans* isolates at 20°C. Isolates are sorted in ascending order.

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Variation in LR organ arrangement across the *C. elegans* population

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2.3 MY16 vs N2 RILs SHOW GREAT VARIATION IN HERMAPHRODITE LR GUT/GONAD HETEROTAXY

The phenotypic variation we observe in LR gut/gonad heterotaxy appears to be widespread in the natural population of *C. elegans* represented in the wild isolates (Callander *et al.*, 2014; Alcorn *et al.*, 2016) (Figure 3). In spite of the great toolkit that the set of wild isolates represents, several recent studies rely on the use of Recombinant Inbred Lines (RILs) or Recombinant Inbred Advanced Intercross Lines (RIAILs). This new toolkit for dissecting quantitative traits in *C. elegans* relies on the genetic information of just two parental strains. While it may not represent the rich heterogeneity of natural wild isolates, it allows a finer mapping for developmental biology analysis (Ayyadevara *et al.*, 2001; Li *et al.*, 2006; Kammenga *et al.*, 2008; Harvey, Shorto and Viney, 2009; Rodriguez *et al.*, 2012). An example of the use of RILs in *C. elegans* developmental studies can be found in a study by Ayyadevara and colleagues (Ayyadevara *et al.*, 2001), where an intercross of the strain RC301 and N2 was used to study nematode longevity. Using RILs, the authors successfully identified several significant QTL, most of which were not observed in previous crossings. Also, recombinant inbred strains obtained from the crossing of N2 and CB4856 (Hawaiian strain) were used in a study of gene expression patterns comparing phenotypic characteristics such as body size, lifespan and reproduction (Li *et al.*, 2006). The use of recombinant lines allowed the authors to generate the first comprehensive map of the genetic polymorphisms underlying differences in expression plasticity. Further, the use of recombinant lines has allowed important advances in the study of the genetic and molecular control of the dauer larvae development (Harvey, Shorto and Viney, 2009). The authors analysed N2 vs DR1350 RILs as they represent the highest significant difference in plasticity of dauer formation. Using
these, the authors determined how dauer development and population growth varied among RILs and mapped the QTL that have a significant influence on them.

In summary, the use of recombinant lines entails a great improvement in dissecting genetic traits in developmental studies in *C. elegans* compared to studies restricted to the natural *C. elegans* wild isolates. Importantly, recombinant lines allowed several authors to successfully identify significant QTL influencing the observed phenotype.

### 2.3.1 Creating RILs

The creation of RILs starts with the selection of two natural wild isolates, which are generally selected as representatives of two genetic or ecological extremes of *C. elegans*, *i.e.*, high vs low expression of a gene; high vs low prevalence of a given phenotype; positive vs negative observation of a trait; etc. (Rockman and Kruglyak, 2008, 2009). Once the two candidate strains are selected, they are crossed. As a general rule, one hermaphrodite from one isolate is crossed with 1-4 males from the other isolate. Then, after 2 generations, around 100 F2s hermaphrodites are individually isolated and let to self-replicate for 10 generations or more. Single worm isolation is performed at every generation. This long self-replication process allows the consolidation of Recombinant Inbred Lines (RILs) which carry two identical copies of a unique combination of parental genomes (*Figure 4*).

### 2.3.2 N2 vs MY16 RILs

As described before, we observe a great variation in LR gut/gonad reversal among 91 wild isolates. The two extremes for this phenotype are strains N2 and MY16, with total heterotaxy frequencies of 0.00% and 10.5% respectively (**p<0.0001 based on Fisher’s...**
exact test). A genetically unique 95-set of RILs obtained from a N2 hermaphrodite and a
MY16 male was previously created by the Rothman Lab (UCSB, USA) for another study.
Hence, I have taken advantage of this set of RILs created from two phenotypic extremes
to characterize the variation in gut/gonad heterotaxy.

2.3.3 Results - RILs hermaphrodite scoring

The 99 RILs that were scored showed a widespread variation in the propensity of
gut/gonad heterotaxy in hermaphrodites (Figure 5) (Table 2). Seven RILs showed 100%
prevalence of dextral gut/gonad configuration (0.0% heterotaxy; sample size 141-507),
comparable to the parental strain N2. Most RILs (89) showed variation of gut/gonad
heterotaxy ranging from the N2 value at 0.0% to not significantly different from the other
parental strain MY16 (values up to 11.7%). Furthermore, and importantly, three RILs
showed gut/gonad heterotaxy frequencies significantly higher than the parental strain
MY16: RILs JR3512, JR3533, and JR3513 showed total frequencies of heterotaxy of 15.4%
(78 in 507; *p<0.005 based on Fisher’s exact test), 17.0% (86 in 505**p<0.001 based on
Fisher’s exact test), and 18.8% (95 in 505; ***p<0.0001 based on Fisher’s exact test for
JR3513) respectively (Figure 5). The formation of these extreme phenotypes, known as
transgressive segregation, will be considered in the discussion of this chapter.

Similarly to the natural wild isolates study, we observed that those classes of
gut/gonad heterotaxy involving only one gonad arm were significantly more prevalent
than those involving both (***p<0.0001, based on T-test). Additionally, posterior
heterotaxy was observed to be significantly more frequent than anterior heterotaxy
(**p<0.0001, based on T-test). This is additional evidence supporting an independence
of anterior-posterior gonad arm development.
Variation in LR organ arrangement across the *C. elegans* population

**Figure 4.** Schematic of creation of recombinant inbred lines (RILs). Starting with a simple cross between two phenotypically distinct parental strains (A and B), 100 distinct F2 recombinants are isolated and RILs are created by selfing for ~10 generations.
Variation in LR organ arrangement across the C. elegans population

Figure 5. Percentage of LR gut/gonad heterotaxy in L4 hermaphrodites in the different N2vsMY16 RILs grown at 20°C. Each RIL is labelled below and sorted on total heterotaxy frequency observed. The sample size per wild isolate ranges from 141 to 507 (average 219). The frequency of each class of heterotaxy represented within each column by colour code. The sum of the three classes of heterotaxy represent the total frequency of gut/gonad abnormalities in L4 hermaphrodites. The wild isolate MY16 gut/gonad heterotaxy frequency of 10.52% (sample size 504) is represented with the red line. The N2 gut/gonad heterotaxy frequency of 0.00% (sample size of 300) not shown. Transgressive segregation is shown by RILs JR3512, JR3533, and JR3513, with frequencies of gut/gonad heterotaxy statistically greater based on Fisher’s exact test than the parental strain MY16 (*p<0.005 for JR3512; **p<0.001 for JR3533; and ***p<0.0001 for JR3513).
**Table 2.** Complementary data for Figure 5: The propensity for L/R gut/gonad reversals varies widely in hermaphrodites of C. elegans MY16 vs N2 RILs at 20°C. RILs are sorted in ascending order.

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### Variation in LR organ arrangement across the C. elegans population

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2.4 HERMAPHRODITE HETEROTAXY IS INDEPENDENT OF EMBRYO CHIRALITY

Similarly to what has been previously done in *C. elegans* males (Callander *et al.*, 2014), the overall anatomical LR asymmetry needs to be assessed to confirm that the abnormal LR gut/gonad positioning is indeed heterotaxy and not a result of full embryo chirality reversal. As previously explained in Chapter 1 that embryo chirality is established in the 4-cell stage embryo, where ABa and ABp cells divide skewed along the LR axis in a clock-wise direction (from a dorsal view), producing a dextral animal with a stereotyped LR organ and cell organization (Wood, 1991) (*Figure 1d*). Previous studies (Callander *et al.*, 2014) took advantage of the predicted gcy-5 expression by the ASER and ASEL neurons. To assess whether the gut/gonad reversals we observe are independent of the embryo chirality, I took advantage of the predicted LR asymmetry in the position of the coelomocytes.

2.4.1 Coelomocytes as LR asymmetry markers

Coelomocytes are macrophage-like cells distributed in three pairs in the *C. elegans* pseudocoelom. Their primary function is endocytosis and acting as a primitive immune system of the animal (Fares and Greenwald, 2001). Two pairs of coelomocytes are located on the ventral left, near the vulva, and another pair is located dorsally, in the posterior side of the body. Our interest focuses on the first pair of ventral coelomocytes, which are located on the right side of the pseudocoelom, for both males and hermaphrodites, between the posterior end of the pharynx and the anterior end of the anterior gonad arm (Sulston and Horvitz, 1977) (*Figure 6b, c*). Coelomocytes ccAR and ccPR are used here as an internal marker of the overall chirality of the animal, as has been done previously (Wood, Bergmann and Florance, 1996), and they can be easily examined in anesthetized animals using Nomarski microscopy. Some animals carrying a *gpa-16(ts)* mutation,
present *situs inversus totalis*, i.e., a complete LR reversal of all organs and cells (Bergmann *et al.*, 2003). As a result, the gut/gonad arrangement is sinistral and I observed that the anterior coelomocyte pair (ccAR and ccPR) was invariably located on the left in ten such LR reversed *gpa-16(ts)* animals observed (*Figure 6a*). In contrast, I analysed coelomocyte position in more than 43 animals from 10 different isolates (including dextral, anterior heterotaxy, posterior heterotaxy and complete reversal animals) and found an invariant ccAR and ccPR location on the right side of the pseudocoelom (*Figure 6d, e*). These observations strongly suggest that LR gut/gonad heterotaxy in both hermaphrodites and males occur independently of the anatomical handedness of the animal established in early embryo development.
Figure 6. DIC images showing relative position of the anterior pair of coelomocytes ccAR and ccPR (red) and the gonad arm (yellow). All animals are shown in a ventral view with anterior up. Adjacent cartoons allow a general reference a) gpa-16(it143) hermaphrodite with situs inversus totalis. Both anterior gonad arm and coelomocyte pair are on the left side. b) Dextral (normal) hermaphrodite from isolate JU778. c) Dextral (normal) male from isolate JU778. d) Complete reversal hermaphrodite from isolate JU778. Coelomocyte pair remains on the right side following embryo chirality while the anterior gonad arm is on the left side. e) Reversal male from isolate JU778. Coelomocyte pair remains on the right side following embryo chirality while the unique gonad arm is on the left side.
2.5 HERMAPHRODITE HETEROTAXY IS A TEMPERATURE SENSITIVE EVENT

An initial study involving ten wild isolates (JU1088, CX11292, JU360, JU1530, DL200, CX11315, JU778, JU310, ED3012 and MY16) was performed to assess if hermaphrodite heterotaxy was significantly more frequent at 25°C than at 20°C (Alcorn et al., 2016). My results showed that this was the case, as was particularly evident for the isolates JU1088, JU1530 and JU778 (*p<0.05; **p<0.01; ***p<0.001 respectively, based on Fisher’s exact test) (Table 3) (Figure 7a). This may be evidence suggesting that the gonad migration process is temperature sensitive. In most of the isolates studied, the temperature-dependant LR gut/gonad reversals was not significantly higher, and in some cases it even showed a slightly lower rate of reversals at 25°C (although these cases may simply reflect imprecision in the estimates given the low sample size). In summary, there is a complex relationship between the LR gut/gonad establishment and temperature.

**Table 3. Selection of 10 wild isolates showing total frequencies of hermaphrodite gut/gonad heterotaxy. Comparison between 20°C and 25°C.**

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</tr>
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<td>20/550; 3.64%</td>
</tr>
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<td>47/432; 10.88%</td>
</tr>
<tr>
<td>MY16</td>
<td>24/200; 12.00%</td>
<td>40/541; 7.39%</td>
</tr>
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</table>

The observation by which the frequency of LR gut/gonad heterotaxy in hermaphrodites of at least some strains is positively influenced by temperature increase,
Variation in LR organ arrangement across the C. elegans population gives us the opportunity to assess and identify the developmental stage at which the temperature sensitivity occurs. Hence, isotype JU778 was selected, as it is a wild isolate that revealed to be among the strains with the highest rates of frequency of gut/gonad heterotaxy at both 20°C and 25°C (4.5%; 35 in 772 and 14.7%; 85 in 575 respectively) and because the difference was the most substantial (**p<0.001 based on Fisher’s exact test) among the ten isotypes studied (Figure 7a) (Table 3).

2.5.1 Results - Temperature shift

JU778 animals were continuously cultured at either 20°C or 25°C for two generations. At the point at which the first embryos could be observed inside the body of the young adults, these individuals were either down-shifted or up-shifted to 20°C or 25°C. Embryos were left to hatch and grow at the shifted temperature until L4 stage, where LR gut/gonad heterotaxy in hermaphrodites was scored.

Results showed that JU778 hermaphrodites developed from embryos continuously grown at 20°C and those up-shifted pre-gastrulation to 25°C showed similar rates of gut/gonad heterotaxy (4.5%; 35 in 772; and 4.7%; 14 in 300 respectively) (**p>0.05 based on Fisher’s exact test). On the other hand, JU778 hermaphrodites developed from embryos continuously grown at 25°C and those down-shifted pre-gastrulation to 20°C showed similar rates of gut/gonad heterotaxy (14.8%; 85 in 575; and 15.5%; 39 in 251 respectively) (**p>0.05 based on Fisher’s exact test) (Figure 7b).

Thus, there is evidence that the temperature-sensitive molecular and cellular events that lead to LR gut/gonad heterotaxy in hermaphrodites occurs before gastrulation, in either early embryogenesis or even in the maternal germline (Figure 7c).
Figure 7. Temperature effect on hermaphrodite heterotaxy. a) Temperature effect on a subset of ten C. elegans isolates cultured at 20°C and 25°C. The frequency of each different heterotaxy phenotype is represented. Sample size is noted above each column. Fisher exact test was used to compare frequencies of heterotaxy for each isolate. (*p<0.05, **p<0.01, ***p<0.001). b) Temperature sensitive period influencing heterotaxy coincides with pre-gastrulation embryo development. Embryos continuously cultured at either 20 or 25°C show similar rates of heterotaxy to those up-shifted or down-shifted pre-gastrulation to 25 or 20°C respectively. Pie charts show the proportion of hermaphrodite heterotaxy (turquoise), with the percentage of hermaphrodites and the sample sizes of hermaphrodite and normal dextral embryos indicated above. c) Schematic of C. elegans development timeline highlighting developmental period at which temperature has a significant effect on heterotaxy. Key developmental events and timing are included when relevant.
Variation in LR organ arrangement across the C. elegans population

2.6  **C. ELEGANS HETEROTAXY IS INDEPENDENT OF MATERNAL EFFECT**

We observed that the temperature-sensitive period for hermaphrodite LR gut/gonad heterotaxy in JU778 occurs before embryo gastrulation, prior to the 26-cell stage (Sulston *et al.*, 1983) (Figure 7c). To assess whether this event is likely determined as a response to events in early embryogenesis (zygote-26cell stage) or perhaps in the maternal germline (epigenetics), two isotypes showing extreme rates for LR gut/gonad heterotaxy (low heterotaxy vs high heterotaxy) were crossed. This cross was made in both sex directions, to assess if the identity of the mother influences the LR gut/gonad heterotaxy of the progeny.

2.6.1  **Absence of maternal effect on JU778 natural isotype**

Isotypes JU778 and N2 were selected for this experiment, and populations rich in males were generated. L4 hermaphrodites and L4 males were picked and the following crosses were established with a 20♀:40♂ ratio. 1) JU778♀ vs N2♂; 2) N2♀ vs JU778♂ 3) N2♀ vs N2♂ 4) JU778♀ vs JU778♂. Both males and hermaphrodites in the F1 were isolated at L4 stage and LR gut/gonad heterotaxy was scored.

Results for the **male** F1 showed that cross 4 (JU778♀ vs JU778♂) presented a significantly higher frequency of LR gut/gonad heterotaxy compared to cross 3 (N2♀ vs N2♂) (9.4%; 9 in 96 and 0.0%; 0 in 100 respectively; **p<0.01 based on Fisher’s exact test). Crosses 1 and 2 (JU778♀ vs N2♂; N2♀ vs JU778♂), showed similar rates of heterotaxy in male F1 (2.4%; 2 in 84 and 2.2%; 2 in 92 respectively; n.s.p>0.05 based on Fisher’s exact test) (Figure 8b).
Similarly, results for the hermaphrodite F1 showed that cross 4 presented a significantly higher frequency of LR gut/gonad heterotaxy compared to cross 3 (4.9%; 5 in 102 and 0.0%; 0 in 100 respectively; *$p<0.05$ based on Fisher’s exact test). And in the same way as for males, crosses 1 and 2 showed similar rates of hermaphrodite heterotaxy (2.9%; 3 in 103 and 3.6%; 4 in 111 respectively; $^{nsp}>0.05$ based on Fisher’s exact test) (Figure 8a).

These results suggest that the identity of the maternal germline in JU778 does not have an influence on LR gut/gonad heterotaxy of the progeny in neither males nor hermaphrodites. Therefore, it appears that the cellular and molecular mechanisms determining the adult gut/gonad body plan occur in early embryogenesis, more precisely between fertilization and the 28-cell stage. Also, as a similar effect is observed in both males and hermaphrodites, their different gut/gonad development may share a common (yet unknown) determining mechanism.

2.6.2 Absence of maternal effect in the MY16 natural isotype

In order to assess if the absence of maternal effect on gut/gonad heterotaxy is restricted to the JU778 wild isolate or is shared with other wild isolates, a similar experiment was performed with the MY16 wild isotype, which previous results was shown to have the highest frequency of gut/gonad heterotaxy at 20°C (Alcorn et al., 2016) (Figure 7a). The crosses were as follow: 1) MY16♀ vs N2♂; 2) N2♀ vs MY16♂ 3) N2♀ vs N2♂ 4) MY16♀ vs MY16♂. In this case, only F1 hermaphrodites were scored for gut/gonad heterotaxy.

Results for the hermaphrodite F1 of MY16 vs N2 showed that cross 4 presented a significantly higher frequency of LR gut/gonad heterotaxy compared to cross 3 (10.5%;
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53 in 504 and 0.0%; 0 in 200 respectively (*p<0.05 based on Fisher’s exact test). And similarly, crosses 1 and 2 showed similar rates of hermaphrodite heterotaxy (7.6%; 19 in 251 and 8.7%; 19 in 219 respectively) (ns p>0.05 based on Fisher’s exact test) (Figure 8c).

This result supports the previous observation for JU778 vs N2, by which the identity of the maternal germline does not have an effect on LR gut/gonad heterotaxy of the hermaphrodite’s progeny, restricting the LR gut/gonad determinants to genes acting during early embryogenesis. Moreover, a comparable effect is observed in two distinct wild isolates crossed with the same reference N2 strain, suggesting a conserved pathway for LR gut/gonad organogenesis among natural wild isolates.
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Figure 8. Evidence of absence of parental effect on LR gut/gonad heterotaxy for both males and hermaphrodites for crossings of wild isolates JU778 vs N2 and MY16 vs N2. “H” stands for “hermaphrodite” and “M” stands for “male”. Sample size is noted above each column.

a) JU778 shows significantly higher gut/gonad heterotaxy frequency than N2 (*p<0.05 based on Fisher’s exact test). However, both N2 vs JU778 crossings show similar rates of total heterotaxy independently of the direction of the crossing (ns p>0.05 based on Fisher’s exact test).

b) Similarly, JU778 males show significantly higher gut/gonad heterotaxy frequency than N2 males (**p<0.01 based on Fisher’s exact test). However, results for N2 vs JU778 both direction crossings in males show similar rates of total heterotaxy independently of the direction of the crossing (ns p>0.05 based on Fisher’s exact test).

c) MY16 shows significantly higher gut/gonad heterotaxy frequency than N2 (*p<0.05 based on Fisher’s exact test). However, both N2 vs MY16 crossings show similar rates of total heterotaxy independently of the direction of the crossing (**p>0.05 based on Fisher’s exact test).
2.7 GUT/GONAD HETEROTAXY IN MALES AND HERMAPHRODITES APPEAR TO BE INDEPENDENTLY DETERMINED

As described above, we previously reported a high variation across the wild isolates in terms of LR gut/gonad reversal in the *C. elegans* population (Alcorn *et al.*, 2016) (Figure 3, Figure 5). The 100 wild isolates studied showed frequency of male reversals varying from 0.0% to 11.2%, while in hermaphrodites, the variation among 91 wild isolates ranged from 0.0% to 10.5% (Figure 3). The general similarity in distribution and obvious phenotypic resemblance initially support the possibility of a shared underlying cause of gut/gonad heterotaxy affecting both males and hermaphrodites.

Further consistent with a shared cellular and molecular mechanism was that both the male and hermaphrodite LR gut/gonad heterotaxy frequency is generally increased under temperature stress (Callander *et al.*, 2014; Alcorn *et al.*, 2016). For example, the JU778 wild isolate shows a frequency of male heterotaxy of 9.9% and 16.3% at 20°C and 25°C respectively (**p<0.0001 based on Fisher's exact test). Similarly, JU778 also shows a total hermaphrodite heterotaxy of 4.5% and 13.7% at 20°C and 25°C respectively (**p<0.0001 based on Fisher's exact test). This sensitivity to temperature stress is observed in most natural wild isolates studied (Alcorn *et al.*, 2016).

Additionally, in section 2.5, I reported evidence suggesting that gut/gonad heterotaxy in both males and hermaphrodites may share a common determining mechanism. Evidence of a pre-gastrulation temperature sensitive period led us to assess if the underlying cause was restricted to embryo development (zygote-gastrulation) or occurred before, in the maternal germline. We concluded that the cellular and molecular mechanisms determining the adult gut/gonad body plan occur in early embryogenesis,
between fertilization and 28-cell stage, and the equivalent results were obtained for both males and hermaphrodites.

In order to assess the correlation between LR gut/gonad heterotaxy in males and hermaphrodites, we performed a Kendall rank correlation coefficient (Kendall, 1938) to measure the strength of the relationship between the two variables. Comparing the same subset of wild isolates for both males and hermaphrodites, our results show that, in fact, there is no association in the prevalence of heterotaxy in males and hermaphrodites, with no significant rank correlation between the variables (Kendall's tau coefficient \( \tau = -0.017; \quad ^{ns} p = 0.837 \)). These results suggest that although male and hermaphrodite gut/gonad heterotaxy events may appear to share similar characteristics, they may be directed by independent genetic processes.

2.8 EFFECT OF STRESS ON GUT/GONAD HETEROTAXY

The study of \(~44,000\) individual worms from different natural wild isolates and RILs provided evidence of widespread variation in LR gut/gonad heterotaxy in *C. elegans* hermaphrodites. Some strains show very low frequencies of gut/gonad heterotaxy, while in others \(~1\) in \(10\) animals contained at least one gonad arm in the “wrong” position. Is it possible that gut/gonad heterotaxy events observed in *C. elegans* hermaphrodites are just a reflection of the general stress susceptibility of the animal? Are those strains with high gut/gonad heterotaxy also those that are more sensitive to biological stress?

2.8.1 Biological stress

Biological stress is the response of an organism to an environmental condition (Ulrich-Lai and Herman, 2009). Environmental factors that cause alterations in homeostasis are perceived as stress. Biologically speaking, a stress factor is an
environmental element or internal input that is perceived by the organism as a life-threatening situation. General examples of stressing factors include physical trauma, starvation, extreme temperatures, radiation, dehydration, etc. (Goldstein and Kopin, 2007). Upon the animal’s perception of a stress factor, the organism will attempt to restore conditions to normal homeostasis. This process, often consuming large amounts of energy, is known as stress response (Segerstrom and Miller, 2004).

*C. elegans* has been used to study of biological stress, for example in germline survival and apoptosis (Gartner, Boag and Blackwell, 2007). Germ cells form the tissue responsible for reproduction and that give rise to the next generation of animals. Importantly, the *C. elegans* germline is the only tissue that undergoes apoptosis in an indeterminate way, as an intrinsic part of the oogenesis program (rather than controlled by cell lineage) (Sulston and Horvitz, 1977). A broadly conserved phenomenon in metazoans is apoptosis induced in response to genotoxic stress, as observed in the *C. elegans* germline (Gartner et al., 2000). When the *C. elegans* germline is exposed to genotoxic stress, cells activate checkpoint pathways which may lead to increased apoptosis of damaged cells. Other environmental stress factors may not directly compromise genomic integrity, but nonetheless trigger germ cell apoptosis. Examples of these factors are particular pathogens, oxidative stress, temperature extremes, osmotic stress or starvation. Germline apoptosis by these stress factors do not require the classical apoptosis pathway involving *egl-1* or *cep-1* (Conradt and Horvitz, 1998) suggesting the existence of independent stress response pathways depending on the type of stress.

While germline apoptosis is an extreme response to a stress factor, there is also evidence of the sensitivity of germline function to temperature fluctuations (David et al.,
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2005; Harvey and Viney, 2007; Prasad et al., 2011; Kim, Park and Rhee, 2013). *C. elegans* fertility is highest at 20°C and exponentially declines until total sterility is observed at ~27°C (Gupta, 2007). But between healthy fertility at 20°C and sterility at ~27°C, variation in germline function is observed (Petrella, 2014). While some *C. elegans* strains rapidly decrease fertility as temperature increases, other strains are more tolerant to temperature stress, suggesting that the temperature-dependent loss of fertility in *C. elegans* is a complex trait for both hermaphrodites and males. Interestingly, there is no obvious relationship between the geographical origin of a strain and its environmental response. That is, wild isolates whose origin is from lower latitudes (tropical environment) do not tend to show greater tolerance to temperature stress compared to strains from higher latitudes (cold environment) (Petrella, 2014).

Stress is a natural response in metazoans, and its biological function includes evolutionary benefits. Typically, lifespan-extension mutations confer a fitness reduction compared to wild-type strains. Hence, mutations that promote longevity are unlikely to become established in wild populations (Kirkwood, 1995). However, there is evidence that stressful environments indirectly select for increased lifespans (Savory et al., 2014). Mutants that are defective for the conserved insulin/IGF-1 signalling pathway are observed to be long-lived and present increased tolerance to temperature stress. The authors concluded that certain lifespan-extension mutations confer on *C. elegans* enhanced selective advantage in stressful environments (Savory et al., 2014).

In summary, stress is a natural response in metazoans as a strategy to restore homeostasis conditions. Temperature stress tolerance has shown variation among natural *C. elegans* wild isolates from around the world. In some situations, stress adaptation could entail an enhanced selective advantage compared to wild-type strains.
2.8.2 Results - Stress influence on LR gut/gonad heterotaxy in MY16 animals

From the study of the wild isolates, MY16 was found to show the highest frequency of gut/gonad heterotaxy (10.5%; n=53/504). Does MY16 show reduced tolerance to stress? In order to assess this question, MY16 animals were cultured under different stressful conditions and LR gut/gonad heterotaxy was scored and compared to the normal gut/gonad heterotaxy frequency described above. The stress factors selected were: temperature stress, starvation, constant movement, and oxidative stress.

I found that LR gut/gonad heterotaxy in MY16 animals remains constant regardless of the stress factors animals have been exposed to (Figure 9a). No statistical difference (ns p>0.05 based on Fisher’s exact test) in gut/gonad heterotaxy was observed compared to normal MY16 frequency in either 15°C temperature stress (10.3%; 31/301), 25°C temperature stress (9.1%; 58/641), starvation (8.9%; 18/203), constant movement (11.5%; 23/200), or oxidative stress (8.0%; 16/201).

2.9 STRESS VARIATION IN DEXTRAL/HETEROTAXY ANIMALS

As described previously, LR gut/gonad heterotaxy is represented by an abnormal positioning of the two major organs of C. elegans animals in the body cavity. These organs represent ~80% of the animal total volume (Sulston and Horvitz, 1977), hence any aberrant positioning affecting these organs it is likely to affect the normal healthy state of the animal. Human syndromes associated with heterotaxy lead to a decrease in quality of life (Strouse, 2004; Burn and Hill, 2009; Sutherland and Ware, 2009). Do C. elegans animals that present aberrant gut/gonad organogenesis suffer from defects in structure or function? To evaluate this question, the JR3513 RIL was selected, as it is the strain among all natural wild isolates and among the MY16 vs N2 RILs that shows the highest
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frequency of gut/gonad heterotaxy (18.8%; 95/505). Health indicators selected were lifespan, number of embryos laid and progeny viability.

2.9.1 Results – Stress Variation

JR3513 animals were normally cultured at 20°C and L4 synchronized populations were scored for gut/gonad heterotaxy. 16 dextral L4s and 16 “heterotaxy” L4s were isolated and systematically transferred to fresh plates for a 24h period until the end of the experiment. Every day, death/survival, number of embryos laid, and % of those embryos that hatched into a living worm was assessed.

Results show that there was no statistical difference between the total number of embryos laid between dextral and heterotaxy JR3513 animals (ns p>0.05 based on a Chi$^2$ test) (Figure 9d). Progeny viability (% of embryos that hatched into a living worm) also showed similar results between dextral and heterotaxy JR3513 animals (ns p>0.05 based on a Chi$^2$ test) (Figure 9c). Finally, no significant difference was observed between the mean lifespan of dextral and heterotaxy JR3515 animals (ns p>0.05 based on a Chi$^2$ test), although some dextral animals appear to live ~5 days longer than the longest lived worms that showed heterotaxy (Figure 9b).

In conclusion, there is no statistical difference in fitness or lifespan between dextral and heterotaxy JR3515 animals.
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Figure 9. Effect of stress on MY16 and JR3513. a) MY16 LR gut/gonad heterotaxy is independent of stress factors. 5 different stressful situations (in red) described below each column are compared to the normal hermaphrodite heterotaxy frequency at 20°C (in green) the difference is non-significant in every case (p>0.05 based on Fisher’s exact test). Sample size is noted above each column. b) Survival of JR3513 animals is independent of LR gut/gonad heterotaxy (p>0.05 based on Chi square test). c & d) JR3513 dextral and heterotaxy animals show similar progeny viability (c) and total number of embryos laid (d) (p>0.05 based on Chi square test). e & f) Comparison of progression of egg-laying in JR3513 dextral (e) and heterotaxy (f) animals through time.
2.10 STRESS VARIATION IN HERMAPHRODITES IS INDEPENDENT OF LR GUT/GONAD HETEROTAXY

There is strong evidence supporting variation in temperature stress tolerance in *C. elegans* wild isolates (Petrella, 2014). Under the same stress factor (high temperature) different isotypes respond differently. This response can be quantified by measuring animal fertility as a proxy for the fitness of the animals. In this section, the question that will be addressed is whether variation in LR gut/gonad heterotaxy in hermaphrodites depends on the temperature stress tolerance among the RILs obtained from crosses of MY16 and N2.

Four RILs were selected for this experiment based on their frequency of gut/gonad heterotaxy, with two RILs showing low frequency of heterotaxy (JR3564; 0.0%; 0 in 200 and JR3568; 0.0%; 0 in 201) and two RILs showing high frequency of heterotaxy (JR3513; 18.8; 95 in 505 and JR3533; 17.0; 86 in 505) (Figure 5). *C. elegans* has been shown to present highest fertility and fitness at 20°C reaching sterility at ~27°C (Petrella, 2014). To quantify fertility and survival, animals were normally cultured at 20°C for two generations, and L4 hermaphrodites were then shifted to target temperatures of 20, 25, 26 and 27°C for a total period of 16 days.

2.10.1 Results - Fertility

No significant differences were observed between the selected RILs in the number of embryos laid at 20, 25, 26 or 27°C (Figure 10a) ($p>0.05$ based on Chi square test). The average total number of embryos laid per worm until sperm depletion at 20°C were 130.5, 89.6, 108.6, and 118.0 for JR1513, JR3533, JR3564, and JR3568 respectively. At 25°C results were similar among RILs (76.8, 54.9, 58.2, and 49.3 for JR1513, JR3533, JR3564, and JR3568 respectively). At 26°C results were also similar among RILs (23.9,
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24.9, 56.9, and 24.4 for JR1513, JR3533, JR3564, and JR3568 respectively). Finally, at 27°C results were also similar among RILs (3.1, 10.5, 12.5, and 1.7 for JR1513, JR3533, JR3564, and JR3568 respectively). Although the embryo yield decreased with temperature, there is no apparent difference in temperature tolerance between high/low heterotaxy RILs at a given temperature.

Similarly, progeny viability was negatively influenced as temperature was increased, but same temperature tolerance was observed in high/low heterotaxy RILs (Figure 10b). Average progeny viability at 20°C was 79.6, 93.3, 95.6, and 93.9% for JR1513, JR3533, JR3564, and JR3568 respectively. At 25°C, viability was 83.5, 90.3, 86.5, and 79.1% for JR1513, JR3533, JR3564, and JR3568 respectively. At 26°C it was 52.7, 74.1, 76.2, and 68.3% for JR1513, JR3533, JR3564, and JR3568 respectively. Finally, at 27°C, viability was 0.0% for all four strains. (ns p>0.05 based on Chi square test).

2.10.2 Results - Survival

As expected, survival of JR1513, JR3533, JR3564, and JR3568 was the highest at 20°C, decreasing with temperature increase, and generally reaching the lowest values at 27°C (Figure 11a). When observing the survival of JR1513, JR3533, JR3564, and JR3568 RILs at 20, 25, 26 and 27°C, with a focus on comparing differences between high/low heterotaxy (JR1513-JR3533; and JR3564-JR3568 respectively), I observed no significant differences in survival of animals associated with thermo-tolerance (Figure 11b). In other words. RILs with a high frequency of gut/gonad heterotaxy do not appear to be more thermo-sensitive than RILs with low gut/gonad heterotaxy frequency.
2.10.3 Results - Variation in egg laying and embryo survival

Average egg laying and average % of embryo survival was graphed as function of temperature (Figure 12b). For all RILs included in the study, as culturing temperature increases, average egg laying gradually decreases, while for the same RILs the % of embryo survival remains constant at ~90% and decreases sharply at 26°C reaching 0.0% at 27°C.

2.10.4 Conclusions

Our stress variation experiment results show that high/low frequency of gut/gonad heterotaxy does not appear to be associated with low/high tolerance to temperature stress. RILs presenting high frequency of gut gonad heterotaxy (JR1513 and JR3533) show similar stress response to high temperatures as RILs presenting low frequency of gut gonad heterotaxy (JR3564 and JR3568). Evidence supporting this can be found in no statistical differences in fertility, survival or stress variation among high/low heterotaxy RILs under the same temperature-stress conditions.
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Figure 10. Temperature stress variation on fertility. High-heterotaxy RILs JR3513 and JR3533 are marked in green and low-heterotaxy RILs JR3564 and JR3568 are marked in orange. Every point represents one day of embryo laying. Days without no eggs laid are not represented. Embryo laying calculated as follows: a) Total number of eggs laid is divided by total number of alive animals and then plotted. No statistical difference is seen comparing high-heterotaxy and low-heterotaxy RILs in any case (p>0.05 based on Chi square test). b) Progeny viability calculated as follows: Total number of F1 animals divided the sum of animals plus non-hatched embryos. No statistical difference is seen comparing high-heterotaxy and low-heterotaxy RILs in any case (p>0.05 based on Chi square test).
Figure 11. Impact of temperature stress variation on survival. High-heterotaxy RILs JR3513 and JR3533 are marked in green and low-heterotaxy RILs JR3564 and JR3568 are marked in orange. a) Representation of survival of every RIL included in the study at 20, 25, 26 and 27°C. b) Survival of RILs JR3564, JR3568, JR3513, and JR3533 at 20, 25, 26 and 27°C. High-heterotaxy RILs JR3513 and JR3533 are represented in green and low-heterotaxy RILs JR3564 and JR3568 are marked in orange. No statistical difference is seen comparing high-heterotaxy and low-heterotaxy RILs in any case (p>0.05 based on Chi square test). p value obtained from Chi square test for each temperature represented where applies.
Figure 12. Stress variation experiment. a) Schematic of stress variation experiment performed on RILs JR3564, JR3568, JR3513, and JR3533 at 20, 25, 26 and 27°C. A total of 20 L4 hermaphrodites are transferred from 20°C to the target temperature 20, 25, 26 or 27°C. Alive animals are transferred daily and accounted for survival measure. Embryos laid are let to freely hatch for a 24 hour period and then F1 animals and non-hatched embryos are quantified. Total duration of the experiment is 16 days. b) Egg laying variation under temperature stress. The average of the total number of eggs laid per animal are plotted allowing comparison between high/low-heterotaxy. No difference was observed (p>0.05 based on two way ANOVA test). c) Variation in embryo survival under temperature stress. The averages of progeny viability are plotted allowing comparison between high/low-heterotaxy. No difference observed (p>0.05 based on two way ANOVA test).
2.11 CONCLUSIONS CHAPTER 2

The study of *C. elegans* internal organ arrangement has been extensively studied, and the conclusion of many authors has been that *C. elegans* adopts an invariant body plan, arbitrarily referred as dextral. *C. elegans* males develop the unique gonad arm on the right side of the pseudocoelom, while the intestine occupies the remaining space on the left. On the other hand, *C. elegans* hermaphrodites present the anterior gonad arm on the right side of the pseudocoelom while the posterior gonad arm shifts to the left side. For many years, it was believed that the *C. elegans* population remained constant in this gut/gonad arrangement (Wood, 1991, 1998; Wood and Kershaw, 1991; Wood, Bergmann and Florance, 1996; Bergmann *et al.*, 2003). However, those studies were focused on the laboratory strain N2, and in general, they did not analyse the whole spectrum of *C. elegans* worldwide populations we have at our disposal today.

2.11.1 Variation in LR gut/gonad body plan

This view changed when the Rothman Lab (UCSB, USA) reported the first cases of *situs inversus incompletus* in *C. elegans* – Left-Right gut/gonad heterotaxy. Initially, this was discovered in males from the laboratory strain N2, but it was later extended to a wide range of *C. elegans* natural wild isolates, revealing widespread variation in LR gut/gonad heterotaxy in males, with frequencies of animals showing gut/gonad heterotaxy ranging from 0.0 to 11.1%.

I addressed whether LR gut/gonad heterotaxy could be present in hermaphrodites and discovered that indeed LR gut/gonad heterotaxy is found in natural wild isolates of *C. elegans* in three different heterotaxy phenotypes: posterior heterotaxy, anterior heterotaxy and complete reversal. Thus, *Caenorhabditis elegans* gut/gonad
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development does not always adopt and invariant dextral body plan, but shows substantial variation in this trait.

In this chapter I have made the following key conclusions:

— LR gut/gonad heterotaxy is independent of embryo chirality
— LR gut/gonad heterotaxy is positively influenced by temperature
— LR gut/gonad heterotaxy is affected by an event in early embryo development
— Widespread variation of gut/gonad heterotaxy among wild isolates
— Widespread variation of gut/gonad heterotaxy among N2vsMY16 RILs
— LR gut/gonad heterotaxy appears to be independently determined in males and hermaphrodites
— LR gut/gonad heterotaxy is independent of strain stress tolerance

2.12 DISCUSSION CHAPTER 2

In this chapter I highlight three major insights:

First: I reveal that LR development of the two major organs of Caenorhabditis elegans animals is subject to widespread variation across both wild isolates and MY16 vs N2 RILs. Some wild isolates present isotypes showing normal dextral arrangement while others show frequent gut/gonad reversals (up to 10.5%). Similarly, some RILs present lines showing normal dextral arrangement while others show frequent gut/gonad reversals (up to 18.8%). This reversals are confirmed to be independent of embryonic chirality of the animal established at 6-cell stage embryo. Rather, they are established
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during early embryo development. Thereby, these reversals reflect an abnormal organ
development known as heterotaxy.

Second: temperature has been seen to positively influence the propensity of
gut/gonad heterotaxy in some isolates. Study of isolate JU778 restricted the temperature
sensitive event to a relatively short embryo developmental period from fertilization to
gastrulation. Thereby, a temperature sensitive event occurring in early embryo
development has a direct effect on gut/gonad post-embryonic development, long before
primordial germ cells or intestinal primordium cells are developed. What kind of event
occurring long before gut/gonad organogenesis could have such a strong influence?
Developmental mistakes during their formation will influence the cues that posteriorly
guide gut/gonad organogenesis during the first larval stages of post-embryonic
development.

Third, and contrary to what we expected, gut/gonad heterotaxy occurs
independently in males and hermaphrodites of the same *C. elegans* species. Statistical
comparison of gut/gonad heterotaxy rates of the same sub-set of wild isolates strongly
supports this. Thereby, although they show similar organ rearrangements, have similar
temperature sensitivity, and share developmental characteristics, they must be treated
independently. This implies that genomic regions that influence male gut/gonad
heterotaxy (Alcorn *et al.*, 2016) may not be necessarily shared with those of
hermaphrodites.
Importantly, I found that gut/gonad heterotaxy in hermaphrodites is presented in three morphologically distinctive forms, depending on the combination of abnormal development in the anterior and posterior gonad arms. Posterior heterotaxy is more prevalent than anterior heterotaxy. And complete reversal (reversal of both gonad arms) is much less frequent than those reversals affecting only one gonad arm. This is true for the studied sets of both wild isolates and RILs (Figure 13).

This is the reversals of the two gonad arms occur independently in hermaphrodites. There may be a single determining process influencing the probability of each gonad arm occupying the normal space in the body cavity. In that case, the probability of complete reversal organ would equal the product of the probabilities combined of posterior and anterior heterotaxy, which is what I have observed.

The MY16 vs N2 RILs showed extreme phenotypes, characteristic of transgressive segregation. Three RILs (JR3513, JR3533, and JR3512) presented rates of gut/gonad heterotaxy significantly higher than the high-heterotaxy parental strain MY16 (Figure 5).
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(Table 2). Transgressive phenotypes are usually observed in segregated hybrid populations where a favourable combination of both parents‘ alleles results in a higher prevalence of a quantifiable phenotype (in this case hermaphrodite gut/gonad heterotaxy) (Hegarty, 2012). There are many causes for natural transgressive segregation, such as elevated mutation rate, reduced developmental stability, or epistasis. But in this case, transgressive segregation was the result of the recombination of MY16 and N2 genomes induced by manually-generated crosses (Rockman and Kruglyak, 2008). Evidence of transgressive segregation in MY16 vs N2 RILs may suggest that hermaphrodite gut/gonad heterotaxy is a complex trait, with numerous loci underlying the gut/gonad heterotaxy phenotype. Further analyses will be required to dissect this suggested genetic complexity.

Temperature stress is widely known to lead to developmental errors during both embryo and post-embryonic development in both C. elegans and humans (Segerstrom and Miller, 2004; Goldstein and Kopin, 2007). Temperature stress sensitivity varies among wild isolates (Petrella, 2014). That is, physiological response to the same stressing condition varies from one isotype to another. Variation in gut/gonad heterotaxy observed among wild isolates and RILs could be a reflection of variation in temperature stress sensitivity. That would imply that those strains showing extremes rates of heterotaxy correlated with extreme physiological response to temperature increase.

I found that the high/heterotaxy wild isolate MY16, which is one of the parental strains in which RILs are based, show the same gut/gonad heterotaxy rate regardless the stress input. Further, I found that within a same RIL JR3513, animals presented the same physiological response to temperature increase regardless their internal gut/gonad organisation (dextral/heterotaxy). And finally, I provided strong evidence by which two
groups of RILs with extreme rates of gut/gonad heterotaxy, showed the similar physiological response to temperature stress. These results show that gut/gonad heterotaxy in hermaphrodites is independent of a given strain’s temperature stress sensitivity.

In the course of the stress variation experiments described in section 2.10, I found that JR3513 animals showed similar total number of eggs laid regardless of whether they presented dextral or heterotaxy gut/gonad arrangement (Figure 9d). However, and notably, I observed differences in the rate at which the eggs were laid (Figure 9e, f). Normal dextral JR3513 animals followed a general trend by which embryo laying increased, reaching the egg-laying peak at ~day 3 and then gradually decreased as sperm were depleted. In contrast, heterotaxy JR3513 animals presented a clearly uneven egg-laying rate throughout the experiment. Egg-laying peak-day presented substantial variation among the animals studied, and at least 6 individuals presented a highly atypical egg-laying rate: high on the first days of the experiment, then sharply decrease, then increase again before stopping due sperm depletion. This variation in egg-laying rate observed in animals presenting heterotaxy in at least one gonad arm could be explained by a general lack of health in these abnormal animals. However, this general state is not reflected in the fitness measures studied (progeny viability and survival). Another possible cause could be mechanics: dextral gonad configuration presents the optimum structure for smooth and effective egg-laying. Abnormal positioning of at least one gonad arm could be eliciting embryo jam at vulva stage, like cars in a Y intersection. Further studies need to be done in this direction to assess if gut/gonad heterotaxy has any unforeseen consequences in life factors of these animals.
In this chapter, I focused on the LR asymmetry breaking of the two major organs in *C. elegans*: the intestine and the gonad, which is independent of embryonic chirality but apparently established during early embryo development. Other examples of symmetry breaks independent of embryonic chirality suggest a multiplicity of independent symmetry-breaking events (Poole and Hobert, 2006; Sutherland and Ware, 2009; Downes *et al.*, 2012). This means that several events leading to LR asymmetry breaks may not be controlled by a single or centralised determining factor.

Many LR establishment processes occur throughout *C. elegans* development that are independent of embryo chirality. Understanding of all these apparently independent determining systems is key for understanding variation in bilateral structures behind the widespread variation observed in the development of bilaterian metazoans.

### 2.13 MATERIALS AND METHODS

#### 2.13.1 Scoring of gut/gonad heterotaxy in *C. elegans* males

*C. elegans* animals were cultured under normal conditions (Brenner, 1974). When a population with a significant number of males was obtained, *C. elegans* males were picked individually and placed on an agar pad with 10μL of Levamisole 5mM in order to anesthetize them. After they were totally sedated, *C. elegans* males were moved individually with an eyelash pick and positioned with the ventral side up. Finally they were observed with Nomarski DIC to perform the scoring.

#### 2.13.2 Scoring of gut/gonad heterotaxy in *C. elegans* hermaphrodites

In order to assess the gut/gonad orientation in hermaphrodites, the following procedure was used: *C. elegans* populations were grown at 20°C for a minimum of 2 generations with constant supply of OP50 *E. coli* food. Then, after an egg-prep (Brenner,
Variation in LR organ arrangement across the C. elegans population

1974) embryos were left to freely hatch in M9 solution overnight at room temperature. The next day, synchronized L1 worms were placed in a fresh Nematode growth media (NGM) plate with full food supply. After usually 48h, most populations reached a synchronized stage of L4. The reason this is the larvae stage selected, is because it is the ideal maturity phase in which fully transparent gonad arms can be observed, and it is easier to differentiate from the opaque and rugged intestine. A chemotaxis NGM plate is then prepared. Droplets of 5µL of sodium azide 1M are placed on 3 equidistant points 1 cm from the edges of the plate. Once soaked, 10µL of OP50 E. coli bacteria is pipetted on top. Then, the synchronized population of L4 worms is cleaned of any OP50 residue with water, and 60µL of suspended nematodes is pipetted on the centre of the chemotaxis NGM plate. Animals are then left to freely chemotax towards a spot of OP50 E. coli for 1 hour. Anesthetized worms are then rolled using an eye-lash tool and scored ventral side up on a Motic dissecting scope and imaged at 45X. Real visuals, representations and cartoons of gut/gonad heterotaxy in C. elegans hermaphrodites can be seen in (Figure 14).
This method has been proved to be the most efficient to score the highest number of animals in the fewest time possible (300worms/hour; 5worms/minute). The most significant advantage is that animals become anesthetised separated from one another, i.e., they do not from clusters, significantly decreasing the time spent manipulating each worm to obtain an accurate score.

It is important to keep wild isolates, RILs, and the whole preparation process until animal scoring free of fungi and bacterial contamination. When sporadic cases of contamination appeared, the target sample was set aside from the experiment and cleaned before being reintroduced. Additionally, non-published observations of the
Rothman Lab (UCSB, USA) warn on the ability of *C. elegans* animals to “escape” from their culturing plates. This would create a catastrophic heterogeneity in *C. elegans* inbred populations. Hence, every different wild isotype in a NGM plate was carefully sealed with Parafilm M to avoid any undesirable crossings.

### 2.13.3 Generation of male stocks

The procedure to generate males was adapted from (Lyons and Hecht, 1997). 20-40 L4 hermaphrodites were isolated into a 7% ethanol solution and rotated for 60 minutes. Anesthetised “drunk” animals were then transferred to a fresh NGM plate, with full supply of OP50 *E. coli* food. Males found in the F1 generation were mated with L4 hermaphrodites to establish a male stock.

### 2.13.4 Stress influence on LR gut/gonad heterotaxy in MY16 animals

Temperature stress was achieved by culturing MY16 animals at either 15°C or 25°C for two generations and scoring gut/gonad heterotaxy in the F3. Starvation was achieved by letting MY16 animals run out of food and become arrested in dauer state for 15 days. After that period dauer animals were re-introduced to the food supply and the F1 let to run out of food and enter dauer state again for 15 more days. Finally, F1 dauer animals were re-introduced to food and the F2 was scored for gut/gonad heterotaxy. Stress by constant movement was achieved by culturing MY16 animals for two generations in a rotary shaker at 140 rpm and scoring gut/gonad heterotaxy on the F3. Finally, oxidative stress was achieved by egg prep (Sulston *et al.*, 1983) with slightly increased hypochlorite and KOH concentration (21.4% and 8.5% compared to 16.0% and 5.7% respectively).
2.13.5 Stress Variation - Dextral/Heterotaxy animals

Synchronized L4 JR3513 hermaphrodites were anaesthetized with Levamisole 1mM to score gut/gonad heterotaxy. 16 dextral L4s and 16 “heterotaxy” L4s were isolated in separate NGM plates. Animals were cultured normally in NGM plates and transferred on a daily basis.

2.13.6 Stress Variation – High/Low heterotaxy RILs

JR3564, JR3568, JR3513, and JR3533 animals were normally cultured at 20°C for two generations, then 20 L4 hermaphrodites were transferred to a fresh NGM plate and immediately shifted to target temperature of 20, 25, 26 and 27°C. After a 24 hour period, alive animals were counted and transferred to a fresh NGM plate for a total period of 16 days. Also, 24 hours after parental animals were removed, the total number of F1 embryos and F1 animals were quantified (Figure 12a). In order to calculate progeny viability, number of F1 animals was divided by the sum of F1 animals and F1 embryos. Embryo laying was calculated by dividing the total number of F1 embryos by the total number of parental animals alive on the plate, hence obtaining an approximation of the number of eggs laid per parental animal.
CHAPTER THREE

Dissecting Quantitative Traits

How do genes dictate form and behaviour? Although a number of researchers had begun to address this question in model animals, predominantly using the fruit fly *Drosophila melanogaster* as a model organism, Sydney Brenner in 1963 recognised that a simpler animal could enable a powerful understanding of the underlying genetic basis of traits. Therefore, he selected *Caenorhabditis elegans* for his studies (Brenner, 1974). Since its selection as a genetic ‘model organism’, *C. elegans* has proven to be of great importance for many researchers for a large variety of investigations.

3.1 GENETIC CHARACTERIZATION OF GUT/GONAD HETEROTAXY IN WI

The natural wild isolates (WI) *C. elegans* population collected of 97 haplotypes from around the globe (Félix and Braendle, 2010), and their available genotypes (Andersen et al., 2012), provide an exceptional toolkit that may allow us to dissect developmental processes. In this section, I will focus on dissecting the genetics behind hermaphrodite gut/gonad heterotaxy observed across the wild isolates.

3.1.1 GWAS identifies significant loci for male reversals

Previously, there has been a successful attempt to characterize the genetic complexity influencing gut/gonad heterotaxy found across *C. elegans* males (Alcorn et al., 2016). The GWAS study was performed using efficient mixed-model analysis (EMMA) (Kang et al., 2008).

Initially, the frequencies of gut/gonad heterotaxy were compared against each other for the 100 isotypes phenotyped. Results showed significant differences between
Dissecting Quantitative Traits

phenotypical extremes; i.e., wild isolates with male gut/gonad heterotaxy frequencies of 0% were statistically significantly different from wild isolates with male heterotaxy of ~8-11%, based on Fisher’s exact test.

In order to identify the genetic loci associated with male gut/gonad heterotaxy, the authors separated the phenotyped wild isolates in a binary way. Males from a given wild isolate either present gut/gonad heterotaxy (1), or they do not (0). A group of 12 wild isolates presented 0% gut/gonad heterotaxy, and this group was compared with the remaining 88 wild isolates showing up to 11% of gut/gonad heterotaxy. GWAS results identified one significant major region on chromosome II containing 23 SNPs and two other significant regions on chromosome III, containing nine SNPs. These three regions significantly associated with male gut/gonad heterotaxy were found to be in near-perfect linkage disequilibrium (Alcorn et al., 2016).

In conclusion, GWAS analysis has proven to be of great usefulness for dissecting the genetic variability behind gut/gonad heterotaxy in males. Small genetic differences in three different genomic regions appear to explain the differences between wild isolates showing 0% frequencies of heterotaxy and those that show some.

3.1.2 CeNDR, the *C. elegans* natural diversity resource

For many years, and the totality of this thesis, *C. elegans* has proven to be a powerful model to learn about human development, complex behaviours, and evolutionary processes. Now, the natural diversity of *C. elegans* wild isolates is being used worldwide for population genetic studies (McCombie et al., 1992; The C. elegans Sequencing Consortium, 1998; Li et al., 2006; Kammenga et al., 2008; Weber et al., 2010; Nuzhdin, Friesen and McIntyre, 2012; Queitsch, Carlson and Girirajan, 2012; Alcorn et al.,
However, in spite of the great potential that GWAS mappings provide, it has not been as widely adopted as it was expected. Probably, because in order to perform a well-powered GWAS, researchers need to acquire the wild strains, then they must be genotyped, and the genotypic variation correlated with an observable phenotype to identify one or more quantitative trait loci (QTL). This last part of correlation, requires computational and statistical genetic skills that usually take a considerable effort to perform, and are often achieved in collaboration (McGrath et al., 2009).

Recently, the Andersen group in Northwestern University, USA, created a comprehensive database for rapid screening of genetically different populations aimed to dissect complex traits. They created the *C. elegans* Natural Diversity Resource (CeNDR) (Cook et al., 2017). Briefly, CeNDR represents the distribution of all natural wild isolates around the globe. Second, they provide sequence of the whole genomes of those wild *C. elegans* strains, and finally, provide an easy virtual toolkit to perform GWAS to correlate the genotype of target wild isolates with a quantitative phenotype aimed to identify genetic variation underlying target traits (Cook et al., 2017).

### 3.1.3 Results - GWAS identifies no significant loci associated with hermaphrodite reversals among wild isolates

The objective of this section is to characterize the genetic complexity behind gut/gonad heterotaxy in hermaphrodites. In section 2.2, I analysed a panel of 91 wild isolates, obtaining the frequency of gut/gonad heterotaxy for each one. This will be the phenotype data I will use. Firstly, I compared the frequencies of the 91 wild isolates scored against each other, which range from 0.00% in 17 strains to 10.52% in the strain MY16. Wild isolate strains with 0.00% (0 in 200) heterotaxia are significantly different from all strains with at least 2.48% (5 in 202) heterotaxia (*p*<0.05 based on Fisher’s exact
test). Wild isolates could be sorted into two groups: 17 wild isolates that never showed gut/gonad heterotaxy (0.00%) and 74 wild isolates showing up to 10.52% of gut/gonad heterotaxy.

To facilitate association testing, I first simplified the phenotype into two groups: a given animal can be normal (dextral) or abnormal (with at least one gonad arm reversed). Hence, I do not distinguish between anterior heterotaxy, posterior heterotaxy or complete reversal.

Unfortunately GWAS mapping of the whole 91 wild isolate panel for hermaphrodite gut/gonad heterotaxy resulted in no significant association with genetic variation present in the *C. elegans* population (Figure 15a).

I repeated the analysis with a selection of 30 wild isolates: 17 wild isolates that never showed gut/gonad heterotaxy versus the top 13 wild isolates showing the highest rates of gut/gonad heterotaxy. GWAS mapping of this 30 wild isolate selection for hermaphrodite gut/gonad heterotaxy also resulted in no significant association with genetic variation present in the *C. elegans* population (Figure 15b).

Finally, I repeated the analysis with the selection of 30 low and high heterotaxy wild isolates previously described, but considering only those reversals including the posterior gonad arm (posterior heterotaxy). GWAS mapping of this 30 wild isolate selection for hermaphrodite gut/gonad heterotaxy resulted in no significant association with genetic variation present in the *C. elegans* population (Figure 15c).
Figure 15. Genome-wide mapping representation of the association between genetic variation in the natural C. elegans wild isolate population and hermaphrodite gut/gonad heterotaxy. Comparison of two groups: one group containing wild isolates showing 0.00% of heterotaxy against the other including variation of heterotaxy up to 10.52%. The x-axis corresponds to genomic position with chromosome number indicated as a roman numeral above each box. Dots represent single-nucleotide variants (SNV) present in the C. elegans population. The y-axis corresponds to the level of significance for the association test. The red line corresponds to the Bonferroni-corrected significance threshold. a) GWAS of the whole 91 wild isolate panel (17 vs 74 respectively). b) GWAS of a selection of 30 wild isolates (17 vs 13 respectively). c) GWAS of a selection of 30 wild isolates (17 vs 13 respectively) excluding those gut/gonad heterotaxy involving the anterior and both gonad arms (only includes those involving the posterior gonad arm).
3.2 GENETIC CHARACTERIZATION OF GUT/GONAD HETEROTAXY IN RILs

GWAS analysis of the wild isolates revealed no loci significantly associated with hermaphrodite gut/gonad heterotaxy. However, GWAS limitations include that it is only able to identify significant loci if they differ in a substantial number of wild isolates. Hence, in this section I will investigate the genetic complexity of hermaphrodite gut/gonad heterotaxy by taking advantage of the phenotyped N2 vs MY16 RILs. Phenotype details can be found in section 2.4 of this thesis. Genotype details can be found in Yamila N. Torres PhD thesis (Torres, 2016).

Recombinant inbred lines do not represent the rich heterogeneity of natural wild isolates, but they allow a finer mapping for developmental biology analysis. Several authors successfully identified significant QTL influencing an observed phenotype by using RILs (Ayyadevara et al., 2001; Li et al., 2006; Kammenga et al., 2008; Harvey, Shorto and Viney, 2009; Rodriguez et al., 2012).

3.2.1 Results - QTL mapping of N2 vs MY16 RILs

Of 99 RILs scored for gut/gonad heterotaxy, only 7 never showed gut/gonad heterotaxy (0.00%), in contrast with the other 89 RILs, which showed up to 18.81% of gut/gonad heterotaxy. Three RILs with the highest rates of heterotaxy presented evidence of transgressive segregation, showing gut/gonad heterotaxy frequencies significantly higher than the high-heterotaxy parental strain MY16 (p<0.005 based on Fisher’s exact test) (Figure 5).

Evidence of transgressive segregation may be evidence of a distribution of phenotypes which is not normal. A shapiro-Wilk test for normality supports a non-normal distribution (W=0.9761, p>0.05) (Wilk and Gnanadesikan, 1968) (Figure 16a).
However, the shapiro-Wilk test is highly dependent on sample size, hence a quantile-quantile plot was generated (Figure 16b). In general, the line of statistical normality is followed, except for a small group at the lower extreme. This may be because the lower bound set by the parental isotype N2 is very close to 0. Excluding a few exceptions, the great majority of the populations does follow the normal distribution. Hence, the data was considered normal and parametric statistical tests were used for QTL mapping (Lumley et al., 2002).

A linkage group is defined as a set of genes (or loci) in a single chromosome. Neighbouring markers are usually inherited as a group: during cell division they act and move as a unit rather than independently, and are therefore said to be linked (Rockman and Kruglyak, 2009). A linkage map represents the likelihood of recombination between pairs of markers, with markers further away from each other having a higher change of recombination between them (Stapley et al., 2017). I calculated linkage groups using R/qtl software and they can be visualized in (Figure 16c), where the LOD score represents the statistical estimate of whether the two markers are likely to be inherited together.

The available genotype data of the RILs includes 2000 genetic markers distributed along the 6 *C. elegans* chromosomes. The genetic linkage map with genetic distances represented in centimorgans (cM) were calculated based on recombination rates in R/qtl. Full details on the genetic data of MY16vsN2 RILs can be obtained from Wormbase or from the Rothman Lab (UCSB, USA) (Figure 16d).
Figure 16. QTL mapping of N2 vs MY16 RILs. a) Histogram of the distribution of heterozygosity in the RILs. b) Quantile-quantile plot showing the distribution of phenotypes (open circles) relative to a normal distribution (solid line). c) Estimated recombination fractions and LOD score for all pairs of markers. Yellow indicates high linkage and blue represents "not linked". d) Genetic map for RILs including 1770 SNPs. Genetic distances represented in centimorgans (cM).
3.2.2 Results – QTL mapping identifies no significant loci associated with hermaphrodite reversals in RILs

The objective of this section is to dissect the genotype associated with gut/gonad heterotaxy in *C. elegans* hermaphrodites using the phenotype data obtained from N2 vs MY16 RILs. Details of the phenotype data can be read in detail in section 2.4 of this thesis. Genetic interactions are not fully represented in RILs as it is in the wild isolates natural population, but the use of QTL mapping tends to have increased power to detect regions of the genome influencing a quantitative trait (Korte and Farlow, 2013).

Quantitative Trait Loci (QTL) analysis was undertaken. Evidence of linkage between genotype and phenotype was measured by LOD score, which is defined as “*The log\(_{10}\)* likelihood ratio comparing the hypothesis that there is a QTL at the marker to the hypothesis that there is no QTL anywhere in the genome*” (Broman, 2010). High LOD scores for a given marker suggest the presence of a QTL, while low LOD score suggest absence of QTL. To establish a significant correlation between a given marker and its relevance to the observed phenotype, LOD scores need to meet statistical significance. Two genome-wide LOD thresholds were calculated for each QTL mapping, according to those established by the R/qtl guide used in this thesis (Lambot *et al.*, 2004), “suggestive linkage” and “significant linkage”, defined by linkage with statistical evidence expected to happen 0.05 and 0.001 times respectively by chance in a genome scan. Suggestive linkage must be taken as a “suggestion” rather than association, as it is expected to appear randomly (false positive) in a QTL mapping with a 20% probability.

First, I performed single-QTL analysis method using marker regression. Here, a t-test was performed for every marker. The LOD score (output) for each marker is plotted
in Figure 17a. Second, I performed a single-QTL analysis method using interval mapping. (Figure 17b). Details can be found in the methods section of this chapter.

Unfortunately, there are no QTL peaks overtaking any significance threshold established. Not even the most permissive “suggestive linkage”, for any QTL mapping method performed (Figure 17a, b). Results for the QTL mapping of the MY6 vs N2 RILs did not reveal any significant loci associated with gut/gonad heterotaxy in hermaphrodites.

3.2.3 Results – No clustering of significant SNPs associated with hermaphrodite reversals in RILs

An advantageous tool available in the R/qtl package is the plotting of how parental loci are distributed among RILs. Any given RIL’s genome is composed of a mosaic of parental genomes. Generally, when observing similar plots for candidate SNPs, it is often observed that a group of strains sharing a similar phenotype will also share alleles from one parent. Genotype data for all RILs colour coded based on parental origin, and the significance of correlation between similar phenotypes sharing genomic regions from one parent was tested in R/qtl. Visual examination of the raw data is strategic in detecting informal evidence for QTL. However, statistical methods are required.

Although visually it appears to be a clustering of MY16 parental genome on those RILs with high gut/gonad heterotaxy in chromosome II (Figure 17c), this association does not reach the significance thresholds (data not shown) (Lambot et al., 2004). Therefore, results suggest no evidence that RILs with high/low frequency of gut/gonad heterotaxy share a similar genomic mosaic on a given locus (Figure 17c).
Figure 17. QTL mapping. LOD scores for each genomic region of the MYvSN2 RILs set. a) Single-QTL marker regression. b) Single-QTL interval mapping. Genome-wide LOD thresholds are: suggestive linkage (LOD score: 1.87) and significant linkage (LOD score: 2.53), thresholds are not shown as QTL peaks do not reach significance. c) Colour-coded SNP values for MY16vSN2 RILs (blue and red respectively). Each horizontal line represents the genotype of a given RIL for all 6 chromosomes. RILs are sorted from high to low frequency of gut/gonad heterotaxy as represented in the plot on the left. This image visualizes whether large regions of the genome are shared among RILs sharing similar gut/gonad heterotaxy frequencies, especially phenotype extremes. No association was observed.
3.3 CONCLUSIONS CHAPTER 3

The complete genome of *C. elegans* has been available since 1998 (The *C. elegans* Sequencing Consortium, 1998), and from this point onwards, genotype information from natural wild isolates and many recombinant inbred lines have become available. This massive genotype data allows genome-wide association studies (GWAS) to be performed, aimed to connect a given genotype to an observable and quantifiable phenotype.

3.3.1 GWAS identifies no significant loci associated with hermaphrodite reversals among wild isolates

Three independent GWA studies were performed using phenotype data from the wild isolates gut/gonad heterotaxy frequency. A first study including all wild isolates; a second study including a sub-set of 30 wild isolates (phenotypic extremes); and a third study including the same 30 isolates sub-set but considering only gut/gonad heterotaxy involving the posterior gonad arm (which is the most frequent form of heterotaxy). GWAS mapping for all studies described for hermaphrodite gut/gonad heterotaxy resulted in no significant association with genetic variation present in the *C. elegans* population. It appears that there is no association between genomic data and gut/gonad heterotaxy in hermaphrodites.

3.3.2 QTL mapping identifies no significant loci associated with hermaphrodite reversals in RILs

Two single-QTL analyses were performed on RILs gut/gonad heterotaxy data: marker regression and interval mapping (Lambot *et al.*, 2004). Results from both methods resulted in very similar outcomes, with similar peaks and general structure. Neither marker regression nor interval mapping QTL analyses resulted in any peak overtaking any significance threshold established: suggestive linkage (LOD score: 1.87)
and significant linkage (LOD score: 2.53). Single-qtl analysis did not reveal any loci significantly associated with gut/gonad heterotaxy in hermaphrodites.

3.4 DISCUSSION CHAPTER 3

In Chapter 2, I described the phenotype data obtained from 91 wild isolates and 99 MY16vsN2 RILs. Fortunately, the genotype data for both wild isolates and RILs was available at the present time (Andersen et al., 2012) (Torres, 2016). The objective of this chapter was to dissect the genetic complexity behind hermaphrodite gut/gonad heterotaxy. GWAS analysis of the wild isolates and QTL mapping of the RILs resulted in the same conclusion: there are no loci significantly associated with hermaphrodite gut/gonad heterotaxy. This suggests that gut/gonad heterotaxy in hermaphrodites is a complex and polygenic trait: propensity of developmental errors in gut/gonad organogenesis is controlled by broad and widespread regions of the genome rather than being restricted to just a couple of genes or one genomic region.

This result was highly predictable, as gut and gonad are the two major organs in the adult nematode, representing ~70% of the total body volume, with a complex network of signals and cell-cell communications that need to perfectly synchronize for a complete and normal development (Sulston and Horvitz, 1977; Sulston et al., 1983). Errors in any step of the chain of development may elicit abnormal gut/gonad organogenesis. Focusing on hermaphrodite gut/gonad heterotaxy, it appears clear that its widespread variation cannot be explained by a single mutation or errors in the expression of a single gene.

*C. elegans* is particularly well-suited for QTL and GWAS analyses as nematodes can be maintained as inbred lines due their self-fertilization. Therefore, it is possible to use a
same genotype data for multiple phenotype studies around the world and in different times, with very little variation among replicates. Unpublished observations of *C. elegans* phenotyping of same inbred lines in two trans-Pacific laboratories (New Zealand and USA) revealed robustness in experiment replicates using the same *C. elegans* strains. Importantly, statistical methodology has improved substantially in the recent years, including several models implemented in R packages like R/qtl (Lambot et al., 2004) or online resources for rapid and easy GWAS (Cook et al., 2017). Analysis of a few thousand SNPs runs on a normal PC within a few minutes.

QTL mapping has proved its value in genomic region identification. But also implies two significant disadvantages: first, allelic diversity can only be assessed from the segregation originated from the parental of targeted F2 or within the RIL population (Korte and Farlow, 2013). And second, mapping resolution is determined and limited by the recombination rate raised from the RILs creation (Rockman and Kruglyak, 2008).

GWAS overcomes the QTL disadvantages described above, but implies others, like small effect size and rare variants: *The phenotypic variance is determined by how strongly the two allelic variants differ in their phenotypic effect (the effect size), and their frequency in the sample* (Asimit and Zeggini, 2010). Compared to QTL mapping, one major difference is that GWAS is typically performed on “unrelated” individuals, while QTL mapping uses recombination within families: in our case of study, RILs represent “children” of the parent strains N2 and MY16. As a general rule, GWAS works often as a complementary of QTL mapping. Significantly associated loci detected with QTL can be further analysed with GWAS for improved resolution. For hermaphrodite gut/gonad heterotaxy analysis, no significant loci were detected with any method.
Why did we not detect any significant loci associated with nematode heterotaxy? Did we have enough power to detect an association or linkage? It is likely that this was the case as GWAS included a panel of 91 wild isolates, with sample size ~300 and ~40,000 SNPs; further, QTL mapping was performed with a panel of 99 RILs, with sample size ~200 and ~2,000 SNPs. Other QTL and GWAS analyses have been performed with fewer strains, lower sample size and much fewer SNPs, and resulted in detection of significant loci associated (Ayyadevara et al., 2001; Cheng et al., 2010; Bendesky et al., 2012; Queitsch, Carlson and Girirajan, 2012; Andersen et al., 2014; Alcorn et al., 2016).

Considering our sample provides enough power, the lack of detection of significant loci associated with the phenotype may be due to the missing heritability problem, or most likely, a polygenic phenotype. Hermaphrodite gut/gonad heterotaxy is controlled by too many regions and a combination of too many small effects contributing to differences between wild isolates and between RILs.

3.5 MATERIALS AND METHODS
3.5.1 Wild isolates GWAS - CeNDR

Genome wide association mapping was carried out using the Caenorhabditis elegans Natural Diversity Resource (CeNDR), a free and easily accessible online resource for C. elegans research that does not require any registration (https://www.elegansvariation.org/) (Cook et al., 2017). CeNDR organises the collection, maintenance, and distribution of wild C. elegans strains, whole-genome sequences and enables gene mapping of phenotypes, with the software used open source and available (https://www.elegansvariation.org/help/Software/). The interface for gene mapping via GWAS allows the submission of multiple traits, and results are organized in a report, which presents figures, tables, and interactive elements. In the event of a QTL
identification, the system allows the user to browse the genes associated with the quantitative trait studied (Cook et al., 2017).

GWAS is performed on cloud-based virtual machines, with statistical analysis using the R package rrBLUP (Endelman, 2011) and graphics generated using the R package ggplot2R. rrBLUP fits a mixed model to account for population structure, before testing each marker for association with the trait (Yu et al., 2006), with population structure estimated from the kinship matrix between strains, calculated from genome-wide SNP data. To run GWAS on CeNDR, strain names and their corresponding phenotypes (% heterotaxy) were input at https://www.elegansvariation.org/genetic-mapping/submit/. Three analyses were run: first, all strains and their combined heterotaxy values (total % of anterior, posterior and complete reversal) were submitted on CeNDR. Second, extreme phenotypes only were selected, with the lowest 17 strains (all with 0.00% total heterotaxy) and highest 13 strains (ranging from 7.92% - 18.81% total heterotaxy) were tested. Finally, the same 17 + 13 strains with extreme phenotypes were selected, but only their values those reversals including the posterior gonad arm (posterior heterotaxy) were entered into the CeNDR portal, in order to test whether a discrete phenotype (rather than the combination of posterior heterotaxy, anterior heterotaxy and complete reversal) would yield a clearer signal of association.

3.5.2 Single-QTL analysis - Marker regression

QTL analysis by marker regression considers each marker individually, splits the individuals into groups, and compare phenotype averages between groups. The linkage of a given market to a QTL is made by T-test. Evidence of linkage to a QTL is measured by a LOD socre (log_{10}), which is calculated as follows. Where RSS_{0} is the null residual sum of squares, RSS_{1} is the residual sum of squares under the alternative, and n is sample size.
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\[ LOD = \frac{n}{2} \log_{10} \left( \frac{RSS_0}{RSS_1} \right) \]

After loading R/qtl package and data, single QTL by marker regression is accomplished by the following commands.

```r
> out.mr <- scanone(mydata, method="mr")
> out.mr[out.mr$chr == 1,]
> out.mr
> par(mfrow=c(1,1))
> plot(out.mr, ylab="LOD score", main="QTL mapping with marker regression")
```

3.5.3 Single-QTL analysis – Interval mapping

QTL analysis by interval mapping is often considered an improvement compared to marker regression as this method takes into account the missing genotype data at a putative QTL. There are several interval mapping options available. In this thesis I used standard interval mapping. LOD score is calculated as follows, and details can be consulted in (Lambot et al., 2004):

\[ LOD = \log_{10} \left( \frac{\prod_i \Sigma_j p_{ij} \phi(y_i; \mu_j, \sigma^2)}{\Pi_i \phi(y_i; \mu_j, \sigma^2)} \right) \]

Among the advantages of interval mapping compared to marker regression is that LOD curves are smoother, and the estimate of the effect of a QTL is improved.

```r
> out.em <- scanone(mydata, method="em")
> out.em[out.mr$chr == 1,]
> out.em
> par(mfrow=c(1,1))
> plot(out.em, ylab="LOD score", main="QTL mapping with interval mapping")
```
Gut twist influence in anatomical handedness

In chapter 2, I presented evidence supporting the existence of a temperature-sensitive molecular and cellular event in early embryogenesis that conditions the post-embryonic gut/gonad arrangement. In other words, something happens in the early embryo that appears to set the stage for LR gut/gonad organogenesis in hermaphrodites.

In this chapter I explore candidate developmental processes occurring in early embryo development with the aim of finding candidate embryonic processes with a direct or indirect consequence on LR gut/gonad heterotaxy in hermaphrodites. I will focus on the embryonic and post-embryonic development of *C. elegans* for two major organs.

4.1 GASTRULATION

In section 2.5, I performed a temperature shift experiment that pointed at a temperature-sensitive event leading to LR gut/gonad heterotaxy in hermaphrodites occurring before embryo gastrulation.

4.1.1 Evolutionary approach of gastrulation

Gastrulation is an early embryo development event by which the blastula cells, the blastocysts, re-arrange their position, resulting in the establishment of the three germ layers (ectoderm, endoderm and mesoderm) in triploblastic organisms (Hall, 2012). Gastrulation sets the opening of the archenteron (primary intestine) known as the blastopore. Depending on the developmental fate of the blastopore, animals can be divided into deuterostomes and protostomes:
In metazoan taxonomy, under the animal kingdom, we find the clade bilaterians, which is defined by animals with a head (anterior), a tail (posterior), a back (dorsal), and a belly (ventral). The bilaterians are contrasted with animals with radial symmetry, such as jellyfish (echinoderms such as sea urchins or sea stars have a bilateral larval development). At the same time, those bilaterians with coelom, excretory organs and nerve chords are organised under the major clade Nephrozoa. Nephrozoa include the two superphylums deuterostomia and protostomia.

Deuterostomes are defined by the developmental fate of the blastopore, which will become the future anus of the animal. The blastopore sets the entrance of the primitive intestine, the archenteron, which eventually tunnels through the embryo to make another opening, which forms the mouth. Deuterostomes often present indeterminate cleavage, by which the first cells in the developing embryo retain their totipotency: each cell is capable of forming a complete organism (Martín-Durán et al., 2016). Deuterostome animals include some worms, echinoderms, and vertebrates (chordata), including humans.

Protostomes are also defined by the developmental fate of the blastopore, which will, in this case, become the future mouth of the animal. Protostome diversity includes most invertebrates, including arthropods. As coleoptera (beetles) constitute almost 40% of described insects and 25% of all known animal life-forms, protostomes are the largest
animal superphylum, including the majority of species, animal diversity and animal biomass (Hunt et al., 2007). It was recognised many years ago by Lake and colleagues (Aguinaldo et al., 1997) that the protostome clade can be divided into the lophotrochozoa and ecdysozoa, this last including nematodes and arthropods. While this did not result in the obsolescence of the protostome/deuterostome dichotomy, it is still accurate to refer to both lophotrochozoa and ecdysozoa as protostomes. Two of the most famous protostomes (ecdysozoa) in experimental biology are the fruit fly Drosophila melanogaster and the nematode Caenorhabditis elegans.

4.1.2 C. elegans gastrulation

The biological importance of gastrulation is universal in the animal kingdom, and C. elegans presents its own gastrulation process. However, C. elegans gastrulation involves relatively little movement of blastocysts and little blastocoel space compared to other protostomes. The first germ layer to internalize is the endoderm. Blastocysts Ea and Ep (endoderm precursor cells) internalize by constriction of their apical surfaces (Sulston et al., 1983). As a result of this, 6 neighbouring blastocysts are drawn towards each other pushing Ea and Ep into the embryo core (Figure 18).

![Figure 18](image.png)

*Figure 18. Schematic of C. elegans gastrulation. The black arrows show constriction of the apical surface of Ea and Ep cells. The blue arrow shows movement of neighbouring blastocysts following ingestion of Ea and Ep cells.*
Cell movement in gastrulation begins at the 26-cell stage of embryo development. The blastocoel is formed when the basal membranes of cells separate from their neighbouring blastocysts while the lateral membranes remain connected. These movements are coordinated by PAR-3 proteins and myosin activity (Nance and Priess, 2002). Rather than invagination of the epithelial sheets, *C. elegans* presents ingression of individual cells. This form of gastrulation is highly atypical in metazoans outside the phylum nematoda (Joshi and Rothman, 2005). It has been argued that gastrulation in *C. elegans* followed the nematode general evolutionary trend of simplification and optimization.

From this point onwards, the endodermal precursor cells Ea and Ep will develop dorsally and anteriorly forming the endoderm and future intestine of the *C. elegans* animal.

### 4.2 C. ELEGANS GONAD DEVELOPMENT

The *C. elegans* hermaphrodite gonad arms develop during larval stages from the gonad primordium located in the mid-body. The route and positioning of each of these gonad arms is determined by the activity of the two distal tip cells (DTCs). First, each DTC at the tip of each elongating gonad arm leads the gonad development on opposite anterior-posterior directions away from the mid-body. Second, they undergo a $90^\circ$ turn from the ventral to the dorsal side of the body. And finally, DTCs turn $90^\circ$ again and start migrating towards mid-body along the dorsal body side. As a result of this, two mirror-image gonad arms are formed (Sulston and Horvitz, 1977).
4.2.1 Role of uncoordinated (unc) genes in gonad development

Timing for each DTC turn is key, and *C. elegans* mutants of *unc-5, unc-6*, and *unc-40* present an abnormal DTC turns (Hedgecock *et al.*, 1987). Abnormal gonad development phenotypes result from abnormal DTC guidance, suggesting that the second phase of DTC guidance (ventral to dorsal) requires activation or disinhibition of the UNC-6 guidance system. Also, this phase is initiated by MIG-8 and DAF-12, which at the same time depend on the up-regulation of *unc-5* (Su *et al.*, 2000). DTC ventral-to-dorsal migration depends on the successful expression of *unc-5, unc-6*, and *unc-40*. UNC-6 is expressed by neurons in the ventral nerve cord during the DTC migration process. UNC-40 is expressed in the DTCs during the entire migration process. And *unc-5* is expressed in DTCs and all five classes of motorneurons (Su *et al.*, 2000). Thus, *unc-5* activity is required for the proper UNC-6 guidance system. Failure in any step of this chain results in abnormal DTC guidance, and therefore, abnormal gonad development and positioning in the body cavity.

Additional temperature sensitive gonadal migration defects have been observed in mutants for *unc-84* (Malone *et al.*, 1999). UNC-84 affects nuclear migration and anchoring of various cell types (Worm Base). Among other effects, defects in *unc-84* affect coordinated locomotion, vulval formation, egg laying, and hermaphrodite gonadal shape.

It is possible that the hermaphrodite *C. elegans* gut/gonad heterotaxy observed as described in this thesis, could result from variation in activity of the UNC-6 guidance system. Although the phenotype I observed may share similarities with abnormal DTC guidance described in Ming *et al.*, and Malone *et al.* (Malone *et al.*, 1999; Su *et al.*, 2000), this hypothesis has not been addressed in this thesis.
4.2.2 PGCs development is partially controlled by endodermal inputs.

*C. elegans* primordial germ cells (PGCs) are the precursors of the germ line, closely associated with the gonad (Cinalli, Rangan and Lehmann, 2008). Also, PGCs have a close association with endodermal cells as PGCs have been seen to extend lobes into the adjacent endodermal cells (Abdu *et al.*, 2016). Until recently, the function of these lobes was poorly understood, but there is now evidence that PGC size and content is controlled by the adjacent endoderm.

PGCs in the *C. elegans* embryo are the Z2 and Z3 cells. Before the embryonic comma-stage (Sulston *et al.*, 1983), Z2 and Z3 change their shape from spherical into dumbbell by extending a large lobe which entwines into the surface of the adjacent endodermal cell (Abdu *et al.*, 2016). This lobe has a similar volume to the cell body where the nucleus remains. Endodermal cells digest these lobes after an extended period of time: lobes and their contents are lost. Lobe formation is autonomous and does not require external inputs. Evidence suggests that these lobes attach to the adjacent endodermal cells before detaching from the main PGC body and being digested by the endoderm. Lobe digestion and scission is developmentally regulated and involves intracellular communication (Abdu *et al.*, 2016).

Lobe digestion implies a significant loss of mitochondria in PGCs. Therefore, the authors hypothesized that lobe formation and digestion protects PGCs from mitochondrial oxidative stress. These findings also provided evidence of PGC-endoderm signalling.
4.3 C. ELEGANS INTESTINE DEVELOPMENT

The *C. elegans* intestine in the first larval stage is composed of 20 cells with the shape of a narrow and twisted tube (Sulston and Horvitz, 1977), all derived from the E blastomere. Intestinal development is largely completed in the embryo. LIN-12 NOTCH signalling has been shown to be essential for normal intestine development, occurring in two distinctive developmental checkpoints.

4.3.1 LIN-12/NOTCH signalling

*C. elegans* NOTCH is composed by two proteins encoded by the genes *lin-12* and *glp-1*. Double mutants show the distinctive Lag phenotype, which is characterised by the absence of rectum, anus and excretory cell (Greenwald, 2005). LIN-12 NOTCH is required for cell fate decisions occurring during embryogenesis. However, *lin-12* and *glp-1* appear to be functionally redundant, as GLP-1 can substitute LIN-12 in some cell fate decisions. Therefore, it appears that the two genes have arisen by gene duplication.

LIN-12/NOTCH is a member of the type I transmembrane proteins that respond to ligands LAG-2, APX-1 and DSL-1: all members of the “DSL” family (Greenwald, 2005). Receptor-ligand interaction elicits cleavage of LIN-12/NOTCH receptor releasing the intracellular domain, which will translocate into the cell nucleus. In the nucleus, LIN-12/NOTCH intracellular domain acts as a transcriptional activation complex triggering target gene expression.

LIN-12/NOTCH proteins have been seen to mediate lateral cell-cell interactions involved in cell fate specification. For example, GLP-1 (receptor) is expressed during early embryogenesis in ABa and ABp cell, while APX-1 (ligand) is expressed by the P2 blastomere. This induces ABp (in direct contact with P2) to follow a distinct cell fate than
the equivalent blastomere ABa (not in contact with P2) (Greenwald, 1998) (Figure 1).

Also, GLP-1 is expressed in the germline, while the ligand LAG-2 is produced by the somatic gonadal cell, the DTC, which triggers germ line mitosis.

4.3.2 First LIN-12/NOTCH interaction – LaR → RaL transition

LIN-12 expression is first detected in the E4 intestinal primordium, symmetrically in all the cells. However, as the intestine develops, LIN-12 expression appears to increase on the right half of the primordium as it decreases on the left. This asymmetric LIN-12 expression becomes evident at E8 and persists until the E16 stage (Neves and Priess, 2005; Neves, English and Priess, 2007). Between E4 and E8 stages, the first LIN-12/NOTCH interaction occurs: non-intestinal cells in contact with left side of the primordium activate the expression of REF-1, which is a bHLH transcription factor targeting LIN-12, downregulating its expression on the left cells of the primordium (Neves and Priess, 2005). As a result, only the cells on the right side of the primordium continue to express LIN-12. Asymmetric expression of LIN-12 on the right cells of the intestinal primordium is a requisite for the second LIN-12/NOTCH interaction, as failure in establishing LIN-12 asymmetric expression elicits failure in the second LIN-12/NOTCH interaction, explained in the next section.

Intestinal primordial cells are organised in two distinctive LR longitudinal rows. Every ring has one intestinal primordial cell on the right and its sister on the left (the first ring presents two cells on each side). Intestine is flanked by the ventral nerve cord, four muscle groups, and by a single layer of five hypodermal cells (Sulston et al., 1983). Boundaries between cells are jagged, forming a complex ladder-like appearance (Figure 19).
However, this structure has a marked LR polarity: if a cell on the right (R) contacts the cell on the left (L) of the anterior intestinal ring, we refer to the contact as RaL. On the other hand, if a cell on the left (L) contacts the right cell (R) of the anterior ring, we refer the contact as LaR (Asan, Raiders and Priess, 2016). In the E4 intestinal primordium, most intestinal ring contacts are LaR (Figure 19a). But a transition to RaL is observed starting at the E8 stage, with a majority of RaL interactions occurring one hour before E16 stage (Figure 19b, c). This LaR → RaL transition requires LIN-12/NOTCH signalling, as *lin-12(n941)* mutants lack RaL interactions in E16 intestinal primordium cells. It has been observed that those embryos that fail to successfully perform a RaL transition, also fail in the second LIN-12/NOTCH: the gut twist. Therefore, it appears that a RaL (rather than LaR) cell contact configuration in the intestinal E16 primordium is a requisite for normal intestinal primordium development.

In summary, the first LIN-12/NOTCH interaction has two functions: first, to generate the asymmetric LIN-12 expression (high on the right and low on the left), and second, to elicit morphological changes in intestinal primordium cells summarised in the LaR → RaL transition. Both events are required for the second LIN-12/NOTCH interaction (Neves, English and Priess, 2007).
4.3.3 Second LIN-12/NOTCH interaction – Gut twist

The second LIN-12/NOTCH interaction occurs at 345 minutes after embryo fertilization, the embryo development phase known as comma-stage. At this point, intestinal primordium is a shrimp-like structure composed of nine intestinal rings. Each ring has one intestinal primordium cell on the left and its sister on the right. The first intestinal ring is composed by four intestinal cells: two on the left and two on the right. All cells of intestinal rings 2-9 are in contact with both dorsal and ventral midlines.

Upon LIN-12/NOTCH signalling, intestinal rings 2, 3, and 4 twist clock-wise 90° (viewed from an anterior view of the primordium). The other intestinal rings remain static. As a result of this, intestinal primordium cells originally located on the right, are now in touch with the dorsal midline and dorsal to its sisters, which are now in contact with the ventral mid-line and ventral to its sisters (Figure 20a, b). The event that precedes and is pre-required for the intestinal gut twist is an asymmetric shift of
intestinal ring cell 2L (second ring, cell on the left) known as “pre-rotation shift of 2L”. This event occurs 30 minutes or more before the gut twist of rings 2, 3, and 4, and it requires LIN-12/NOTCH. In lin12(n941) mutants, the pre-rotation shift of 2L does not occur (Asan, Raiders and Priess, 2016).

Before the gut twist, each intestinal ring contains a lumen, which is not circular but rather flat. In intestinal ring 1 (composed by four cells) the lumen is horizontal, while the rest of the rings have a vertical lumen (Figure 20c). After the gut twist, lumens for intestinal rings 2, 3, and 4 appear to align with the horizontal intestinal lumen in ring 1. This suggests that at least one function of the gut twist is the alignment of intestinal ring lumens (Asan, Raiders and Priess, 2016). Interestingly, while intestinal rings 2 and 3 perform a complete 90° twist, the intestinal twist in ring 4 is often incomplete, at ~60-70°. This may be evidence supporting the hypothesis by which the gut twist is partially aimed to align intestinal ring lumens.

Complete LIN-12/NOTCH (consisting of the LIN-12 receptor, effector protein LAG-1 and ligands LAG-2 and APX-1) is required in the intestinal primordium during embryo development for first, pre-rotation shift of 2L, second, LaR → RaL transition, third, asymmetric LIN-12 expression, fourth, twist of intestinal rings 2, 3, and 4, and fifth, intestinal ring lumen alignment. In lin12(n941) mutants, LIN-12/NOTCH pathway is absent and therefore, all of these events do not occur. As a result, intestinal primordium cells remain in their original position (Figure 21a, b) and intestinal lumens remain un-aligned (Figure 21c) after 401 minutes (comma-stage).
Figure 20. Normal intestinal primordium gut twist. Two developmental checkpoints are represented for comparison purposes: 345 and 401 minutes after fertilization. LIN-12/NOTCH signalling is occurring between these two checkpoints. 

a) DIC images of normal embryos from the N2 strain. Intestinal rings 1-9 are outlined in white. Intestinal rings 2, 3, and 4 are highlighted and colour coded: left intestinal cells in purple and right cells in red.

b) Pictures of clay models of C. elegans intestinal primordium. Colour coded for clarification purposes. Note how intestinal cells of rings 2, 3, and 4 located on the right at 345 minutes, move dorsally after 401 minutes.

c) Schematic of intestinal primordium rings (Int), highlighting lumen orientation. Note how lumens of intestinal rings 2, 3, and 4 twist aligning with the horizontal lumen of intestinal ring 1.
Figure 21. Absence of intestinal primordium gut twist. Two developmental checkpoints are represented for comparison purposes: 345 and 401 minutes after fertilization. LIN-12/NOTCH signalling is not active. a) DIC images of lin-12(n941) mutant embryos. Intestinal rings 1-9 are outlined in white. Intestinal rings 2, 3, and 4 are highlighted and color coded: left intestinal cells in purple and right cells in red. b) Pictures of clay models of C. elegans intestinal primordium. Colour coded for clarification purposes. Note how intestinal cells of rings 2, 3, and 4 located on the right at 345 minutes, remain static after 401 minutes. c) Schematic of intestinal primordium rings, highlighting lumen orientation. Note how lumens of intestinal rings 2, 3, and 4 do not twist. No alignment with the horizontal lumen of intestinal ring 1 is observed.
4.4 GUT TWIST ROLE IN GUT/GONAD DEVELOPMENT

Importantly, while reading about intestine primordium organogenesis, I found a revealing statement in Hermann et. al., (Hermann, Leung and Priess, 2000) which I quote below:

"What are the consequences of this incomplete and abnormal intestine development in the absence of LIN-12/Notch function? Is normal gut twist in the embryo a requisite for normal post-embryonic gonad development? One possible hypothesis is that normal intestinal gut twist is required for cell-cell communication between endoderm and gonadal tissue, resulting in normal gonadal placement. Abnormal gut twist may alter the cellular and molecular cues received by gonadal tissue, eliciting abnormal gonad positioning, resulting in variation in the gut/gonad heterotaxy phenotype discussed in this thesis."

4.4.1 Results – lin-12(n941) mutants show both abnormal gut twist & gut/gonad heterotaxy

The first step in assessing the possible role of intestinal primordium cell organogenesis in gut/gonad heterotaxy, is to evaluate the "aberrant positioning of the gonad relative to the intestine" Hermann et. al., (Hermann, Leung and Priess, 2000) mentioned. Did these authors observe gut/gonad heterotaxy?

For this experiment, I took advantage of the strain MT1965: a C. elegans strain with a lin-12(n941) null allele carried on a genetic balancer eT1, constructed in an N2
background. This last detail is key, as the strain is genetically identical to N2 except for a target loci carrying a lin-12(n941) null allele. As noted earlier among more than 25,000 N2 hermaphrodite animals scored, all showed 0.0% gut/gonad heterotaxy.

I found that MT1965 L4 animals showed 14.5% (44/303) gut/gonad heterotaxy in hermaphrodites when cultured 20° and 18.5% (37/200) at 25°. The gut/gonad heterotaxy phenotype was morphologically identical to the one observed among natural wild isolates and RILs. I confirmed this situs inversus incompletus by observing coelomocyte positions (not shown). Finally, relative frequencies for each phenotype followed the general trend observed among wild isolates: posterior heterotaxy is the most frequent (7.9%; 24/303) followed by anterior heterotaxy (5.0%; 15/303), and complete heterotaxy being the least frequent (1.7%; 5/303). As expected, I also observed that MT1965 embryos showed high frequency of abnormalities in intestinal primordium organogenesis during comma-stage. 66.7% of embryos observed showed either incomplete or abnormal gut twist (12/18). Although I cannot confirm that all L4 heterotaxy animals scored and all embryos with abnormal gut twist observed were carrying the lin-12(n941) null allele, these results present sufficient evidence to suspect a correlation between abnormal intestine organogenesis during embryo comma-stage and post-embryonic abnormal gonad development.

I next assessed the variation in abnormal intestine organogenesis during comma-stage in those strains already phenotyped for gut/gonad heterotaxy (wild isolates and RILs). Do those strains with high gut/gonad heterotaxy show similar high frequencies of defects in embryo intestine organogenesis?
Results – High gut/gonad heterotaxy wild isolates show defects in embryo intestine organogenesis

To address whether there was a correlation between LR gut/gonad reversals and defects in gut twist, I selected two wild isolates with high frequencies of gut/gonad heterotaxy in hermaphrodites: JU778 (35/772; 4.5%) and MY16 (53/504; 10.5%). One wild isolate with no gut/gonad heterotaxy was selected as a negative control: N2 (0/300; 0.0%). Frequencies of embryo defects and gut/gonad heterotaxy in MT1965 from the previous section were included as a positive control.

Embryos cultured at 20°C were mounted for Nomarski microscopy observation. A significant number (~60) comma-stage embryos were observed and the intestinal primordium was analyzed to assess normal/abnormal gut twist. Among 71 comma-stage embryos observed in N2, none of them showed abnormal gut twist (0/71; 0.0%) (Figure 22). Among 66 comma-stage embryos observed in JU778, 4 showed abnormal gut twist (4/66; 6.0%) (Figure 23a). Among 57 comma-stage embryos observed in MY16, 3 showed abnormal gut twist (3/57; 5.3%) (Figure 23b). Results are summarised in the table below (Table 4).

Table 4. Comparison of frequencies of hermaphrodite gut/gonad heterotaxy and defects in embryo intestine organogenesis in 3 wild isolates and MT1965.

<table>
<thead>
<tr>
<th>WILD ISOLATE</th>
<th>HERMAPHRODITE GUT/GONAD HETEROTAXY AT 20°C</th>
<th>DEFECTS IN EMBRYO INTESTINE ORGANOGENESIS AT 20°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>0/300; 0.00%</td>
<td>0/71; 0.00%</td>
</tr>
<tr>
<td>JU778</td>
<td>35/772; 4.53%</td>
<td>4/66; 6.06%</td>
</tr>
<tr>
<td>MY16</td>
<td>53/504; 10.52%</td>
<td>3/57; 5.26%</td>
</tr>
<tr>
<td>MT1965 [lin-12(n941)]</td>
<td>44/303; 14.52%</td>
<td>12/18; 66.67%</td>
</tr>
</tbody>
</table>

Interestingly, defects in embryo intestine organogenesis have shown variation: while all 4 abnormal embryos in JU778 showed total absence of gut twist (rings 2, 3, and
4 remain static after comma-stage), abnormal embryos from wild isolate MY16 showed partial or incomplete gut twist. Embryos only presented successful twist in intestinal ring 3, while rings 2 and 4 remained static (Figure 23).
Figure 22. DIC images of comma-stage embryos from wild isolate N2. Intestinal rings 1 to 9 are outlined in white. Intestinal rings 2, 3, and 4 are highlighted and colour coded: left intestinal cells in purple and right cells in red. Note how intestinal gut twist in rings 2, 3, and 4 resulted in intestinal ring sisters positioned on a dorsal-ventral axis and visible from a lateral view.
Gut twist influence in anatomical handedness

Figure 23. DIC images of the comma-stage embryos from wild isolates a) JU778 and b) MY16 showing gut twist defects. Intestinal rings 1 to 9 are outlined in white. Intestinal rings 2, 3, and 4 are highlighted and colour coded: left intestinal cells in purple and right cells in red. a) All four of the defective JU778 embryos show absolute absence of intestinal gut twist in rings 2, 3, and 4. This resulted in intestinal ring sisters positioned on a left-right axis and only one sister is visible from a lateral view. b) The three defective MY16 embryos show incomplete gut twist. Embryos only present twist in intestinal ring 3 while rings 2 and 4 remain static.
4.4.3 Results – Defects in embryo intestine organogenesis correlates with gut/gonad reversals in adults

The previous results suggested a possible correlation between variation in defects in embryo intestine organogenesis and variation in gut/gonad heterotaxy observed in hermaphrodites. However, it is possible that these phenotypes could be independent of one another and it was critical to test whether there is a direct correlation between these two developmental events. Wild isolates MY16 and N2 showed significant differences in both frequencies of defects in embryo intestine organogenesis and hermaphrodite gut/gonad heterotaxy. Therefore, I took advantage once again of the phenotyped 99 panel of MY16 vs N2 RILs. Ten RILs were selected for this experiment: five RILs showing 0.0% gut/gonad heterotaxy and five RILs showing between 11.0 and 18.8%. The intestinal primordia of comma-stage embryos for each RIL were observed and scored for gut twist defects. Importantly, this experiment was performed blindly. Each RIL was randomly assigned a code name and embryo scoring was performed without knowledge of whether the target embryo belonged to a high or low heterotaxy RIL.

The results revealed that those RILs showing low rates of hermaphrodite gut/gonad heterotaxy in L4 adults (0.0%) also show low frequencies of defects in rotation of the embryonic gut cells, ranging between 0.0% and 4.9%. On the other hand, those RILs showing high rates of hermaphrodite gut/gonad heterotaxy in L4 adults (11.0-18.8%) also showed high frequencies of defects in rotation of gut cells between 10.7% and 16.4% (Figure 24). Results are summarised in the table below (Table 5).
Table 5. Comparison of frequencies of hermaphrodite gut/gonad heterotaxy and defects in embryo intestine organogenesis in a selection of 10 RILs. Code names were assigned to enable defects to be scored blindly.

<table>
<thead>
<tr>
<th>RIL</th>
<th>CODE NAME</th>
<th>HERMAPHRODITE GUT/GONAD HETEROTAXY AT 20°C</th>
<th>DEFECTS IN EMBRYO INTESTINE ORGANOGENESIS AT 20°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>JR3557</td>
<td>Delta</td>
<td>0/209; 0.00%</td>
<td>2/41; 4.88%</td>
</tr>
<tr>
<td>JR3564</td>
<td>Hotel</td>
<td>0/200; 0.00%</td>
<td>0/19; 0.00%</td>
</tr>
<tr>
<td>JR3568</td>
<td>Juliet</td>
<td>0/201; 0.00%</td>
<td>0/16; 0.00%</td>
</tr>
<tr>
<td>JR3570</td>
<td>Foxtrot</td>
<td>0/203; 0.00%</td>
<td>0/28; 0.00%</td>
</tr>
<tr>
<td>JR3580</td>
<td>Lima</td>
<td>0/208; 0.00%</td>
<td>1/46; 2.17%</td>
</tr>
<tr>
<td>JR3513</td>
<td>Mike</td>
<td>95/505; 18.81%</td>
<td>12/73; 16.44%</td>
</tr>
<tr>
<td>JR3533</td>
<td>Echo</td>
<td>86/505; 17.03%</td>
<td>3/28; 10.71%</td>
</tr>
<tr>
<td>JR3512</td>
<td>Kilo</td>
<td>78/507; 15.38%</td>
<td>5/33; 15.15%</td>
</tr>
<tr>
<td>JR3567</td>
<td>Golf</td>
<td>59/505; 11.68%</td>
<td>4/35; 11.43%</td>
</tr>
<tr>
<td>JR3498</td>
<td>India</td>
<td>56/506; 11.07%</td>
<td>5/43; 11.63%</td>
</tr>
</tbody>
</table>

A comparison between high/low groups was performed by two-way ANOVA test. The result was statistically significant for both hermaphrodite gut/gonad heterotaxy (**p<0.0006) and defects in embryo intestine organogenesis (**p<0.0003) (Figure 24). Therefore, this result provides evidence that the rotation of intestinal primordium cells during mid-embryogenesis is correlated with, and may influence, the variation in the anatomical handedness of *C. elegans* hermaphrodites.
Figure 24. Defects in embryo intestine organogenesis correlates with gut/gonad reversals in adults. Comparison of low (orange) and high (blue) for a) hermaphrodite gut/gonad heterotaxy (***p<0.0006 by two-way ANOVA test) and b) defects in embryo intestine organogenesis (***p<0.0003 by two-way ANOVA test).
4.4.4 Results – Individual embryo isolation confirms direct correlation

While I observed a correlation in the rates of heterotaxy and defects in intestinal cell rotation, it was critical to obtain a direct relationship between these two events to support a direct cause-effect between them. For this analysis I selected the strain that has shown the highest frequency of gut/gonad heterotaxy in hermaphrodites: JR3513 (95/505; 18.8%). Animals were assessed for defects in intestine primordium twist by DIC microscopy and target embryos were individually isolated and allowed to hatch and grow to adults, which were scored for gut/gonad heterotaxy.

A total of 35 comma-stage embryos from RIL JR3513 were successfully isolated and scored, eight of which showed defects in gut twist. The other 27 presented normal gut twist. Therefore, JR3513 showed 22.8% (8/35) defects in intestine morphogenesis. Among the abnormal embryos, two showed total lack of gut twist, five showed gut twist of only two rings; and one showed gut twist of only one ring (Figure 25). The frequency of defects in gut twist in JR3513 (22.9%; 8/35) is statistically similar to the frequency of hermaphrodite gut/gonad heterotaxy (18.8%; 95/505) (ns \( p > 0.05 \) based on Fisher’s exact test).

All eight abnormal embryos hatched and developed into adults that showed abnormal LR positioning of at least one gonad arm. Five embryos developed into adults showing posterior heterotaxy and three embryos developed into adults showing anterior heterotaxy (Figure 25). In contrast, all 27 normal embryos, which showed total successful gut twist of intestinal rings 2, 3, and 4, hatched and developed into adults that showed normal LR positioning of both gonad arms, i.e., they all developed into dextral animals (Figure 26).
Results are summarised in the table below (Table 6).

Table 6. JR3513 embryo isolation experiment. 35 comma stage embryos from RIL JR3513 assessed for both intestinal primordium twist defects and gut/gonad heterotaxy in adult hermaphrodites.

<table>
<thead>
<tr>
<th>EMBRYO CODE</th>
<th>NORMAL GUT TWIST?</th>
<th>INTESTINAL RINGS ROTATED</th>
<th>GUT/GONAD HETEROTAXY?</th>
<th>GUT/GONAD HETEROTAXY PHENOTYPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abnormal 1</td>
<td>no</td>
<td>none</td>
<td>yes</td>
<td>Posterior</td>
</tr>
<tr>
<td>Abnormal 2</td>
<td>yes</td>
<td>2, 3</td>
<td>yes</td>
<td>Anterior</td>
</tr>
<tr>
<td>Abnormal 3</td>
<td>no</td>
<td>3, 4</td>
<td>yes</td>
<td>Posterior</td>
</tr>
<tr>
<td>Abnormal 4</td>
<td>no</td>
<td>3</td>
<td>yes</td>
<td>Posterior</td>
</tr>
<tr>
<td>Abnormal 5</td>
<td>no</td>
<td>none</td>
<td>yes</td>
<td>Posterior</td>
</tr>
<tr>
<td>Abnormal 6</td>
<td>no</td>
<td>3, 4</td>
<td>yes</td>
<td>Anterior</td>
</tr>
<tr>
<td>Abnormal 7</td>
<td>no</td>
<td>3, 4</td>
<td>yes</td>
<td>Posterior</td>
</tr>
<tr>
<td>Abnormal 8</td>
<td>no</td>
<td>2, 3</td>
<td>yes</td>
<td>Anterior</td>
</tr>
<tr>
<td>Normal 1-27</td>
<td>yes</td>
<td>2, 3, 4</td>
<td>no</td>
<td>Dextral</td>
</tr>
</tbody>
</table>

This result is highly significant (**p<0.0001 based on Chi square test) and strongly suggests that intestinal primordium configuration during comma-stage affects the LR post-embryonic gut/gonad position.
Figure 25. DIC images of comma-stage embryos from RIL JR3513 showing abnormal gut twist. Intestinal rings 1 to 9 are outlined in white. Intestinal rings 2, 3, and 4 are highlighted and colour coded: left intestinal cells in purple and right cells in red. Each DIC picture is labelled on the left and accompanied with a worm cartoon representing the post-embryonic gut/gonad development of the given individual embryo. Note how all 8 embryos showing variation in defects in intestine primordium embryogenesis then developed into an adult animal showing abnormal gut/gonad development.
Gut twist influence in anatomical handedness
**Figure 26.** DIC images of comma-stage embryos from RIL JR3513 showing normal gut twist. Intestinal rings 1 to 9 are outlined in white. Intestinal rings 2, 3, and 4 are highlighted and colour coded: left intestinal cells in purple and right cells in red. Each DIC picture is labelled on the left and accompanied with a worm cartoon representing the post-embryonic gut/gonad development of the given individual embryo. Note how all 27 embryos show normal gut twist in intestine primordium embryogenesis and developed into an adult animal showing normal dextral gut/gonad development.
4.5 CONCLUSIONS CHAPTER 4

The results in this thesis suggest evidence of an early embryonic event that influences hermaphrodite gut/gonad development. Previous studies (Hermann, Leung and Priess, 2000; Su et al., 2000) reported abnormal gut/gonad development in some strains. The objective of this chapter was to assess those observations further to assess whether abnormal gut morphogenesis correlates with aberrant LR gut/gonad positioning.

In this chapter I have made the following key conclusions:

— lin-12(n941) has an influence on hermaphrodite gut/gonad heterotaxy

— Wild isolates variation in gut/gonad heterotaxy correlates with variation in gut twist defects

— RILs variation in gut/gonad heterotaxy correlates with variation in gut twist defects

— Individual embryo isolation demonstrates one-one correlation between embryo intestine defects and gut/gonad heterotaxy in hermaphrodites

The intestinal primordium configuration therefore has a direct effect on the post-embryonic gut/gonad development in hermaphrodites.

4.6 DISCUSSION CHAPTER 4

Through evolution, C. elegans has evolved towards optimization and simplification which can be seen, for example, in the parsimonious cell movements during gastrulation (Joshi and Rothman, 2005) and in the optimization of organ functions, such as the C. elegans kidney, which is composed of just three cells (Nelson and Riddle, 1984). The two
major organs in a *C. elegans* adult (gut & gonad) arise from intestinal and gonadal primordium cells respectively, and their development start in the embryo. Gastrulation initializes gut development, with the ingestion of the endoderm precursor cells Ea and Ep (Asan, Raiders and Priess, 2016) (Figure 18). Embryo endoderm development requires NOTCH signalling for proper arrangement of intestinal cells. After egg hatching, at the beginning of post-embryonic development, the *C. elegans* intestine is fully functional, while primordial germ cells (gonad precursors) have only begun to develop (Abdu *et al.*, 2016). It appears that this order of events may be essential: proper intestine development is dependent on early and mid-stage embryonic cell-cell signalling (Greenwald, 2005), and then gonadal development is dependent on endodermal cues (Abdu *et al.*, 2016). In other words, correct gut development may be required for the gonad to properly grow on their normal assigned body cavity, entwining around a fully-formed intestine.

The role of cell-cell communication between endodermal cells during organogenesis is key, involving signalling between non-equivalent cells (Greenwald, 1998). LIN-12/NOTCH proteins function as signalling receptors activated by the binding of DSL ligands (Greenwald, 2005). This signalling has been studied as an asymmetry modulating factor and mediator of cell-fate decisions. For example, the SOP lineage development in *Drosophila* produces 4 different cell lineages (hair, socket, neuron, and sheath). If LIN-12/NOTCH is interrupted, the SOP lineage produces 4 neurons (Greenwald, 1998). Nematode LIN-12/NOTCH studies can be directly applied to studies involving vertebrates by evidence of mammalian homologues NOTCH1-NOTCH4 (Greenwald, 1998). It is possible that the role of LIN-12/NOTCH pathway in *C. elegans*
heterotaxy may have a similar role in human organogenesis, leaving an open door for gene therapy and personalised medicine.

In *C. elegans* development, LIN-12/NOTCH is required for intestine organogenesis, and its effect is translated in gut asymmetry modulation in two developmental checkpoints: LaR → RaL transition & gut twist (Asan, Raiders and Priess, 2016) (Figure 19, Figure 20). A tantalising observation (Hermann, Leung and Priess, 2000) raised the possibility that two events (gut twist & gut/gonad heterotaxy) may be related.

Three experiments supported a connection between gut twist and heterotaxy. Finally, a direct and completed correlation was assessed in RIL JR3513 by embryo isolation, providing strong evidence that defects in gut twist in the embryo influences gut/gonad heterotaxy in *C. elegans* adults (Figure 25, Figure 26). Further embryo isolation experiments aimed to increase sample size or include more strains to consider evolutionary variation will help to clarify the underlying cause for hermaphrodite gut/gonad heterotaxy.

After the successful gut twist, only intestinal primordial cells located on the left of rings 2, 3, and 4 are in contact with the ventral midline, while their sisters on the right are not (Figure 20). Therefore, only those left cells are in contact with PGCs 401 minutes after fertilization onwards. Abnormal gut twist means that a different set of cells are in contact with PGCs 401 minutes after fertilization onwards, presumably resulting in misplacement of the gonad and gut.

I propose that exclusive physical contact of left intestinal primordial cells of rings 2, 3, and 4 is required for normal LR gonad placement. I propose that specific and controlled cues are sent by the intestine via cell-cell contacts that establish the proper
gonad positions. Interference in this precise cell-cell contact by absence of target cells and/or substitution by others leads to interference in gonad handedness establishment cues, eliciting gut/gonad heterotaxy reflected in posterior heterotaxy, anterior heterotaxy, and complete reversal phenotypes (Figure 2).

4.7 MATERIALS AND METHODS

4.7.1 Intestine primordium scoring

Animals were cultured normally and embryos were cleaned by egg-prep (Brenner, 1974) before mounting them on a 10% agar pad on a microscope slide and observed by DIC Nomarski microscopy at 1000X using a Leica DMR® fluorescence microscope. Comma-stage embryos were selected for microscopic photographs using Spot Pursuit® camera (greyscale, cooled, 1.4 megapixel) and AnalySIS LifeScience® software.

4.7.2 Individual C. elegans embryo isolation

Embryos were assessed for defects in intestine organogenesis as described above. Then, a general picture of the embryo was taken at 50X to ascertain the relative position of the target embryo, which was then manually isolated with an eye-lash tool under Motic® dissecting scope and imaged at 50X. Finally, individual embryos were transferred to a NGM plate and cultured normally at 20°C.
CHAPTER FIVE

Final Discussion and Conclusion

Handedness of *Caenorhabditis elegans* animals is determined in the 6-cell stage embryo, where the relative position of AB blastomeres determine the handedness of all subsequent asymmetries throughout embryo and post-embryonic animal development (Wood and Kershaw, 1991). Previous evidence, including the work performed in this thesis, support an invariant dextral *C. elegans* development (Wood, Bergmann and Florance, 1996). Complete reversal of all organs and cells can only be accomplished by gpa-16 mutants or micromanipulation – no natural cases of *situs inversus totalis* are found to occur naturally. However, the work in this thesis revealed a novel developmental error affecting the LR position of the intestine and gonad in hermaphrodites, or gut/gonad heterotaxy: while the overall handedness of the animal remains dextral, target organs have their relative position within the body cavity reversed. Further, this study revealed a likely causal basis for this process during embryogenesis.

5.1 FINDINGS OF THIS THESIS

Initial observations of JU778 hermaphrodites revealed abnormal gut/gonad development that did not correspond with either normal dextral organ configuration or total reversal described by Wood (Wood, 1991). I observed animals showing both goad arms on the same side of the body. This discovery lead to further screening and resulted in this remainder of this study, organised in three distinctive parts. First, I describe the phenotype (Chapter 2). Second, I attempt to characterise the underlying genetics (Chapter 3). And third, I describe an early embryonic cellular event which correlates with the heterotaxy phenotype (Chapter 4).
5.1.1 Variation in LR organ arrangement across the *C. elegans* population

In chapter 2, I expose the widespread variation in hermaphrodite gut/gonad heterotaxy among natural wild isolates and among RILs generated from two strains at the extreme ends of the distribution of phenotypes: N2 and MY16. Widespread variation plus generation of transgressive segregation in three RILs suggest a polygenic effect. Gut/gonad heterotaxy appears to be a complex and poorly understood developmental event. In addition, it appears in three distinctive forms: posterior heterotaxy, anterior heterotaxy, and complete reversal. Posterior heterotaxy is overwhelmingly more prevalent than anterior heterotaxy. This suggests that the LR misplacement event appears to occur independently for each gonad arm. Prevalence of complete reversal appears to be the result of the combined probabilities of posterior and anterior gonad arm misplacement, consistent with the events occurring independently.

Also, in chapter 2 I took advantage of the variation in gut/gonad heterotaxy expressed by strain JU778 under temperature stress to determine the possible stage at which the variation occurs. In addition to bi-directional crosses with strain N2, I delimited the temperature sensitive period of hermaphrodite gut/gonad heterotaxy between fertilization and gastrulation (26-cell stage, 150 minutes). It is notable that an event occurring as early as gastrulation, when the animal is no more than a ball of cells, influences the striking post-embryonic heterotaxy development of the animals’ two major organs. Cytoskeletal activity acts in the establishment of asymmetric gene expression in the embryo (Nance and Priess, 2002) and partitioning defective proteins (PAR) have been shown to promote actomyosin cortical contractions and cytoplasmic fluxes eliciting asymmetric cytoplasm composition in the P0 embryo. Further, cytoskeletal rearrangements elicit the very first division of the *C. elegans* embryo,
resulting in two dramatically different cells in both size and cell lineage (Sulston et al., 1983). Might cytoskeletal activity be part of the underlying cause for hermaphrodite gut/gonad heterotaxy? Evidence of the role of cytoskeletal rearrangements in LR establishment in Xenopus and snails may be evidence suggesting a similar effect in *C. elegans* (Schweickert et al., 2007). Understanding of a conserved LR establishing mechanism influenced by cytoskeletal activity and shared among bilaterians may improve our understanding in human LR syndromes, like Kartagener’s syndrome (Shapiro et al., 2016).

5.1.2 Dissecting Quantitative Traits

In chapter 3, I used phenotype and genotype data in an attempt to dissect the genetic complexity of hermaphrodite gut/gonad heterotaxy. I obtained the phenotype data from wild isolates and RILs, with a sample size of more than 40,000 animals from ~200 strains. Genotype data was obtained from online resource CeNDR (Cook et al., 2017) and from a PhD thesis pending publication (Torres, 2016). Bioinformatic analyses resulted in no significant association of hermaphrodite gut/gonad heterotaxy with genetic variation present in the *C. elegans* population. Although this is a negative result, it suggests that the genetic complexity underlying hermaphrodite heterotaxy is not restricted to a single gene or only a few genes. It is possible that there are many loci distributed along all 6 *C. elegans* chromosomes that influence this process. Further analyses will be required to dissect the genetic complexity of this event.

5.1.3 Gut twist influence in anatomical handedness

In chapter 4, I take a different approach, and attempt to find a cellular event occurring in embryo development that influences hermaphrodite gut/gonad heterotaxy. For obvious reasons, embryo development of the intestine and the gonad are the main
target. What comes first, the gut or the gonad? *C. elegans* larval stage 1 hatches from the egg with a fully formed and functional intestine, but the animal still requires 4 larval stages to develop the gonadal tissue required for reproduction (Sulston and Horvitz, 1977; Sulston *et al.*, 1983). Therefore, the intestine is fully formed when the gonad arms start to develop and grow. Perhaps abnormal placement of gonad arms in hermaphrodites is not the result of abnormal gonad development, but rather the consequence of abnormal intestine development, which then biases gonad arms to develop abnormally. I reported a direct and perfect correlation in RIL JR3513 between embryo intestine defects and adult gonad defects. Therefore, it appears that gonadal LR development is influenced by LR intestine architecture. Perhaps DTCs take advantage of unknown intestinal cues as a guidance system to guide the gonad arms – like beacons indicating the right path. Could abnormalities in intestine architecture alter this guidance system and mislead the DTCs eliciting heterotaxy? Further analyses will be required to address this question.

## 5.2 LIMITATIONS OF THIS STUDY

### 5.2.1 Variation in LR organ arrangement across the *C. elegans* population

In the study of hermaphrodite gut/gonad heterotaxy, 91 wild isolates were successfully scored. However, more isotypes are being currently isolated, catalogued and sequenced (Cook *et al.*, 2017). This will expand the ecological information currently available with the *C. elegans* natural isotypes diversity. Higher number of available isotypes will expand phylogenetic studies and provide more evolutionary background for dissecting quantitative traits.

The scoring method described in this thesis and previously (Alcorn *et al.*, 2016) allows one to score ~300 worms/hour. Optimization of scoring method to achieve
scoring rates ~1000 worms/hour could significantly increase sample size resulting on higher refinement in QTL mapping and GWAS. Important improvements have been recently made in this area (Keil et al., 2017), substituting the tedious manual observation with immobilization techniques using microfluidic devices. Larvae are grown in microchambers and periodically immobilized by compression to allow high-quality imaging. The authors successfully generated time-lapse movies of complex neural arborization through automated image registration (Keil et al., 2017). The use of microfluidics on a synchronized hermaphrodite L4 population, with an additional method to orientate the tubular nematodes into a ventral position would significantly increase the scoring rate of gut/gonad heterotaxy. In the meantime, tedious manual gut/gonad heterotaxy scoring must be performed.

5.2.2 Dissecting Quantitative Traits

By definition, QTL mapping and GWA studies are limited by the information available for carrying out the association. Stronger phenotype data (in the form of more strains and/or higher sample sizes) and more refined SNP maps can increase the sensitivity of the analysis (Lambot et al., 2004; Nuzhdin, Friesen and McIntyre, 2012). In this thesis, wild isolates and RILs were analysed using a 4,690 and 2,000 SNPs maps respectively (Andersen et al., 2012; Torres, 2016). These markers are evenly distributed along the genome, but their density often does not provide in-depth information of the regions (Torres, 2016). Important advancement are being made to generate further statistical models adjusted to C. elegans developmental studies (Cook et al., 2017). Hence it is highly possible than in the future additional analyses of the same hermaphrodite gut/gonad heterotaxy data, combined with refined statistical models, will reveal the underlying genetic cause of hermaphrodite heterotaxy.
5.3 PROSPECTIVE AND FUTURE DIRECTIONS

In the course of this study, many advances have been made towards finding the underlying cause for hermaphrodite gut/gonad heterotaxy. However, many questions have arisen throughout the course of this work that need to be addressed.

RILs created from two wild isolates at the extreme ends of the distribution of phenotypes, N2 and MY16, showed transgressive segregation in some strains. Is it possible that further generation of a second set of RILs from the extreme ends of the distribution of MY16 vs N2 RILs could generate even more extreme transgressive segregation? Is it possible to repeat the process until a strain is identified with 50% hermaphrodite gut/gonad heterotaxy (i.e., randomization of gut/gonad handedness)?

Genes responsible for an observable phenotype can be mapped to chromosomes by analysing Near Isogenic Lines (NILs) (Kooke, Wijnker and Keurentjes, 2012). To create a near isogenic line, an organism with the phenotype of interest is crossed with a standard line or reference strain of the same organism. The F1 generation is then selfed to produce the F2 generation. F2 individuals with the target trait are selected for crossing with the reference strain (the recurrent parent). This process is repeated for ~10-20 generations. This process of repeatedly crossing with the recurrent parent is called backcrossing. It should result in gradual loss of donor chromosomal segments that are not involved in the trait (Kooke, Wijnker and Keurentjes, 2012).

Further analyses to dissect the genetics underlying hermaphrodite gut/gonad heterotaxy may include MAGMA. "MAGMA is a tool for gene analysis and generalized gene-set analysis of GWAS data. It can be used to analyse both raw genotype data as well as summary SNP p-values from a previous GWAS or meta-analysis" (de Leeuw et al., 2015).
This analysis is characterized for taking $p$-values from GWAS, but without taking into account their significance. It asks if genes in a particular set (e.g., a biochemical pathway) are more strongly associated with a trait than by chance.

Finally, although individual embryo isolation provided strong evidence of the impact of embryo gut twist in adult hermaphrodite gut/gonad heterotaxy, an increase of sample size to ~100 would be desirable. Also, it would be worthy to expand the experiment to other high-frequency phenotype strains, such as other RILs such as JR3512 or JR3533 or wild isolates like MY16 or CB4857.

### 5.4 CONCLUDING STATEMENT

A developmental error affecting the relative position of the intestine and the gonad, known as gut/gonad heterotaxy, affects *C. elegans* hermaphrodites. This event is temperature sensitive and it is influenced by developmental events occurring in the early embryo. Genetic analyses reveal that this form of heterotaxy is polygenic and cannot be restricted to just a couple of genes or one genomic region. However, strong evidence suggests that hermaphrodite gut/gonad heterotaxy is influenced by the relative position of intestinal primordial cells during intestine development in comma-stage embryos. Abnormalities in embryonic gut twist lead to post-embryonic abnormal development of the gut/gonad handedness.


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