Establishing the mangrove killifish, *Kryptolebias marmoratus*, as a model species for developmental biology

Submitted by

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

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Abstract

The mangrove killifish, *Kryptolebias marmoratus*, has the potential of becoming a strong model organism for a range of biological disciplines thanks to its ability to self-fertilise, a process only known to occur in invertebrate animals until its discovery. Selfing, a natural occurrence in this species, has lead to the formation of clonal lineages composed of highly homozygous individuals. The aim of this thesis was to further establish *K. marmoratus* in the field of developmental biology by providing an information infrastructure to help advance research on this peculiar animal and further promote its place in the pantheon of model organisms.

To do so, I first set out to standardise *K. marmoratus* embryology by providing defined developmental stages with clear visual representations of key embryonic structures. This staging series is an essential tool that will ensure repeatability and consistency within and across different laboratories. Secondly, I examined several techniques for embryonic manipulation and for imaging that can be used in an array of experimental designs. Using these techniques I demonstrated microinjection of embryos by monitoring the yolk syncytial layer and its nuclei, and time-lapse analyses of the yolk surface during embryonic development. Finally, I applied the knowledge gained from my first two studies and examined Bmp signalling in *K. marmoratus* embryos and its influence on body patterning. By inhibiting this pathway, I found a new phenotype characterised by an extremely short and split body axis. These data highlighted the importance of studying known signalling pathways in unknown organisms as species-specific differences may improve our understanding of fundamental developmental processes.

This thesis demonstrates that with its easily obtainable and manipulated embryos, *K. marmoratus* can be used for embryological research in the same light as other model organisms such as zebrafish or medaka. The rising amount of information on mangrove

killifish will help further take advantage of this unique and intriguing species, and supports the use of this hermaphroditic vertebrate as a strong comparative model in developmental biology.

Table of Content

General tables and figures

Author's declaration

The experimental chapters in this thesis are presented as a series of publications. The contribution made by the first author and writer of this thesis (SM) are declared below.

Chapter 2: Mourabit, S., Edenbrow, M., Croft, D.P., and Kudoh, T. (2011). Embryonic development of the self-fertilizing mangrove killifish *Kryptolebias marmoratus*. Developmental Dynamics *240*, 1694-1704.

SM planned and carried out the experiments, analysed and interpreted the data, and wrote the manuscript.

Chapter 3: Mourabit, S., and Kudoh, T. (2012). Manipulation and imaging of *Kryptolebias marmoratus* embryos. Integrative and Comparative Biology *52*, 761-768

SM planned and carried out the experiments, analysed and interpreted the data, and wrote the manuscript.

Chapter 4: Mourabit, S., Moles, M., Smith, E. Kudoh, T. Bmp is crucial for epiboly movement and anterior-posterior axis formation in the self-fertilizing mangrove killifish embryo. In preparation.

SM planned and carried out the experiments, analysed and interpreted the data, and wrote the manuscript.

List of abbreviations

11-KT	11-ketotestosterone
17α20 β-P	17α-20 β-dihydroxy-4-pregnen-3-one
ab	air bladder
af	anal fin
AP	anterior-posterior
bd	blastodisc
bm	blastomeres
Bmp	bone morphogenetic proteins
cer	cerebellum
cf	caudal fin
ch	chorion
df	dorsal fin
di	diencephalon
dl	dorsal lip
DM	dorsomorphin
DMSO	demethyl sulfoxide
dpf	days post-fertilisation
DV	dorsal ventral
E2	17β-oestradiol
em	embryo
EVL	enveloping layer
ey	eye
fb	forebrain
Fgf	fibroblast growth factor
fmb	forebrain–midbrain boundary

fr	fin ray
gb	gallbladder
gt	gut
h	heart
H_2S	hydrogen sulphide
hb	hindbrain
HE	hatching enzyme
hpf	hours post-fertilisation
hv	hindbrain ventricle
l	lens
lj	lower-jaw
lv	liver
mb	midbrain
MeOH	methanol
mhb	midbrain–hindbrain boundary
MT	17α-methyltestosterone
n	needle
nd	nephric duct
no	notochord
od	oil droplet
op	olfactory pit
ot	otolith
ov	otic vesicle
p	melanophore
pf	pectoral fin
PFA	paraformaldehyde

ps	perivitelline space
psu	practical salinity units
PTU	1-phenyl 2-thiourea
RA	retinoic acid
s	somite
sc	spinal cord
t	testosterone
tec	optic tectum
tel	telencephalon
uj	upper-jaw
vv	vitelline vessels
y	yolk
YSL	yolk syncytial layer

List of species names

Species Name	Common Name
Artemia	Brine shrimp
Danio rerio	Zebrafish
Drosophila	Fruit fly
Escherichia coli	*E. coli*
Fugu rubripes	Puffer fish
Fundulus heteroclitus	Mummichog
Kryptolebias marmoratus (previously *Rivulus marmoratus*)	Mangrove killifish
Kryptolebias ocellatus	
Mus musculus	House mouse
Oryzias latipes	Medaka
Saccharomyces cerevisiae	Budding yeast
Xenopus	

Chapter 1: General introduction

1.1 Developmental biology

1.1.1 Preface

Embryology, the study of an embryo's development from fertilisation to hatching, and developmental biology, a broader term that also encompasses the study of how genetics control development, are the main disciplines that drive forward the undying need to explain the ancient mystery of how we are made. Aristotle was one of the first known scientists to have pondered on this question. A founder of embryology, he laid out pioneering theories on the subject of developing animals, from the nature of their development (holoblastic *versus* meroblastic cleavage), to the nature of their birth (oviparity, viviparity, ovoviviparity). His theory on epigenesis, which supports the idea that embryos grow their tissues and structures during their development, an apparent concept to researchers of our era, was later challenged by the *humonculus* of preformationism – the notion that organisms are directly produced as fully formed miniatures within the sperm or egg. This concept prevailed in the 18th century, and it is not until the advent of improved microscopy and associated techniques that the end of preformationism unfolded (Gilbert, 2000). Karl Ernst von Baer, in the 1820s, played a major role towards this outcome as his pioneering research led to the documentation of Aristotle's epigenesis, by describing anatomical structures and their formation during embryonic development. Von Baer's laws and the theory of the three germ layers, laid out fundamental principles of development, which propounded the necessity for comparative embryology (Gilbert, 2000).

A long time has passed since the discovery of the mammalian ovum, and a colossal improvement of the techniques used in developmental biology research has led to a greater understanding of the secrets of how a single fertilised cell goes through the orchestrated journey that ultimately creates a new life. Indeed, from live observations

of developing embryos using state of the art microscopy, to cell fate mapping and the creation of online gene libraries available at the ease of a click, developmental biology has entered an age that inspires excitement and fascination more than ever.

1.1.2 The body form and early patterning

The formation of a complex multicellular organism from a single cell and the various mechanisms involved in this process represents the endeavour that is developmental biology. These processes will *in fine* give rise to the finalised form of an organism, composed of specialised structures that all originated from a single cell. This is achieved through fundamental steps of development, determined and driven by gene signalling. These steps can essentially be summarised as: pattern formation, morphogenesis, cell differentiation, and growth, with all the processes that they involve being intimately linked (Wolpert et al., 2011). I will hereby explain some of the developmental processes involved in the formation of the body form in fish embryos, as these notions are of particular interest for the research conducted in this thesis. An essential outcome of embryonic development is the production of a form, which in fish presents itself as an anterior region (the head), a posterior region (the tail), an underside (the ventral side), a back (the dorsal side), and thus ultimately a left and right. Such animals are bilaterally symmetrical, and the axis running from the head to the tail is known as the anterior-posterior axis, whereas the axis dividing the ventral and dorsal sides is known as the dorsal-ventral axis (Wolpert et al., 2011).

Producing the body plan and axes is a complex process achieved early on in embryonic development. In a fish embryo, for example a zebrafish (*Danio rerio*), shortly after fertilisation the first cell (the blastodisc) visible on top of the yolk cleaves several times until it forms a large dome of thousands of cells. These then flatten and spread over the yolk, a process known as epiboly, the first morphogenetic movement

which onsets cell spatial positioning. These cells are collectively known as the blastoderm and are tiered into a protecting layer (the enveloping layer, EVL) and the deep cells. The blastoderm is adjacent to another layer directly in contact with the yolk, known as the yolk syncytial layer (YSL), which forms prior to the onset of epiboly as the marginal tier of blastoderm cells coalesces into one multinucleated syncytium (Fig. 1I shows the position of the YSL by 50% epiboly; Kimmel et al., 1995).

Epiboly of the blastoderm is accompanied with movements of the YSL, both progressing towards the vegetal pole, and the YSL is thought to be a major pulling force for epibolic movements, as it is capable of moving independently from the blastoderm but the latter cannot move without the YSL (Trinkaus, 1951; Betchaku and Trinkaus, 1978; Cheng et al., 2004; Kane et al., 2005; Solnica-Krezel, 2006). E-cadherin (for cell adhesion) and microfilaments and tubules of the cytoskeleton (providing contractile forces) are thought to play an important role in epiboly movements (Lin et al., 2009). For instance, deeps cells of the blastoderm in mutants and morpholino injected embryos for E-cadherin display delayed or arrested epiboly, although both YSL and EVL continue forward irrespectively (Shimizu et al., 2005). In addition, disruption of microtubules during gastrulation severely impairs YSL formation and thus epiboly. In that effect, it was shown that knockdown of *slc3a2* caused an activation of the RhoA/ROCK pathway, which destabilises the microtubule network of the YSL (Takesono et al., 2012). It is noted however that molecular mechanisms and the regulation of epiboly overall is still poorly understood (Lin et al. 2009).

By 50% epiboly, gastrulation begins: a fundamental landmark of development involving other morphogenetic movements (internalisation, conversion and extension) (Kimmel et al., 1995). Internalisation is the process by which mesodermal and endodermal cells at the margin of the blastoderm internalise, i.e. they move underneath the main sheet of cells back towards the animal pole. Alongside, convergence will occur

which causes tissues to converge laterally towards the dorsal side of the gastrula. In addition to extension, which causes these tissues to elongate anterior-posteriorly (towards both animal and vegetal pole) thus forming the anterior-posterior axis (Fig. 1II; Schier and Talbot, 2005). These processes of gastrulation lead to the production of the three germ layers - the ectoderm (the outer layer, e.g. skin and nervous system), the mesoderm (the middle layer, e.g. muscle, heart, blood, bones), and the endoderm (the inner layer, e.g. gut, lungs, liver) - and are essential in establishing the body plan of the embryo (Fig. 1I, II; Leptin, 2005). Indeed, gastrulation leads to the spatial repartition of differentiating cells, forming tissues and structures in their correct location. As such, by the end of gastrulation, a zebrafish embryo possesses clear anterior-posterior and dorsal-ventral axes.

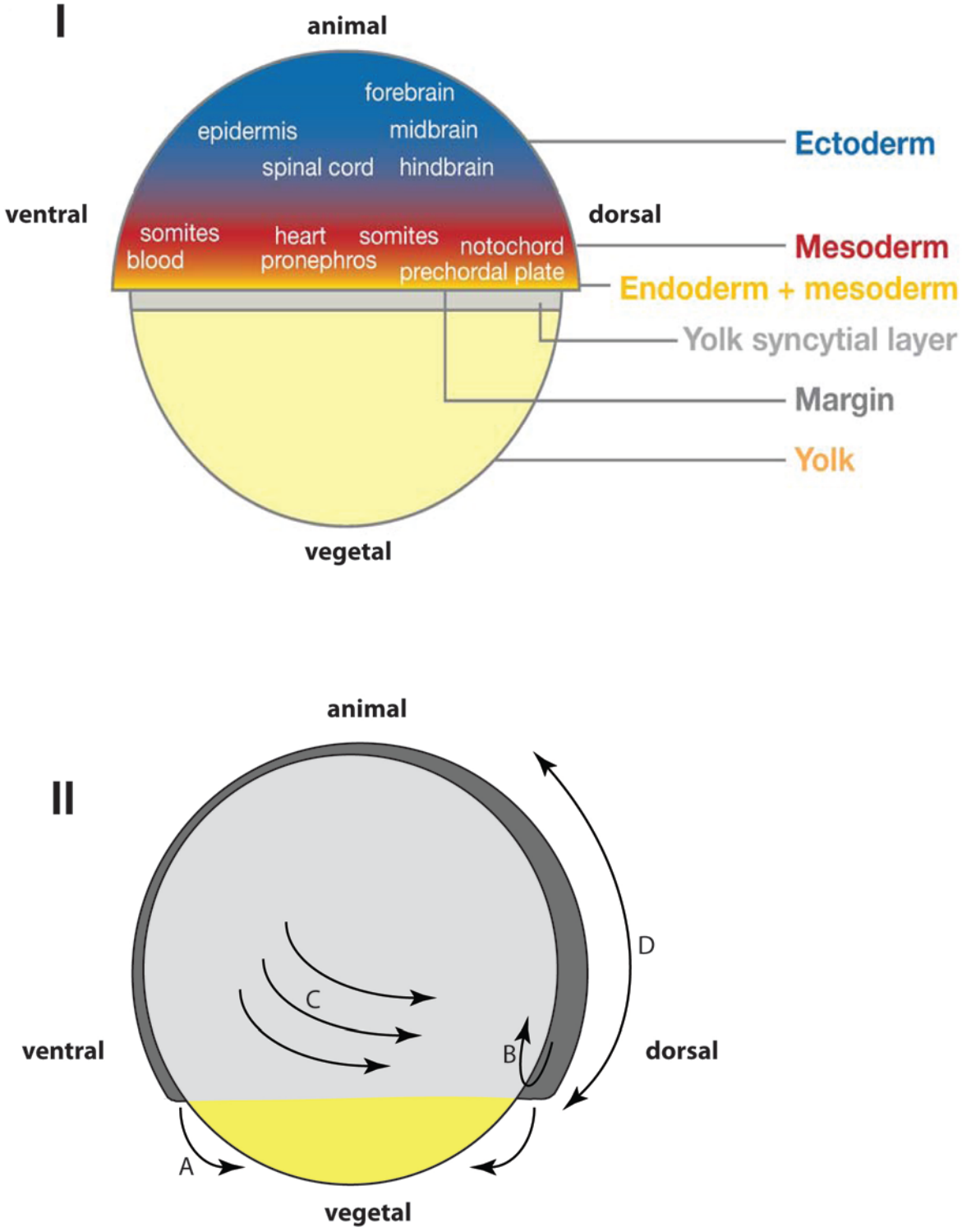

Figure 1. I: Zebrafish fate map at 50% epiboly displaying cell fates at that time and their respective germ layer. **II**: Direction of morphogenetic movements towards the end of epiboly: **A**, epiboly; **B**, internalisation; **C**, convergence; **D**, extension (modified from Schier and Talbot 2005).

1.1.3 Gene signalling is crucial in regulating early patterning

Morphogenetic movements are governed by timely activated gene expression, which leads to the highly orchestrated behaviour of cells and their differentiation in the three germ layers, ultimately forming the body plan and precursor structures for developing organs. If we focus on the earlier events that involve the formation of the body axis and patterning of the three germ layers, we can point out several major families of signalling molecules and their respective signalling centres that pattern the embryonic fate map. Not all of these will be mentioned here, and I will focus primarily on the pathway involved in research conducted in this thesis. During establishment of the body axes, essential information for spatial organisation and cell differentiation is given by the major signalling pathways including the bone morphogenetic proteins (Bmp), Fibroblast growth factor (Fgf) and the Wnt signalling pathways (Schier and Talbot, 2005).

The Wnt pathway is known to be involved in both morphogenetic movements during gastrulation (non-canonical Wnt), as well as specification of dorsal-ventral and anterior-posterior cell fates (Canonical Wnt). For example zebrafish *silberblick* (*slb*) mutants (*wnt11* mutation, involved in the non-canonical pathway) have been reported to have abnormal extension of axial tissue, resulting in fusion of the eyes later in development (Heisenberg et al., 1997; Heisenberg et al., 2000). The canonical Wnt signalling pathway is known to allow the accumulation of β-catenin in dorsal side of the embryo, which is required for the formation of the dorsal axis (Schier and Talbot 2005). In addition, Wnt signalling is also important for specification of ventral and posterior cell fates in zebrafish. For instance, morpholino knockdowns of Wnt8 have been shown to cause abnormal formation of spinal cord and posterior central nervous system (Erter et al., 2001). The Fgf signalling pathway also plays an important role in dorsal-ventral and anterior-posterior axis formation. Fürthauer et al. (2004) showed that in addition to Bmp antagonists (see below), the early activity of Fgf (pre-gastrula) is crucial for the

restriction of Bmp to a gradually more ventral position during gastrulation. To this effect, they demonstrated that inhibition of the Fgf signalling caused Bmp gene expression to expand towards to dorsal side of the embryo, thus creating an expansion of ventral cell fates.

In vertebrates, Bmp are major players in dorsal-ventral patterning of the early embryonic mesoderm and in the specification of the ectoderm to form epidermis (von Bubnoff and Cho, 2001). They operate by secreted Bmp ligands binding to type I and II Bmp receptors. These are transmembrane proteins, and the resulting complex leads to the type II receptor (a constitutively active kinase) phosphorylating the recruited type I receptor. This activated type I receptors in turn phosphorylates receptor-regulated Smad proteins (Smad1/5/8), which interact with Smad4 (known as co-smad), allowing translocation to the nucleus where they act as transcription factors (with other DNA binding cofactors) and induce a cellular response (Wrana et al., 1994; von Bubnoff and Cho, 2001). There are several inhibitory proteins that regulate this Bmp pathway in different levels, creating a gradient of Bmp expression during gastrulation, such as the antagonists Chordin, Noggin, and Follistatin (Schulte-Merker et al. 1997; Bauer et al., 1998). Patterning of the dorsal-ventral axis is largely the result of this graded spatial production of Bmp, and in fish and amphibian embryos areas of higher concentration induce the formation of ventral tissues, Bmp inhibited areas give rise to the dorsal structures of the embryo, and a graded decrease of Bmp amid these extremities leads to laterally derived structures (Dosch et al., 1997; Neave et al., 1997).

These pathways are the major players of early patterning during the blastula and gastrula stages of the embryo. As organisms are the product of their embryonic development, understanding such pathways has lead to important breakthroughs in various disciplines of biology, and these discoveries all started with experiments on model organisms.

1.2 Model organisms

For most areas of biological research, one needs a tool to answer questions and hypotheses. This tool, which provides the substance for the understanding of specific biological subjects, comes under the flag of a model organism. To summarise, the idea is that the discoveries made in a model organisms can provide information on organisms that are for instance harder to understand or work with. These species can be chosen for various reasons, with perhaps the strongest being a historical choice. Indeed, if decades (or more) of research have already been dedicated to uncovering the fundamental processes governing the development of a specific species, it is easier to further advance research by picking up where this species has brought said scientific advances, rather than starting from scratch with a new model. The house mouse (*Mus musculus*), yeast (*Saccharomyces cerevisiae*), *Escherichia coli* and *Drosophila* are amongst these 'popular' models (Powel, 1997).

Historical preferences aside, model organisms are also selected due to particular requirements for the research in question. We will briefly look at three main categories of model species to provide an understanding of their necessity. These categories are taken from the Wellcome Trust "The Human Genome" website and are described below. Note that species useful in one category may also be useful in another, and there may be other categories depending on one's research interests. History has seen (and is seeing) a great number of model organisms ranging from viruses to vertebrates, and the aim here is to consider some of the reasoning behind selecting a type of a model, and not to review all the existing model species.

1.2.1 Genetic model organisms

When studying genetics (or to put it broadly heredity), it is helpful to use organisms that provide benefits such as large progeny and short generation turnover. Indeed, these

characteristics allow for large-scale experiments where one can look at changes over generations rather than a single individual's life cycle, as seen for instance in experiments using *Drosophila*.

1.2.2 Genomic model organisms

Some species are used due to their place in evolutionary history. In this scenario, their genome is of interest due to the species phylogeny or similarity to another genome. For instance, species with similar genes to humans can be used for biomedical research and studies on human diseases. The example given here is the puffer fish, *Fugu rubripes* as it has a similar gene collection to humans.

1.2.3 Experimental model organisms

The idea here is the ease of manipulation or handling, and other such affinities for practical experimental designs. Great examples for this are small fish species such as zebrafish and medaka (*Oryzias latipes*), which provide large optically transparent embryos that can easily be collected straight after external fertilisation and rapidly develop from one cell to hatching.

1.2.3.1 Zebrafish and medaka, two popular model fish species

Such features prove to be great advantages for embryological research, and small fish species such as zebrafish and the Japanese medaka have grown to be well-established model organisms in developmental biology used in laboratories throughout the world. The Japanese medaka, *O. latipes*, is a small oviparous fish species found in fresh or brackish rivers of Asia, primarily in Japan but also in Korea and eastern China. Medaka are hardy, surviving a wide range of temperatures (10-40 °C) and able to reproduce even in seawater (Sakamoto et al., 2001; Wittbrodt et al., 2002). This species has been

studied considerably over the past 100 years and its physiology, embryology and genetics are well documented (Wittbrodt et al., 2002). The zebrafish, *D. rerio*, has become an eminent model for vertebrate genetics and development as well as human diseases and therapeutic drug screening due to certain genetic similarities with humans (Fishman, 2001, Penberthy et al., 2002; Sumanas and Lin, 2004; Lamason et al., 2005). Zebrafish originate from India, Bangladesh and Nepal, inhabiting freshwater bodies such as rivers or ditches near rice-fields, where temperatures fluctuate from 6 to 38 °C seasonally (reviewed in Spence et al., 2008).

Both zebrafish and medaka have a short generation turnover of 2-3 months, which is comparable to the mouse but shorter than *Xenopus* (6 months or 3 years depending on the species) (Wittbrodt et al., 2002). They produce a large amount of optically transparent eggs on a daily basis, with rapid development (hatching occurring 2-3 days post fertilisation in zebrafish and 10 days in medaka), making them extremely useful for developmental biology and genetics as previously mentioned (Kimmel et al., 1995; Iwamatsu, 2004). As such, a wide array of tools and methodological approaches has been established for these species, from embryonic manipulation (such as an in-depth staging series) to the availability of full genome sequence and large stocks of mutants (Kimmel et al., 1995; Furutani-Seiki et al., 2004; Iwamatsu, 2004; Jekosch, 2004; Kobayashi and Takeda, 2008; Nusslein-Volhard, 2012).

Although well established model organisms such as these two already exist, it is important to transfer the technical knowledge gained from these widely used models onto new species. Indeed, assembling such information for other closely or distantly related species can help improve our comprehension of evolutionary differences and similarities of embryogenesis between species, and thus our understanding of fundamental molecular mechanisms and developmental processes.

1.3 The mangrove killifish

In 1961 Robert Harrington published a report on an oviparous hermaphroditic fish with internal self-fertilisation. In this study, he described *Rivulus marmoratus* (Poey, 1880), a small Cyprinodontidae observed to lay eggs when kept in isolation. Adults contained eggs within their body, and he observed that they were oviposited at various developmental stages. These eggs produced hatchlings, and 6 months later these would in turn begin to lay eggs. Dissections and histological sections of these individuals revealed functional ovotestes (Harrington, 1961). Now known as *Kryptolebias marmoratus*, the mangrove killifish is a very unique simultaneous hermaphrodite and one of the two known vertebrates capable of self-fertilisation, the other being a closely related species, *Kryptolebias ocellatus* (Tatarenkov et al., 2009).

1.3.1 Geographical distribution

Self-fertilisation, and thus the ability to form a population from a single individual, as well as their amphibious nature allowing them to travel across land, have left mangrove killifish with an incredibly widespread geographical distribution. *K. marmoratus* are found in the mangrove forests of North, Central, and South America. Populations are found in Anguilla, Antigua Barb, Aruba, Bahamas, Barbados, Belize, British Virgin Islands, Brazil, Cayman Island, Cuba, Curaçao Island, Dominica, French Guiana, Grenada, Guadeloupe, Jamaica, Martinique, Mexico, Montserrat, Netherland Antilles, Puerto Rico, Saint Lucia, Saint Kitts and Nevis, Saint Vincent, Trinidad and Tobago, Turks and Caicos Islands, US Virgin Island and Venezuela (Fishbase.org).

1.3.2 Ecology

The mangrove killifish, as it name suggests, is found in mangrove forests of the previously cited locations. Mangrove forests are a variety of tropical inshore ecosystems

where several species of trees and shrubs flourish in saltwater. These tropical communities are found in low-wave, high-sedimentation shoreline estuary habitats where salty anoxic mud, extreme changes in salinity and strong tidal fluctuations are the norm (Nybakken and Bertness, 2004). In this environment, *K. marmoratus* are found in shallow water bodies, often ephemeral, and under logs and leaf litter out of the water (Huehner et al., 1985; Davies et al., 1990). To survive in this harsh environment, *K. marmoratus* have adapted to be extremely euryhaline and can be found in brackish water or hypersaline pools (Lee et al. 2008). King et al. (1989) demonstrated that *K. marmoratus* individuals were capable of withstanding extreme changes in salinity, ranging from 0 to 114 practical salinity units (psu, previously known as parts per thousand or ppt) – over three times the average salinity of seawater. Mangrove killifish can also survive in a broad scope of temperatures, ranging from 7 to 38 °C (Taylor et al., 1995).

These adaptations make *K. marmoratus* particularly easy to keep and grow in laboratory conditions, as husbandry is extremely easy and low maintenance (most laboratories keeping this species change water every 2 months). In laboratory conditions fish are generally kept at 25 °C and between 14-20 psu. They can be fed with *Artemia* daily, and kept isolated in small 1.5 L containers or as groups in larger tanks. They are tolerant to hypoxia (<1 mg/L O_2; Dunson and Dunson, 1999) and external levels of ammonia up to 446 μmol/L in 16 psu seawater (Frick and Wright. 2002).

Another great advantage that *K. marmoratus* possess for life in mangrove forests is their ability to 'breathe' air (breathing used in the sense of acquiring oxygen). This amphibious species is frequently exposed to aerial conditions in its environment, and it is capable of surviving out of water for up to 66 days in laboratory conditions (Taylor, 1990). Most fish rely primarily on their gills for gas exchange in water and respiratory adaptations are required to allow fish to be amphibious, such as specialised structures

for air breathing and gill modifications for their protection during emersion (Graham, 1997; Evans et al., 2005). In mangrove killifish, interlamellar cell mass increases in emersed individuals, preventing desiccation and the collapsing of gill lamellae, and air breathing is achieved *via* a network of cutaneous capillaries and blood vessels in their fins (Grizzle and Thiyagarajah, 1987; Ong et al., 2007). Epidermal capillaries are abundant on the dorsal side of *K. marmoratus* (from nape to trunk then gradually decreasing posteriorly), but rare or absent in other areas, and laid as close as 1 μm to the skin surface. Capillaries in the fins are limited to the dermis. These capillaries assist passive aerial gas exchange in emersed individuals, with their dorsal positioning allowing air breathing when fish are only partly emersed, or even water breathing from the oxygen-rich upper layer of the water surface (Grizzle and Thiyagarajah, 1987). *K. marmoratus* individuals may also be able to regulate blood flow to these epidermal capillaries in order to match aerial respiration demands. In addition, the increase of NH_4+ concentration (ammonia volatilisation) on the cutaneous surface of emersed fish further suggests the skin as the main site of gaseous exchange (Litwiller et al., 2006).

An all-persuasive element of mangrove forests is the presence of hydrogen sulphide (H_2S) produced from bacteria living in the mud. Abel et al. (1987) found that *K. marmoratus* individuals emersed in response to elevated levels of H_2S in the laboratory and in the wild. Other stressors have been shown to induce emersion, such as low water temperature (19-20 °C) or aggression between individuals (Huehner et al., 1985). In addition, *K. marmoratus* have been observed to feed on stationary *Drosophila*, by aligning themselves to flying individuals until they land and subsequently leaping from water to capture their prey (Abel et al. 1987). These adaptations to emersion demonstrate that this species is able to permanently exploit terrestrial resources of the mangrove forests unavailable to other fishes (Abel et al., 1987). The various biological features involved in these amphibious adaptations provide an interesting developmental

model for the study of cutaneous air breathing mechanisms, such as the vast capillary network involved in this process. In addition, the extremely resilient nature of this species coupled with its ability to breathe air makes *K. marmoratus* particularly easy to keep and grow in laboratory conditions, as husbandry is extremely easy and low maintenance. Indeed, most laboratories in the world keep *K. marmoratus* specimens individually in small 1.5 L plastic tanks (with fish either in water or emersed sticking themselves to the lid of the tank) and conduct water changes once every 2-3 months.

1.3.3 Adult morphology

Similarly to zebrafish and medaka, mangrove killifish are small in size, reaching in average a maximum of 75 mm in 2 to 3 years in the laboratory (Lee et al., 2008). This species is androdioecious (see the next section), meaning that populations are composed of males and hermaphrodites (Tatarenkov et al., 2009). Sexual dimorphism may be seen as a difference in coloration, with primary males having orange body coloration and hermaphrodites being brownish with a caudal ocellus (Fig. 2). This difference however is not always clear-cut and in order to accurately distinguish males from hermaphrodites it is best to consider both coloration and the presence of a caudal ocellus jointly (Grageda et al., 2004).

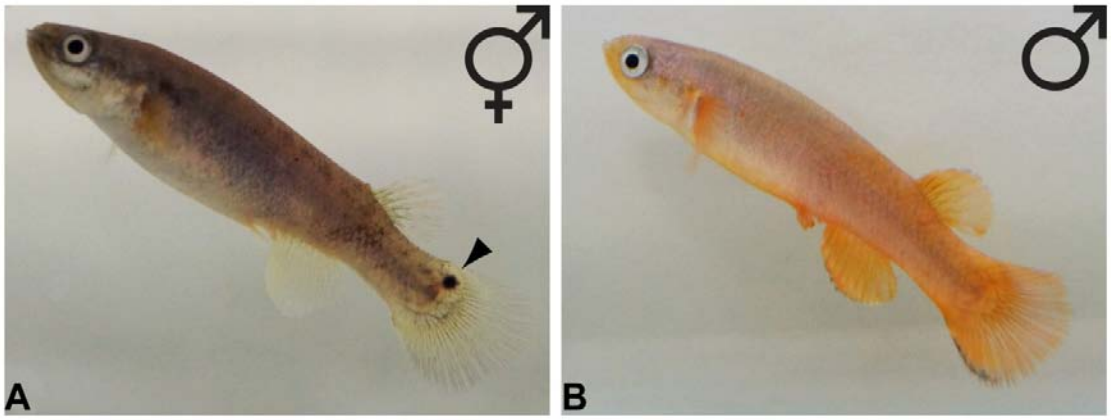

Figure 2. *K. marmoratus* hermaphrodite (**A**) and primary male (**B**) morphology. The arrowhead in **A** indicates the caudal ocellus.

1.3.4 Reproductive cycle

Simultaneous hermaphrodites are well known amongst fish species but until the discovery of *K. marmoratus* self-fertilisation was a process only known to occur in invertebrate animals (Jarne and Aude, 2006). Females are absent in *K. marmoratus* as this species, along with *K. ocellatus*, is the only known vertebrate that shows androdioecy (Tatarenkov et al., 2007). Hermaphroditic *K. marmoratus* can be kept in isolation for egg collection and similarly to zebrafish and medaka the optically transparent eggs are abundant and available daily. The unique reproductive characteristics of the mangrove killifish (discussed below) make this species an exciting and useful developmental model (see section 1.4), for studies on gonadal differentiation and the intrinsic differences in endocrinology of male/female development, but also for the advantages provided by clonal lineages and isogenic individuals.

1.3.4.1 Sexual phenotypes

Three sexual phenotypes are observed in this androdioecious system: primary males, hermaphrodites and secondary males. Primary males, i.e. individuals developed with unisexual male gonads, are rare in most of the wild populations (Turner et al., 2006), and can be the result of low incubation temperatures, no higher than 20 °C (Harrington 1967, 1968). Harrington (1968) narrowed the plastic period of sex determination in *K. marmoratus* to just before hatching, as embryos incubated around 20 °C from stage 22 (which he described as circulation through the dorsal aorta and on yolk vessels) to hatching repeatedly produced males in the laboratory. Furthermore, he found that past the hatching stage *K. marmoratus* were no longer thermolabile and individuals incubated at these low temperatures were all hermaphrodites. Secondary males may arise from hermaphrodites losing their female reproductive function 3 to 4 years in their life cycle, or due to individuals being exposed to higher temperatures and shortened

photoperiod (Harrington, 1967; Harrington, 1971; Lee et al., 2008). Thus, the proportion of males in a population depends not only on environmental temperature but also day length (Harrington, 1975).

Kanamori et al. (2006) reported that primary males could be produced easily and efficiently (97 %) in the laboratory by treating embryos with 17α-methyltestosterone (MT). Their treatment consisted of exposing embryos 12 days after hatching to MT (0.025 μg/ml) for 10 days. They found that in control embryos, oogenesis in the gonads started around 14 days after hatching, whereas spermatogenesis did not occur until two months post-hatch, further indicating the protogynous (born female) nature of this species (Cole and Noakes, 1997). In their MT treated embryos, oogenesis started normally but had completely stopped within a month, and spermatogenesis started earlier than in control individuals.

1.3.4.2 Gonad morphology

Hermaphroditic *K. marmoratus* individuals possess bilobed gonads, consisting primarily of ovarian tissue with testicular tissue closely attached to the dorsal gonadal lumen. Eggs with micropyles and no perivitelline space (unfertilised) are found in the anterior part of the gonadal lumen, the ovarian cavity, whereas eggs with a raised perivitelline space and developing blastodisc are seen in the posterior part of the gonadal lumen, which connects to the genital duct (see Fig. 1 in Sakakura et al., 2006). Oocytes of various maturation stages are found around the ovarian cavity, and mature oocytes are ovulated into the ovarian cavity and transferred into the gonadal lumen. The bilobed gonads do not possess a sperm storage structure, and free spermatozoa are found in the posterior gonadal lumen, suggesting that this region is the scene of internal fertilisation with sperm being directly discharged into it (Sakakura et al., 2006).

1.3.4.3 Endocrinology

Biological functions related to reproduction (from gametogenesis to sexual behavior and courtship) depend on stringent control and regulation of sex hormones by the endocrine system (Devlin and Nagahama, 2002). In teleosts, the ovary secrets 17β-oestradiol (E2) to regulate oogenesis, and testis produce 11-ketotestosterone (11-KT) to regulate spermatogenesis (Nagahama, 1994). In addition, progestin (such as 17α-20 β-dihydroxy-4-pregnen-3-one [17α,20 β-P]) serves as maturation inducing hormone for oocytes (Nagahama, 1997). Whereas these hormones would essentially be produced separately in males and females, or sequentially in hermaphrodites, the mangrove killifish may produce them simultaneously due to its self-fertilisation.

Examining *K. marmoratus* reproductive endocrinology, specifically gonadal plasma sex steroid hormone levels, Minamimoto et al. (2006) found that not only hermaphrodites but also primary males of this species synchronously produced estrogen, androgen, and progestin. In their study, E2 and 11-KT were both detected in the plasma of males and hermaphrodites. In addition, ovarian follicles (from hermaphrodites) and testicular tissue (from males) cultured with the precursors of 17α-hydroxyprogesterone or testosterone (T) produced T, E2, 11-KT and 17α,20 β-P. They concluded that *K. marmoratus* steroidogenesis is distinct from other Teleosts as both ovary and testis are capable of producing oestrogen, androgen and progestin irrespectively of the gonads' functional sex, but they also distinctively stand out as the ovary in hermaphrodites produces all these hormones irrespectively of gametes' maturation stage.

Since many stages of oocytes and spermatocytes can be found at any one time in a hermaphrodite (Soto et al., 1992; Sakakura et al., 2006), this mix of sex hormones is capable of regulating each stage of oogenesis and spermatogenesis synchronously, despite ovarian and testicular tissue lying in close proximity (with no thick connective

tissue separating them). The mechanisms by which this is made possible, and thus the mechanisms involved in the development of the ovotestes, ovulation, internal self-fertilisation, and spawning in the mangrove killifish are still unclear and require further research (Minamimoto et al., 2006; Sakakura et al., 2006).

1.3.4.4 Population dynamics

The selfing ability of hermaphrodites has led to the formation of highly homozygous populations, where genetic diversity either originates from *de novo* mutations or from occasional outcrossing with males, providing the basis for genetic variation in wild populations (Mackiewicz et al. 2006). Outcrossing in this otherwise clonal organism is known to be capable of providing quick and vast genotypic variety (Tatarenkov et al., 2007). The percentage of males in a population varies, although generally males are quite rare with at most 24 % in one Belize cays site (Turner et al., 1992), thus the occurrence of outcrossing events will depend on the population at hand.

The environmental reasons responsible for the production of males in the wild, as well as their relevance at the population level, are still puzzling and are an ongoing area of research in this species. When looking at the constantly moving environment of mangrove forests, due to tidal changes for instance, and the amphibious nature of *K. marmoratus* allowing it to more easily disperse to new areas in this environment, self-fertilisation provides a good means of colonisation for this species, as one individual is able in theory to recreate a new population. Males may provide a way to occasionally introduce genetic variability in these otherwise selfing populations.

1.4 *K. marmoratus* as a model organism in developmental biology

As we previously discussed, an organism is *in fine* the product of complex embryonic development which gives rise to its particular form and characteristics.

Understanding embryonic development, where all the structures present in the adult form are made, will provide key information on the fundamental processes that enable an organism to function. Such understanding is crucial, and its benefits are clearly felt in areas such as medical research with the advances on *in vitro* fertilisation and regenerative medicine with stem cells therapy, or environmental protection of endangered species. Moreover, being able to freely manipulate embryogenesis, and thus the output of embryonic development, allows researchers to produce variations of the adult form of an organism. These variations will have numerous utilities, such as the production of mutants, which help us further our understanding of specific genes and their roles and interactions.

The self-fertilising mangrove killifish has the potential of becoming a strong comparative model for developmental biology due to its selfing ability, which makes it fundamentally inbred. Indeed, certain *K. marmoratus* populations are highly homozygous and thus the existence of clonal lineages is a naturally occurring event, rather than an artificial end point produced by intensive inbreeding in a laboratory (as seen in other species such as zebrafish or medaka). The 'purity' of these available clonal lineages means that we are able to study highly homozygous individuals without the deleterious effects of unnatural inbreeding. In addition, this species has a short life cycle (3-4 months, comparable to zebrafish and medaka), is extremely easy to maintain in the lab, and provides similar advantages to other small fish models species such as a short generation turnover and a daily availability of large transparent eggs that undergo relatively rapid embryonic development (see Table 1 for a comparison). There are currently 21 established and genetically verified clonal lines in the world, of which 11 have been demonstrated to be truly isogenic, thus providing genetically identical individuals (Tatarenkov et al., 2010).

Table 1. Comparison of general features between Zebrafish, Medaka, and Mangrove killifish

	Zebrafish	Medaka	Mangrove killifish
Fecundity (fish/day)	*c.* 200 eggs	*c.* 30 eggs	*c.* 1 to 10 eggs
Generation turnover	2-3 months	2-3 months	3-4 months
Photoperiod	early morning	early morning	all day
Hatching stage	2 days	10 days	2 weeks (although can enter diapause for over a month)
Salinity	freshwater	freshwater/brackish	freshwater/brackish
Temperature	6-38 °C	10-40 °C	7 to 38 °C
Dechorionation time	few minutes	2 hours	4 hours
Transparent chorion	Yes	Yes	Yes
Amphibious	No	No	Yes
Reproductive strategy	Sexual reproduction males/females	Sexual reproduction males/females	Sexual reproduction males/ hermaphrodites (self-fertilising or outcrossing)
Maintenance	Recirculating system	Recirculating system	Standing water

A lot of interest has been shown for the unique ecology and physiology of mangrove killifish, as their adaptations to the stressful environment of mangrove forests makes them an exciting species for the study of stress responses. Such a hardy and well adapted organism may help us further understand how certain species can cope with a changing environment in the light of climate change. The ability to work with isogenic individuals means that researchers will be able to largely avoid the variation attributed to genetic diversity between individuals and focus on the effect that the environment and specific stress queues, such as fluctuating temperatures, have on the developing embryo, from molecular pathways to physiological responses. In addition, the isogenic nature of certain *K. marmoratus* lines can provide a strong model for the study of nature *versus* nurture in vertebrates.

Mangrove killifish embryos have been proposed as a model organism for carcinogenicity and toxicity studies, as the development of tumours is very frequent and only requires a short duration of exposure to carcinogens (Koening and Chasar, 1984;

reviewed in Lee et al., 2008). Davis (1986) suggested that *K. marmoratus* was a suitable model for toxicity studies, as it is used to living in small volumes of water, its amphibious adaptations facilitated the uptake of pollutants from water and possibly air, and isogenic clones would allow interclonal and intraclonal tissue transplants (Davis 1986). Finally, its unique endocrinology has made it an interesting species for the studies on characterising endocrine-disrupting chemicals (Tanaka and Grizzle, 2002; Lee et al., 2006).

Recently, *K. marmoratus* has been the subject of its first symposium and numerous advances have been made on this model organism, for instance with both its genome project and mutant screens currently ongoing (Kelley et al., 2012; Moore et al., 2012). Mutant screening is an extremely promising avenue in *K. marmoratus* research due to the clonal nature of this vertebrate. The availability of known and distinct clonal lines, with reduced genetic variability and polymorphism between individuals, makes the identification of mutations (and more subtle genetic changes) easier (Moore et al., 2012). In addition, using a selfing vertebrate in the laborious task that is mutant screening can help reduce both workload and maintenance of the lines considerably. Indeed, in species such as zebrafish or medaka a great number of individuals are required to maintain a stock for a specific line, whereas in *K. marmoratus* this can be achieved in theory with a single individual. On top of that *K. marmoratus* husbandry only requires minimal effort as fish are kept in stagnant water rather than complex circulating systems such as those used for zebrafish or medaka maintenance. Furthermore, Moore et al. (2012) demonstrated that the process of mutagenesis is one generation shorter thanks to hermaphroditic individuals allowing self-crossing and thus the production of homozygous mutant by F2 rather than F3 (as seen for instance in zebrafish mutagenesis).

The work and publications enclosed in this thesis are part of the developmental biology advances presented at this symposium.

1.5 Aim of the thesis

To study developmental biology in a model organism there are important tools that need to be available to researchers. Some of these tools will allow embryonic manipulation, for instance microinjection techniques allowing the tracing of specific tissues during development or the knockdown of certain signalling pathways. Other more fundamental tools, such as a staging series, provide the means to easily observe and record embryogenesis. The aim of this thesis was to provide some of these embryological manipulation tools and guidelines, as the mangrove killifish is a relatively new model organism lacking several important advancements of developmental biology seen in other well-established models. Establishing the necessary knowledge and information involved in embryonic manipulation of *K. marmoratus* will enable us to further research its development (morphogenesis and gene signalling for instance), and consequently the unique biological facets of this intriguing species.

1.5.1 Staging series

I first set out to standardise *K. marmoratus* embryology by providing key embryonic manipulation tools. An important starting step in this process was the establishment of a clear staging series. Even within eggs of a single clutch, embryos can develop asynchronously and such disparity can be intensified in *K. marmoratus* due to the dynamic of internal self-fertilisation and oviposition at various stages (intra-parental development). Embryonic development expressed as time post-fertilisation offers an approximation of the elapsed time since fertilisation but due to the aforementioned

variability, the need for defined stages depicting the formation of apparent morphological landmarks becomes clear. Previous literature on *K. marmoratus* embryogenesis contained some descriptions of developmental stages (Harrington, 1963, 1968; Koening and Chasar, 1984), but none had provided detailed visual representations of these structures.

1.5.2 Embryological manipulation

Alongside working on a comprehensive photographic staging series with updated descriptions, I examined several techniques for embryonic manipulation and for imaging that can be used in an array of experimental designs to further enhance the availability and efficiency of methodological approaches involved in developmental research with *K. marmoratus*.

1.5.3 Bmp signalling

Ultimately I applied these developed tools and methodological approaches to study Bmp signalling during *K. marmoratus* development, and its effect on gastrulation and the formation of the body plan. As previously mentioned, Bmp are major players in dorsal-ventral patterning of the early vertebrate embryo (von Bubnoff and Cho, 2001). This patterning is largely the result of a graded spatial production of Bmp, where higher levels of Bmp induce ventral tissues and epidermal fates, Bmp inhibited areas give rise to dorsal structures and neural fates, and a graded decrease of Bmp amid these extremities leads to laterally derived structures including the neural crest (Fig. 3; Dosch et al., 1997; Neave et al., 1997; Barth et al., 1999). This ‘default model’ of neural induction thus states that the development of dorsal and anterior cell fates comes by default and the ventralising signal of Bmp activates more ventral cell fates. It is noted however that despite the consensus on this gradient, Bmp signalling combined with Fgf

is required for the specification of trunk and tail central nervous system tissue in more posterior regions (ventral) of the gastrula, highlighting that some neural cell fates are still assigned in high Bmp regions (Fig. 3; Kudoh et al., 2004).

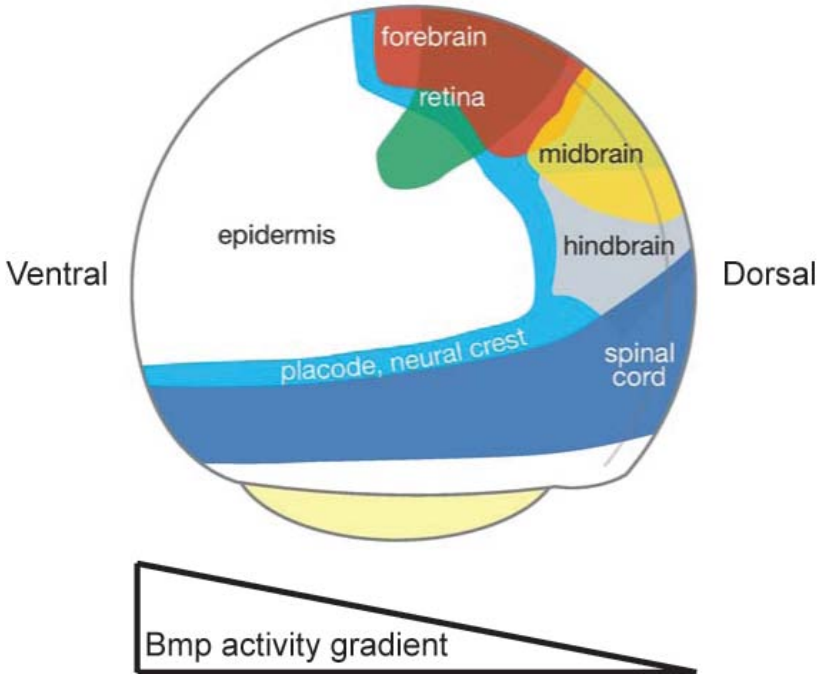

Figure 3. Zebrafish fate map of the ectoderm at 90 % epiboly, and the associated Bmp activity gradient gradually decreasing towards the dorsal side (modified from Schier and Talbot, 2005).

Mutations in genes of this signalling pathway lead to a dorsalised phenotype, i.e. an expansion of dorsal-lateral regions of the blastula at the expense of ventrally derived structures. Weaker phenotypes of mutations in genes involved in bmp signalling display a reduction in the ventral tail fin as seen in recessive phenotypes *mini fin/tolloid* and *lost-a-fin/alk8* (Mullins et al., 1966; Connors et al., 1999; Mintzer et al., 2001). With increasing severity, ventrally derived tissues such as blood and tail are not apparent, and dorsal tissues such as the notochord are expanded. A stronger phenotype, homozygous *snailhouse*/*bmp7*, is characterised by a shortened anterior-posterior axis, which twists

around itself posteriorly like a coiled snail shell (Mullins et al., 1996; Schmid et al., 2000). In the most severe homozygous phenotypes, such as *swirl/bmp2b* and *somitabun/smad5*, anterior somites expand dramatically constricting the yolk and causing it to burst (Mullins et al., 1996; Hild et al., 1999; Schmid et al., 2000).

In my last chapter, I tested if it was possible to apply the knowledge gained from my previous research to study Bmp signalling in *K. marmoratus*, and further assess its suitability as a model for developmental biology. As mutants are currently unavailable for *K. marmoratus*, I used a Bmp specific inhibitory chemical: dorsomorphin (DM). DM is a small molecule capable of blocking Bmp signalling due to its selective inhibition of Bmp type I receptors. In a normal zebrafish embryo, Bmp activates these receptors by facilitating their assembly to type II receptors. Consequently, these active type I receptors phosphorylate SMAD1/5/8 and lead to gene transcription (see section 1.1.3; Yu et al. 2008). Inhibition of Bmp signalling by DM has been observed to induce dorsalisation in a stage specific manner in zebrafish embryos. Mild phenotypes similar to the *lost-a-fin* mutants are induced when adding 10 μM DM between the shield stage and 75 % epiboly, and more severe phenotypes such as *snailhouse* mutants have been observed in response to exposures at 4 to 64-cell stages (Mullins et al., 1996; Yu et al. 2008).

Studying well known pathways in new model species can provide a different angle on research, and assessing similarities or differences will help us better understand fundamental molecular mechanisms and developmental processes.

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Chapter 2: Embryonic development of the self-fertilizing mangrove killifish *Kryptolebias marmoratus*

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Chapter 4: Splitbody: Bmp is crucial for epiboly movement and anterior-posterior axis formation in the self-fertilizing mangrove killifish embryo

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Splitbody: Bmp is crucial for epiboly movement and anterior-posterior axis formation in the self-fertilizing mangrove killifish embryo

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Summary

Bone morphogenetic proteins (Bmp) are major players in the formation of the vertebrate body plan due to their crucial role in patterning of the dorsal-ventral (DV) axis. Despite the highly conserved nature of Bmp signalling in vertebrates, the consequences of inhibiting this pathway can be species-specific. Here, we report that Bmp plays an important role in epiboly, yolk syncytial layer (YSL) movements, and anterior-posterior (AP) axis formation in embryos of the self-fertilising mangrove killifish, *Kryptolebias marmoratus*. Stage and dose specific exposures of embryos to the Bmp inhibitor dorsomorphin (DM) produced three distinctive morphologies, with the most extreme condition creating the "splitbody" phenotype, characterised by an extremely short AP axis where the neural tube, somites, and notochord were bilaterally split. In addition, parts of caudal neural tissues were separated from the main body and formed cell 'islands' in the posterior region of the embryo. This "splitbody" phenotype, which has not been observed in zebrafish, shows that the suppression of Bmp may lead to significantly different consequences during development in other vertebrate species.

Results and Discussion

Stage specific inhibition of Bmp induces a split body axis in *K. marmoratus* embryos

Patterning of the DV axis is the result of a differential spatial activation of Bmp signalling during early embryogenesis. For instance, in zebrafish embryos, areas of high Bmp concentration ventrally induce the formation of tissues such as epidermis and tail spinal cord (Kudoh et al. 2004). Although the Bmp signalling pathway is highly conserved in vertebrates, modified Bmp activity can lead to different consequences depending on the morphological and genetic characteristics of the species. For example, whereas zebrafish chordino mutants, which have a null mutation in the gene coding for the Bmp-antagonist Chordin, have a significantly smaller brain (Schulte-Merker et al. 1997), chordin knock-out mice do not show such clear reduction in brain size, possibly due to a differential redundancy of another Bmp-antagonist, Noggin (Bachiller et al. 2000). Here we show that reduction of Bmp activity in *K. marmoratus* achieved by addition of the specific inhibitor DM, resulted in significantly different morphological defects compared with the phenotypes previously reported in zebrafish (Mullins et al. 1996; Yu et al. 2008).

To examine the evolutionary conserved and divergent role of the Bmp signalling pathway in a new model species, and to further investigate the embryonic stage specific role of Bmp in vertebrates, *K. marmoratus* embryos were treated with DM in a stage specific manner. The *snailhouse* phenotype seen in early DM exposures in zebrafish (Yu et al. 2008) was mimicked in *K. marmoratus* by exposing embryos to 100 μM DM from the late blastula stage (Figure 1 C) (see Mourabit et al. 2011 for *K. marmoratus* developmental stages), displaying the distinctive shortened curled tail morphology (Figure 1 C3 arrowhead) by 3 days post fertilisation (dpf). Embryos treated with the same concentration but starting from late epiboly (Figure 1 D) produced a milder

phenotype characterised by its bent tail (Figure 1 D3 arrowhead). However, DM exposures from the 32-cell stage (Figure 1 B), much earlier in development and comparable to exposure from 4 to 64-cell in zebrafish, produced a distinctive phenotype, hereby referred to as "splitbody", characterised by its short body (Figure 2 B1), morphologically undifferentiated head region (Figure 2 B2 arrowhead), split body axis (Figure 2 B2 arrow) and 'cell island' formations in the posterior region of the embryo (Figure 2 B3 arrowhead). This "splitbody" phenotype has not been observed in other model species including zebrafish, *Xenopus*, chick and mouse.

Bmp is essential for normal epiboly progression in *K. marmoratus*

To determine the cause of a divided AP axis in the "splitbody" phenotype, we first examined differences in early development between wild type and embryos exposed to 100 μM DM at the 32-cell stage (Figure 2 A, B, C). During gastrulation, Bmp inhibition was shown to clearly delay epiboly progression. At 1 dpf, when control embryos reached *c.* 75 % epiboly (Figure 2 A1), DM treated embryos were delayed with epiboly covering *c.* 30 % of the yolk (Figure 2 A2). Similarly at 2 dpf, control embryos entered the otic vesicle formation stage (Figure 2 B1), whilst exposed embryos lagged behind with epiboly covering *c.* 90 % of the yolk (Figure 2 B2). Such a significant delay in epiboly, resulting from Bmp signalling inhibition, has not been observed in zebrafish embryos (Mullins et al., 1996).

Prior to gastrulation, the embryo is composed of 4 layers, the enveloping layer, deep cells, the yolk syncytial layer (YSL) and the yolk (Kimmel et al., 1995). It is known that in zebrafish, during late gastrulation, delays in movements of the deep cells do not equate to delays in the YSL (Kane et al. 2005). Furthermore, research in *Fundulus* has shown that the movements of the YSL are independent from the blastoderm, as the YSL continues its epibolic migration if the blastoderm is removed (Trinkaus, 1951). Thus, in

order to determine if inhibition of Bmp signalling also delayed YSL movements, yolk syncytial nuclei (YSN) were stained by sytox green injection at the late blastula stage. Embryos were then exposed to 200 μM DM, a concentration capable of mimicking the "splitbody" phenotype (see the next section and Figure 3). Embryos were observed the next day, and whilst controls reached the eye formation stage with YSN spread throughout the yolk (Figure 2 C1), both the YSN and the blastoderm of DM exposed embryos were delayed at *c.* 50 % epiboly (Figure 2 C2). This data suggests that the YSL is also affected by the inhibition of Bmp, as YSN were moving relative to the blastoderm margin and displayed the same level of delay.

Laterally derived structures and the notochord are divided in "splitbody"

The severe delay in epiboly movements previously described suggests that during gastrulation, the laterally derived structures at the margin of the blastoderm are unable to correctly merge at the end of epiboly, leading to the formation of a split body axis. To confirm this hypothesis, we examined the spatial arrangement of the neural tube and somites in "splitbody" (Figure 3 & 4), two tissues possibly derived from lateral gastrula domains as in zebrafish (Kimmel et al., 1990), using Hoechst (for the body contour), sox3 *in situ* hybridisation (for the neural tube), and MF-20 immunofluorescence (for somites) staining on the "splitbody" embryos.

Hoechst and *sox3* staining confirmed a split in the body axis and the neural tube for embryos exposed to both 100 μM DM at the 32-cell stage and 200 μM DM at the late blastula stage. Despite this split, some individuals were observed to have an opened end (Figure 3 B1, 2 (19/20) & D1, 2 (12/20) arrowheads) of the two neural tube strands, whilst others had a closed end with both strands joining in their most posterior section (Figure 3 C1, 2 (1/20) & E1, 2 (8/20) arrowheads). Furthermore, the cell 'islands' seen in "splitbody" were observed by Hoechst staining (Figure 3 B3, C3, D3, E3

arrowheads), and displayed *sox3* positive staining suggesting that the island is partly composed of neural plate cells (Figure 3 B4, C4, D4, E4 arrowheads).

The separation of laterally derived structures in "splitbody" was further demonstrated by the staining of somite muscles in embryos 4 dpf using the myosin antibody MF-20. Somites are part of the laterally derived structures of the blastoderm during gastrulation of zebrafish (Kimmel et al., 1990). If epiboly occurs correctly, somites form pairs either side of the neural tube of the developing embryos (Figure 4 A1, 2, 3 arrowheads). In the "splitbody" phenotype, somites were unpaired and appeared divided in the two strands of the embryonic body axis (B1 Hoechst staining showing the clear split of the body axis; B2, 3 arrowheads, somites are present in both strands of the divided body).

As the two body axes in "splitbody" were composed of laterally derived structures unable to merge correctly, the notochord, which arises from the dorsal axis (Kimmel et al., 1990), may either grow in one of the two body strands or split in both. To determine the outcome of notochord development in "splitbody", we stained this tissue with *ntl in situ* hybridisation (Figure 4 C, D) using a medaka probe (Araki et al., 2001). In a normal 4 dpf *K. Marmoratus* embryo, *ntl* stains notochord tissue in the tip of the tail (Figure 4 C2 arrowhead), however in "splitbody" individuals both tips were stained, suggesting that the notochord splits and grows into the two body axes (Figure 4 D2 arrowheads).

DM dose dependence of the "splitbody" phenotype

Given that embryos treated to 100 μM DM from 32-cell and 200 μM DM from late blastula displayed a similar "splitbody" phenotype, we hypothesised that the 100 μM dose took longer to fully suppress Bmp signalling, but as embryos were treated earlier in development Bmp was fully suppressed by the mid-blastula transition and produced

"splitbody". To explore this hypothesis, we examined the level of Bmp signalling activity by measuring phosphorylation of Smad1/5 with Western blotting. Embryos exposed to 100 μM from 32-cell as well as 100 and 200 μM DM from late blastula demonstrated that these treatments equally suppressed phospho-Smad1/5 by late gastrula (Figure 6 A and B). These results confirmed that DM effectively suppressed Bmp signalling during gastrulation, but also suggested that zygotic Bmp is key for normal epiboly movements, as the 100 μM treatment only produced "splitbody" if applied earlier in development.

Conclusions

Here, we report a defect in the merging of laterally/ventrally derived structures at the end of epiboly and a division of the notochord, as a result of Bmp signalling inhibition. Both neural tube and somites were unable to completely merge at the end of gastrulation due to delayed epiboly and YSL movements, thus producing the "splitbody" phenotype.

The YSL plays an important role in epiboly movements of the blastoderm. Studies in *Fundulus* have shown that although epiboly can take place after removal of the blastoderm, the latter is unable to perform this morphogenetic movement without the YSL (Trinkaus, 1951; Betchaku and Trinkaus, 1978). Here, we demonstrate that the delayed blastoderm was accompanied by a delay in movements of the YSN. It is unclear whether the delay in movements of the YSL is triggered by the setback of the blastoderm, but the relative independence of the YSL from the blastoderm discussed previously suggests that Bmp plays a crucial role in movements of the YSL during gastrulation.

It is known from zebrafish research that the Bmp gradient of the zebrafish gastrula regulates convergent extension (CE), as this morphogenetic movement is absent

ventrally whilst lateral tissues display increased CE until the dorsal side where convergence weakens and extension stays strong (Myers et al., 2002). This pattern corroborates with and is driven by the ventrally-high, laterally-gradient, and dorsally-low distribution of Bmp signalling during gastrulation (Myers et al. 2002). Here, "splitbody" *K. marmoratus* embryos experienced low to absent levels of Bmp throughout the gastrula thus disturbing CE movements. In the absence of strong convergence, lumps of cells could be isolated from the main body axis. In addition, the weakened extension movements explain the extremely shortened body axis of the "splitbody" phenotype.

The variation observed in "splitbody", where some individuals exhibited an opened double body axis and others a closed one, resulted from the stage and dose specific exposure of *K. marmoratus* embryos to DM, with the more mild treatments producing the *snailhouse* equivalent. "Splitbody" was only produced when embryos were treated early in development (100 μM at 32-cell stage or 200 μM at late blastula) to ensure a delay in epiboly at gastrula and thus a split in the body axis. These results suggested that zygotic Bmp is key for normal epiboly movements, as the 100 μM treatment only produced "splitbody" if applied earlier in development, thus giving DM the time to fully suppress Bmp by the onset of epiboly. Recent studies have shown the temporal importance of Bmp signalling in patterning DV tissues along the AP axis (Tucker et al., 2008). Our data demonstrate that Bmp signalling is also crucial for the correct timing of epiboly closure and thus the formation of a single anterior-posterior body axis in the mangrove killifish. Such results have not been shown in zebrafish, suggesting that the suppression of Bmp may have different consequences during development in other fish species.

The mangrove killifish produces eggs roughly two times bigger than zebrafish. As the blastoderm is under extreme tension to move over the yolk during epiboly, the larger

yolk of *K. marmoratus* embryos may increase the stretch required for the sheet of deep cells to reach the mid-gastrula point, which may enhance the epiboly defect less obvious in zebrafish. Furthermore, the differential genetic background of this species may result in varying levels of severity of the patterning defect caused by Bmp suppression. Both a genome project and a mutant screen are currently ongoing for the mangrove killifish (Kelley et al., 2012; Moore et al., 20012), and will help further uncover the molecular mechanisms and phenotypic variation of the loss of function of Bmp signalling between different species.

Experimental procedures

Experimental Animals

K. marmoratus of the Hon9 clonal lineage were obtained from an existing stock at the University of Exeter (UK). Hermaphroditic individuals were kept individually in 1.5 L plastic containers (25 °C, 14 psu (practical salinity units), 12:12 h light:dark photoperiod) and were fed daily *ad libitum* on *Artemia* nauplii. Brackish water was made using demineralised water and marine salts (Tropic Marin, Germany). Eggs were collected from aquaria filter pads placed in the containers (Pondmaster filter foams), and provided a substrate for oviposition. Both control and DM treated embryos were reared under the same conditions as adult individuals. Embryonic stages were determined using the staging series in Mourabit et al. 2011.

Experimental protocols

Imaging

Micrographs were taken using a Nikon Digital Sight DS-U2 camera mounted on a Nikon SMZ1500 microscope and an Olympus XC10 camera mounted on an Olympus SZX16 microscope. Imaging of live and fixed *K. marmoratus* embryos was performed

using the Agarose bed and methyl cellulose techniques respectively, described in detail by Mourabit et al. (2011, 2012). For control, drug treated and injected embryos, 10-20 embryos were used for each experiment.

Dorsomorphin exposures

Stock solutions for dorsomorphin (DM) (6-[4-(2-Piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine, Sigma P5499) were prepared as 10mM dissolved in demethyl sulfoxide (DMSO) and diluted in 14 psu brackish water to final concentrations. Embryos at the 32 cell, late blastula and 80 % epiboly stages were exposed to different concentrations of DM (100 μM and 200 μM).

Whole-mount in situ *hybridisation*

K. marmoratus sox3 was cloned by PCR using the following primers: forward GAGTGTGTGAGTGATCACTGA, and reverse TCTGAGAGTGGGACGTGATGG. Primer design was based on *K. marmoratus* sox3 sequence information obtained by Illumina RNAseq sequencing:

GAGTGTGTGAGTGATCACTGAAAGCCGGCCGAATGTATAACATGATGGAAA
CCGAGCTGAAGACCCCGCTCCCGCAGTCCAACTCGGGCTCGGCGCCGGGCG
CGAAGAACAACAGTGCCAGCGACCAGGAGCGGGTAAAGCGGCCGATGAAC
GCCTTCATGGTCTGGTCCCGGGGCCAGCGGAGGAAGATGGCACAAGAGAAC
CCCAAAATGCACAACTCTGAAATCAGCAAGCGGCTCGGCGCTGACTGGAAA
CTTCTGACTGACGCCGAGAAGAGGCCGTTCATCGACGAGGCCAAGCGTCTG
CGCGCGATGCACATGAAGGAGCATCCGGATTATAAATACCGGCCCCGCAGG
AAGACCAAGACCTTGCTCAAGAAAGACAAGTATTCTTTGCCCGGGGGGCTG
CTGGCGCCAGGAACCAATACCGTCAACAACTCGGTGTCGGTGGGGCAGCGC
ATGGACGGTTACGCGCACATGAACGGCTGGACGAAAGCGCGTACTCGCTCA
TGCAGGACCAGCTGGCCTACCCTCAGCATCACGGCATGAACAGCCCGCAGA
TCCAGCAGATGCACCGGTACGAGATGGCGGGCCTGCAGTACCCGATGATGT
CCTCGGCGCAGACCTACATGAACGCGGCGTCCACCTACAGCATGTCCCCGG
CGTACACGCAGCAGAGCCCCAGCGCCATGGGCCTGAGCTCCATGGCGTCCG
TGTGCAAGACCGAGCCCAGCTCACCGCCGCCGGCCATCACGTCCCACTCTC
AGA

The PCR product was inserted into the pGEM-T Easy vector (Promega). *Escherichia coli* colonies containing this plasmid were cultured and the plasmid DNA was then purified using a QIAprep Spin Midiprep (QIAGEN). The plasmids were digested with *PstI*, and Digoxigenin-labelled RNA probe was synthesised by T7 RNA polymerase (Roche).The medaka *ntl* plasmids (Araki et al. 2001) were digested with *SalI*, Digoxigenin-labelled RNA probe was synthesised by T3 RNA polymerase (Roche).

K. marmoratus embryos were fixed at the required stages of development using 4 % paraformaldehyde (PFA) (14 psu brackish water, 20 mM HEPES buffer, pH adjusted to 7) in 1.5 ml Eppendorf tubes (5 embryos/tube) at room temperature for 4 days. Following fixation, these embryos were washed with 1 ml phosphate buffer saline (10 min) then manually dechorionated and dehydrated in 1 ml 100 % methanol at -20 °C for one hour (they can be stored at this step for several weeks). These embryos were then used for whole-mount *in situ* hybridisation, performed according to the method described by Kudoh et al. (2001), with modifications. A full protocol is available in the supplemental information section.

Hoechst and immunofluorescent staining

For MF-20 antibody staining (Hybridoma Bank), fixed embryos stored in methanol (see above for conditions), were rehydrated in PBSTx (PBS + 0.5 % Tritonx, Sigma) and further permeabilised using Proteinase K. Control and DM treated embryos at day 3 post-fertilisation were treated to 10 μg/ml PK for 5 minutes, and day 4 embryos for 10 minutes. These embryos were then washed in PBSTx to stop the digestion and re-fixed with 4 % PFA for one hour at room temperature. Embryos were put in blocking solution for 3 hours at room temperature (1 % skimmed milk and 1 % DMSO in PBSTx), and then incubated in primary antibody overnight (1:20 monoclonal mouse antibody MF-20

in blocking solution). The next day, the primary antibody was thoroughly washed (four series of 30 minute washes in PBSTx), and incubated in Alexa Fluor 546 goat anti-mouse IgG secondary antibody overnight (Invitrogen, A11003). Finally, the secondary antibody was thoroughly washed with four series of 30 minute washes in PBSTx.

For Hoechst staining, *sox3* or MF-20 stained embryos were incubated in a Hoechst solution for 30 minutes (0.5 μg/ml in PBSTx). The solution was then thoroughly washed (four series of 30 minute washes in PBSTx) and embryos were ready for imaging.

Microinjection

Microinjection of sytox green (Invitrogen) into the yolk syncytial layer (YSL) was performed following the procedure described by Mourabit et al. (2011, 2012). Sytox green (0.5 mM) was injected in the YSL at the late blastula stage and fluorescent yolk syncytial nuclei were photographed at day 1 post-fertilisation in control and DM treated embryos.

Western Blotting

Embryos at the late blastula stage were lysed in cold 2x lysis buffer (4 % SDS, 20 % glycerol, 125 mM Tris-HCl pH 6.8, 50 μg/ml BPB, 10 % β-Mercaptoethanol) at 5 embryos/ 400 μl lysis buffer. Lysates were clarified by centrifugation (14.5 Krpm for 5 minutes) and the supernatants were heated at 70 °C for 10 minutes then analysed by SDS-PAGE. Western Blots for Smad1/5/8 (1:200; Santa Cruz Biotechnology, sc-6031-R) and Phospho-Smad1/5 (1:1000: New England Biolabs, 9516S) were performed according to the manufacturer's instructions (blocking solution , for pre-blocking and dilution of all the antibodies, was composed of 2 % bovine serum albumin in Tris buffer saline (20 mM Tris-Hcl pH 7.5 and 150 mM NaCl).

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Figure Legends

Figure 1. Stage specific inhibition of Bmp in *K. marmoratus.* Embryos were exposed to 100 μM dorsomorphin at the 32-cell (**B**), late blastula (**C**) and 80 % epiboly (**D**) stages of development. Photographs of the embryos were taken 3 days post-fertilisation. **A1-3**: Control. **B1-3**: "splitbody", this phenotype is characterised by absence of a distinct tail region (**B1** arrowhead), morphologically undifferentiated head region and split body axis (**B2** arrowheads), and cell 'island' formations, described as isolated lumps of cells, in the posterior region (**B3** arrowhead). **C1-3**: Curled tail, this phenotype resembles *snailhouse* seen in zebrafish and is characterised by its curled tail (**C3** arrowhead). **D1-3**: Bent tail, this phenotype primarily displayed a bent tail (**D3** arrowhead). All images are dorsal views of the embryos. Scale bars: 250 μm.

Figure 2. Bmp inhibition delays epiboly progression in *K. marmoratus.* Epiboly coverage was recorded at day 1 (**A**) and day 2 (**B**) post-fertilisation (dpf) in embryos exposed to 100 μM dorsomorphin (DM) at the 32-cell stage. Progression of the yolk syncytial later (YSL) during gastrulation was assessed via staining the of yolk syncytial nuclei (YSN) using Sytox green Fluorescent YSN were observed 1 dpf (**C**). **A1, 2:** As control embryos reach *c.* 75 % epiboly (**B1** arrowhead), DM treated embryos are delayed with epiboly covering *c.* 30 % of the yolk (**A2** arrowhead). **B1, 2**: Controls reach the otic vesicle formation stage (**B1**) whilst exposed embryos are lagging behind around 90 % epiboly (**B2** arrowhead). **C1**, **2**: Shortly after epiboly closure, control embryos enter the eye formation stage (**C1**) and YSN are spread all over the yolk (**C2**). On the other hand DM exposed embryos are still mid-epiboly and fluorescent YSN are observed near the blastoderm margin (**C2** arrowhead), demonstrating that YSN are also delayed by inhibition of Bmp signalling. All images are lateral views of the embryos. Scale bars: 250 μm

Figure 3. The neural tube is separated in embryos of the "splitbody" phenotype. *K. marmoratus* embryos were exposed to 100 μM dorsomorphin (DM) at the 32-cell stage (**B, C**), and 200 μM DM at the late blastula stage (**D**, **E**) of development. These embryos were then fixed 3 days post-fertilisation and used for *in situ* hybridisation using a *sox3* probe (stains all neural tissue) (A-E2, A-E4) and Hoechst staining (a blue fluorescent DNA stain) (A-E1, A-E3) in order to examine body contour and split neural tube (**A-E1**, **2**), and the nature of the posterior isolated cell lumps or cell 'islands' (**A-E3**, **4**). **A1-4**: Control embryo. **B, D**: "splitbody" individual with an opened end of the body axis and neural tube split (**B** arrowhead; n = 19/20, and **D** arrowhead; 12/20). "splitbody" individuals with a closed end, as both strands of the body axis and neural tube join in their most posterior region (**C** arrowhead; n = 1/20 and **E** arrowhead; 8/20).

All DM embryos presented here generated cell 'islands' (**B3, E3** arrowhead) with distinct *sox3* positive staining (**B4, E4** arrowhead). Photographs were taken 3 days post-fertilisation. All images are dorsal views of the embryos. Scale bar: 250 μm

Figure 4. Somites and the notochord are divided in the "splitbody" phenotype. *K. marmoratus* embryos were exposed to 100 μM dorsomorphin at the 32-cell stage and fixed 4 days post-fertilisation in order to stain somites using the myosin antibody MF-20 (**A**, **B**), and the notochord by *in situ* hybridisation using a medaka *ntl* probe (**C**, **D**). When epiboly unravels correctly, somites are formed as pairs arranged either side of the neural axis (**A1-3**, arrowheads). In the "splitbody" phenotype however, somites were unpaired as they appeared separated in the two strands of the embryos body axis (**B1** Hoechst staining showing the clear split in the body axis; **B2, 3** arrowheads, somites are present in both strands of the divided body). In control embryos, *ntl* stained the notochord in the tip of the tail (**C2** arrowhead). "splitbody" embryos had the tips of both body axes stained with *ntl*, demonstrating that the notochord divides into both strands (**D2** arrowheads). Photographs were taken 4 days post-fertilisation. All images are dorsal views of the embryos. Scale bars: 250 μm

Figure 5. Dorsomorphin inhibits phosphorylation of Smad1/5. Bmp signalling activity was quantified by measuring phosphorylation of Smad 1/5 at the late gastrula stage. Embryos were exposed to 100 μM from 32-cell as well as 100 and 200 μM DM from late blastula. These were then frozen at the late gastrula stage and used for Western Blotting. **A:** Quantification of densitometry results obtained from 3 independent experiments (Mean ± standard error of the mean), normalised to total Smad and indicated as fold increase over the resting control condition. **B:** Representative Western Blot of 3 independent experiments showing the levels of total Smad1/5/8 and phospho-

Smad1/5 in a dose and stage specific manner. These data demonstrate that all three treatments equally suppress phospho-Smad1/5 by late gastrula.

Supplemental Information

Mangrove killifish whole-mount *in situ* hybridization

Embryos were fixed in 1 ml paraformaldehyde (4 % PFA (Sigma), artificial sea salts at 14 psu (Tropic Marin), 20 mM HEPES (Sigma), pH 7) at the desired stage in 1.5 ml Eppendorf tubes for 4 days at room temperature (5 embryos/tube). After fixation, PFA was removed and the embryos were washed with 1 ml of phosphate buffer saline (PBS) for 10 minutes. During this wash the embryos were dechorionated with fine tweezers. Dechorionated embryos were then transferred to methanol by a series of gradual changes (25, 50, 75 % in PBTw (1x PBS, 0.1 % Tween20) for 1 minute each, then left in 100 % methanol at -20 °C for one hour (this step can be used to store embryos for several weeks).

Methanol was then gradually washed (75, 50, 25 % MeOH in PBTw for 1 minute each) and the embryos were then left in 1 ml of PBTw for 10 minutes.

[Optional *stage*: further permeabilisation of the embryos can be achieved by exposing them to proteinase K (Sigma) (10 μg/ml, in PBTw) at room temperature for 5 minutes (3 days post-fertilisation embryos or older, timings may change). Embryos were then washed with PBTw for 5 minutes (twice), fixed in 4 % PFA for 1 hour, then washed with PBTw 5 minutes (twice).]

Embryos were then pre-hybridised in 0.5 ml Hyb buffer at 65 °C for one hour (Hyb buffer: 50 % formamide, 5x saline sodium citrate (SSC), 5 mM EDTA, 0.1 % Tween20, 0.1 % CHAPS, 50 μg/ml heparin, 1 mg/ml torula RNA). Hyb buffer was then replaced with 0.2 – 0.5 ml of probe/Hyb buffer and left at 65 °C overnight (the diluted probe in

Hyb buffer was preheated at 80 - 90 °C for 10 minutes and immediately chilled on ice for 5 minutes before it was used).

The probes were recovered the next day (they can be used a few times), and embryos were washed with 1 ml of washing solution 1 (50 % formamide, 2x SSC, 0.1 % Tween20) at 65 °C for 30 minutes. They were then washed with 1 ml washing solution 2 (2x SSC, 0.1 % Tween20) at 65 °C for 30 minutes. Finally the embryos were washed with 1 ml washing solution 3 (0.2x SSC, 0.1 % Tween20) at 65 °C for 30 minutes (this last was performed twice).

Embryos were then washed with 1 ml PBTw at room temperature for 10 minutes. The samples were then placed in 0.5 ml blocking solution for one hour. Blocking solution is prepared with 1 g blocking reagent (Roche) dissolved in 47.5 ml maleic acid buffer (MAB), and 2.5 ml of lamb serum was then added when the solution had cooled down. MAB: 0.1 M maleic acid, 150 mM NaCl, pH to 7.5.

Embryos were then placed in 0.2 - 0.5 ml anti-DIG solution (diluted 1:100 in blocking solution) for 2 hours. The anti-DIG solution was made by mixing 10 μl Anti-DIG antibody conjugated with alkaline phosphatase (Roche) with 500 μl of blocking solution and 20 fixed zebrafish embryos (bud, somitogenesis or 24 hour stage; these must be PFA fixed, methanol treated then PBS rehydrated). This solution was left to shake gently for 4 hours at 4 °C before use.

The embryos were then washed in 1 ml PBTw for 30 minutes (four times). The last wash can be done overnight at 4 °C. For the last wash, the embryos were placed in a 24 well-plate using a large-mouth glass pipette (heated to polish the rim). The PBTw was then removed and 1 ml alkaline phosphatase (AP) buffer was added for 10 minutes at room temperature on a shaker (AP buffer: 0.1 M Tris pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$, 0.1 % Tween20). AP buffer was then replaced with 0.5 ml BM purple (Roche). The plate was placed in a opaque box to shut off the light and left for 30 minutes to

overnight on a shaker, periodically checking the embryos until the staining comes up (overnight staining should be done at 4 °C). Once the embryos were displaying sufficient staining, BM purple was removed and the embryos washed with 1 ml PBTw for 10 minutes, then fixed in 4 % PFA.

Figures

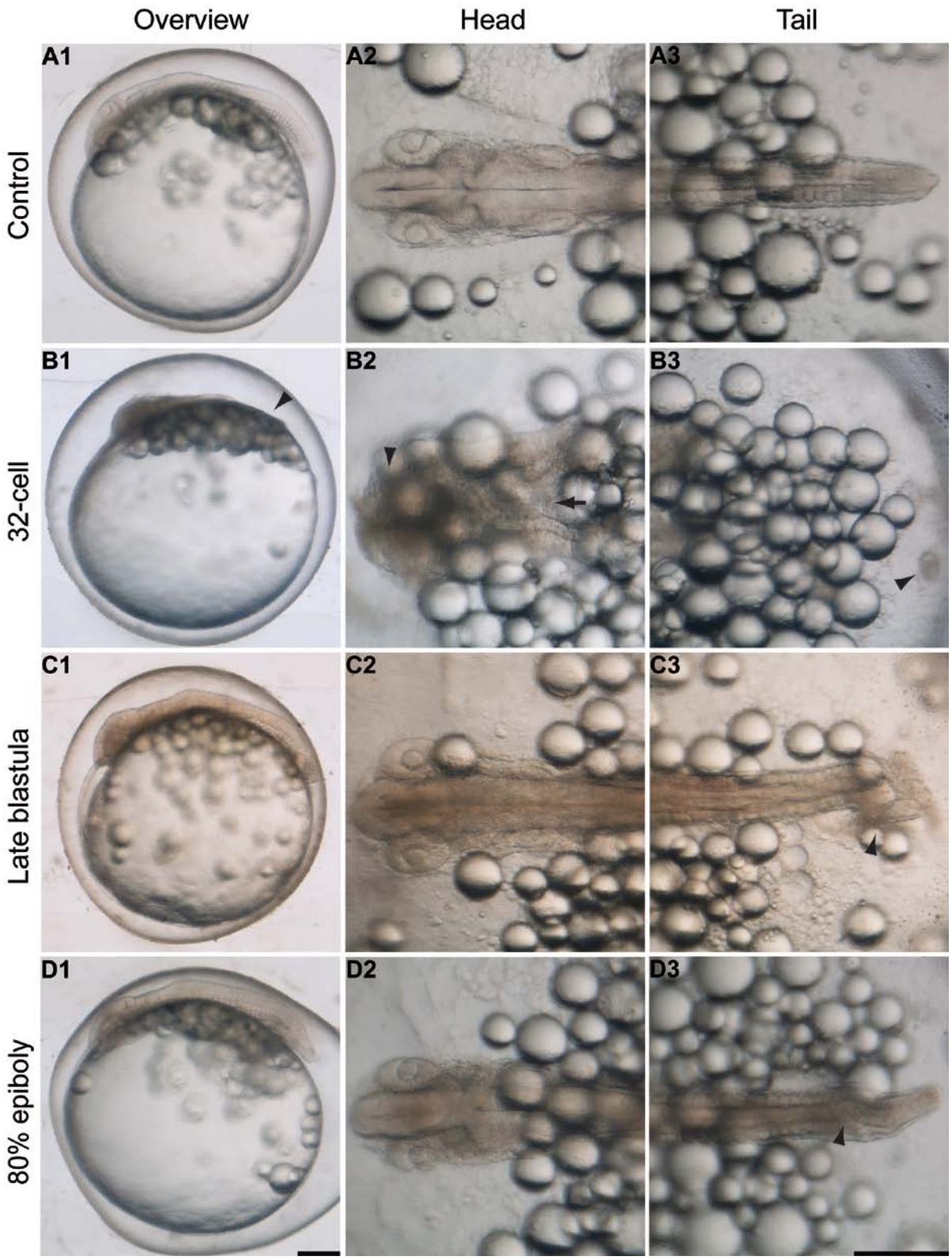

Figure 1.

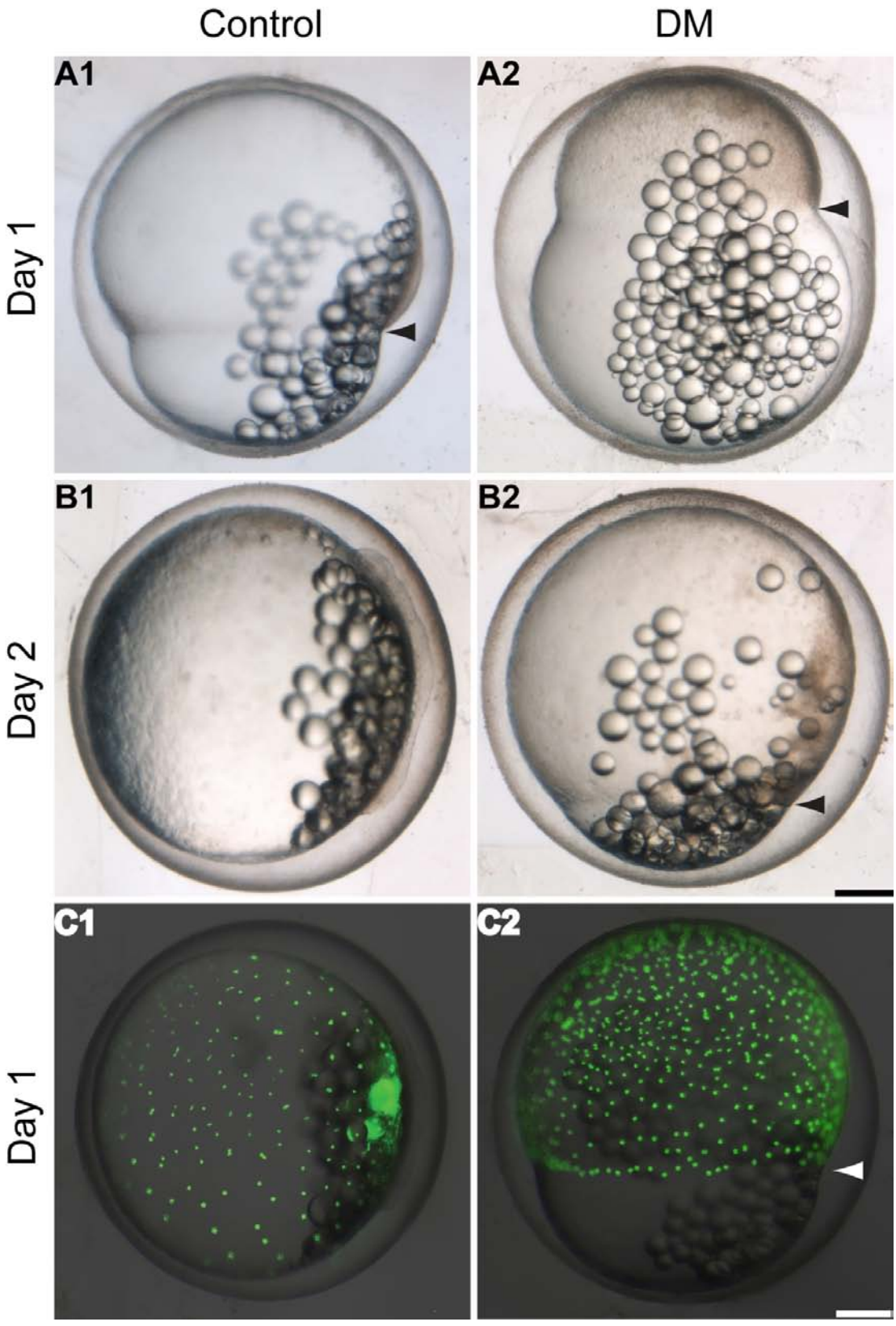

Figure 2.

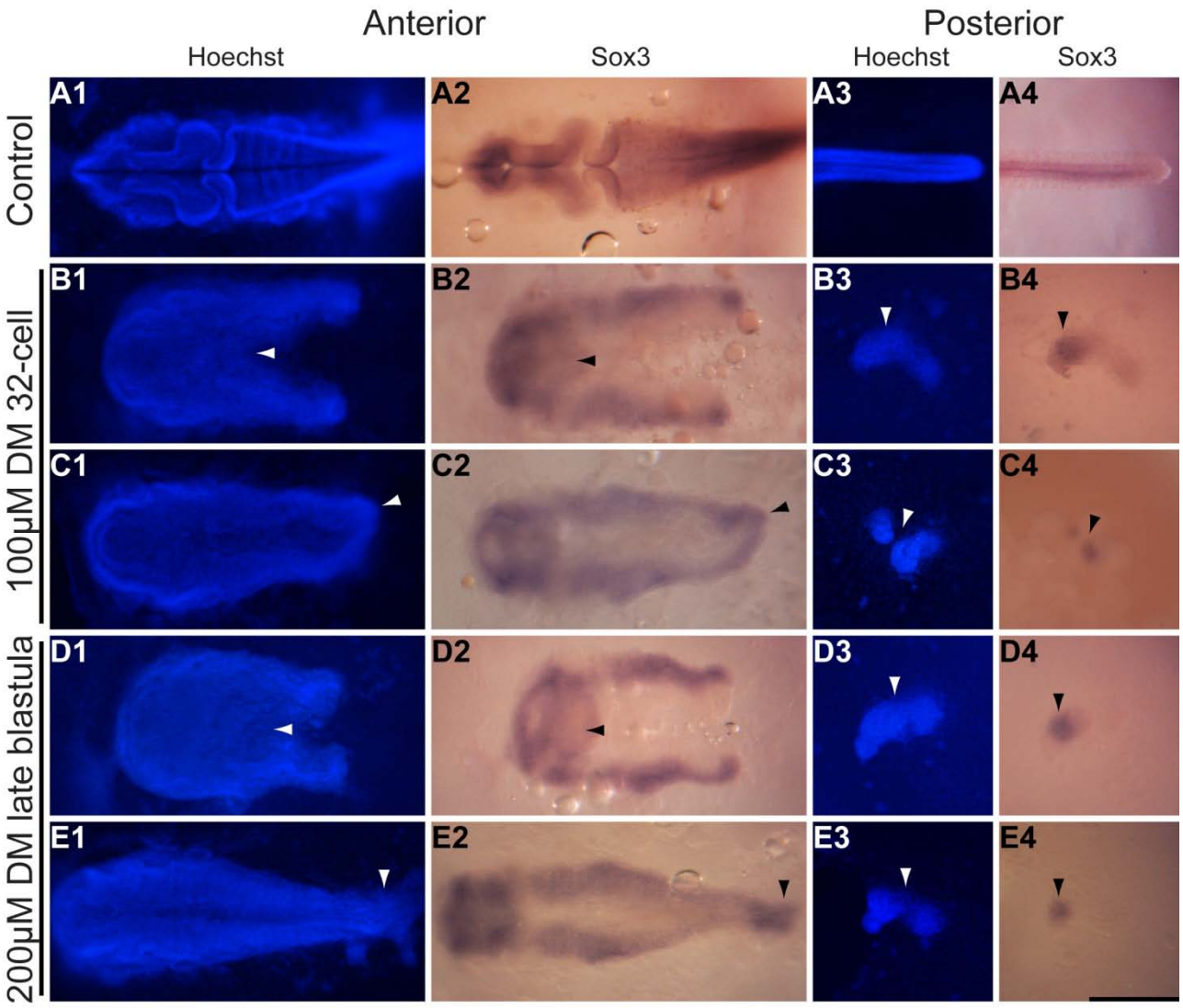
Anterior
Posterior
Hoechst
Sox3
Hoechst
Sox3
Control
100µM DM 32-cell
200µM DM late blastula
A1
A2
A3
A4
B1
B2
B3
B4
C1
C2
C3
C4
D1
D2
D3
D4
E1
E2
E3
E4

Figure 3.

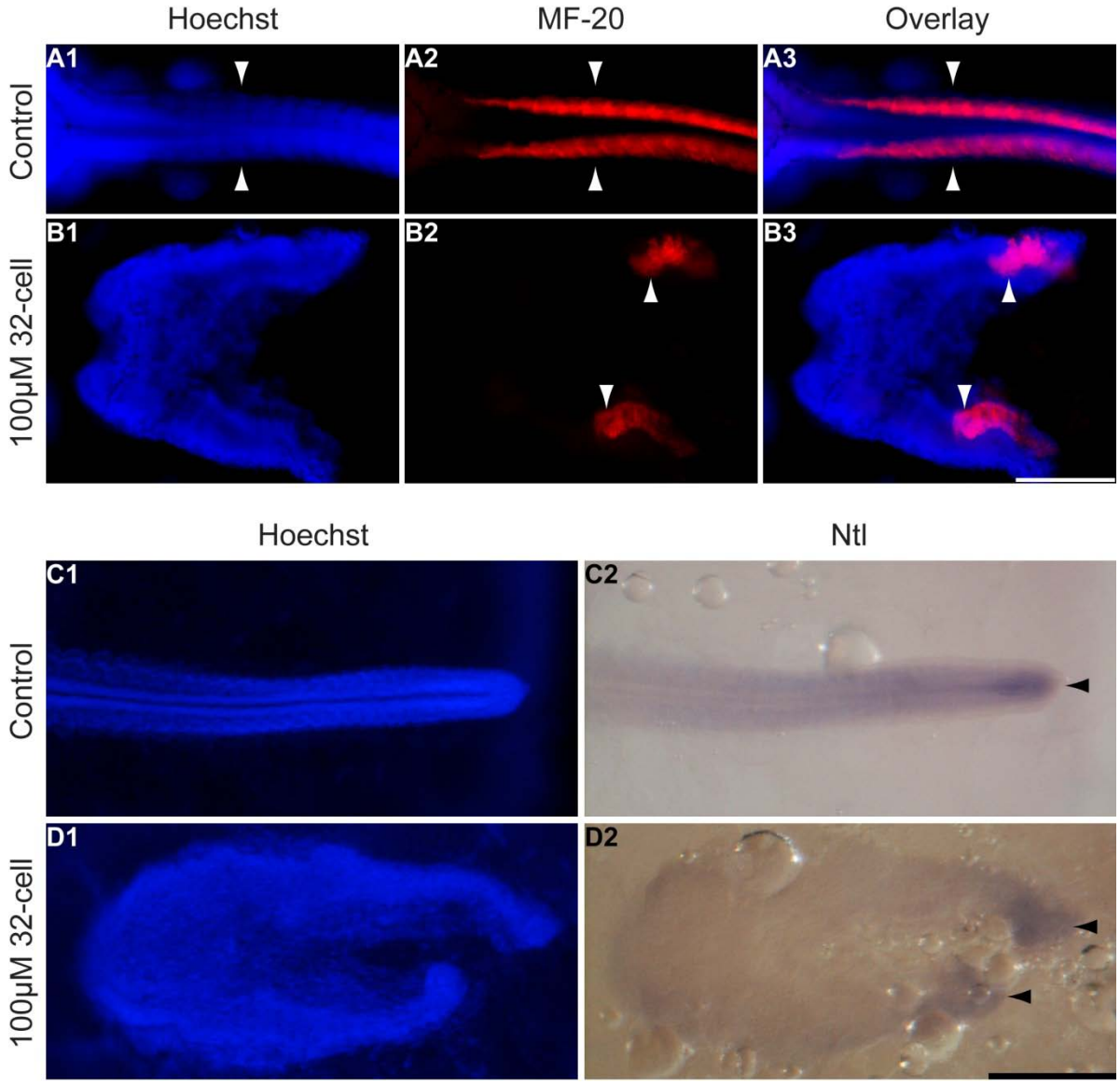
Hoechst
MF-20
Overlay
Control
100µM 32-cell
A1
A2
A3
B1
B2
B3
Hoechst
Ntl
C1
C2
D1
D2

Figure 4.

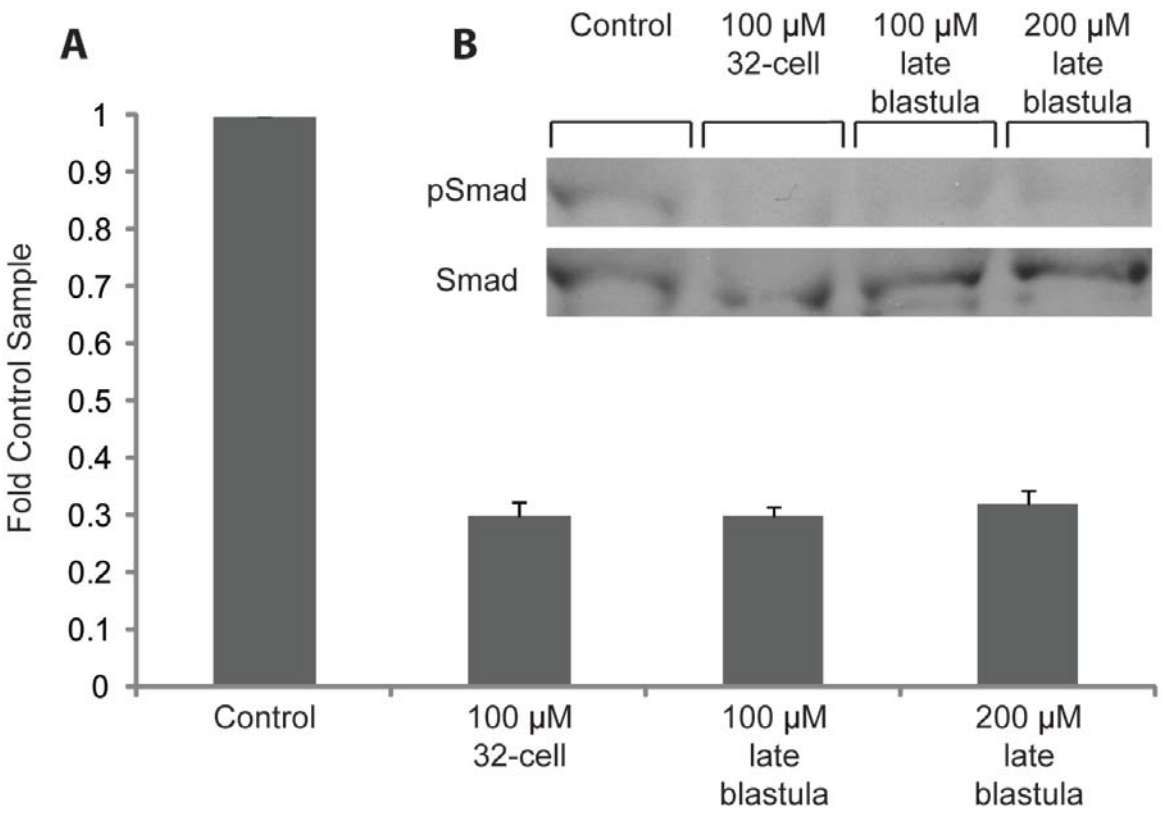
A
B
Control
100 µM 32-cell
100 µM late blastula
200 µM late blastula
pSmad
Smad
Fold Control Sample
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
100 µM 32-cell
100 µM late blastula
200 µM late blastula

Figure 5.

Chapter 5: General discussion

5.1 Overview

The creation of life is an old conundrum that perhaps has driven our curiosity for studying embryogenesis since immemorial times. All the structures and tissues assembling me, the writer, and you, the reader, are essentially composed of a fundamental unit: the cell. The adult form of a multicellular organism is ultimately the product of a single cell, primed for the long and arduous journey that is embryonic development. This process involves countless mechanisms, governed by the fascinating world of gene signalling, that lead to a complex array of behaviour and communication between cells, gradually forming groups, tissues and organs, growing in unison towards a specific form. A convenient way of studying developmental biology is to divide it into stages: steps of development associated with the occurrence of important processes and structures. For instance, gastrulation is a fundamental landmark of embryonic development, constituting a stepping-stone towards the establishment of the form. Indeed, the formation of the three germ layers during gastrulation results in cell differentiation, positioning, and governing, establishing roads and directions for future development. As Lewis Wolpert said, the most important time of your life is not birth, marriage or death, but gastrulation.

Researching the mechanistic of such landmark, or any embryological event for that matter, requires a solid information infrastructure that allows one to easily manipulate embryonic development. When I say manipulate, I mean it in every sense of the term – from handling the embryo (sometimes the most colossal of tasks!), to monitoring its live development, such as to observe morphogenesis, or post-mortem, such as to examine gene expression or stain certain tissues. However manipulation doesn't end at handling, as the techniques involved enable one to influence and direct the development of an organism, by altering gene signalling pathways for instance. Such information

infrastructure has essentially been established for model organisms: popular species in which researchers have gathered, over many years, a wealth of information regarding all aspects of their embryonic development.

The mangrove killifish, *K. marmoratus*, a veteran of survival, is an amphibious little fish species that, along with *K. ocellatus*, is the only simultaneous hermaphroditic vertebrate able to self-fertilise (Tatarenkov et al., 2009). This unique self-fertilisation ability leads to a natural population dynamic that primarily includes (but is not limited to due to outcrossing) the formation of clonal lineages, where individuals can become isogenous – genetically identical (Tatarenkov et al., 2011). Thus, this species essentially provides highly inbred specimens, but without having to conduct unnatural inbreeding in the laboratory; a process that comes with deleterious effects and is an inappropriate representation of an organism's true way of functioning. The intent of this thesis was to further establish *K. marmoratus* in the field of developmental biology, by providing an information infrastructure to help advance research on this peculiar animal and further promote its place in the pantheon of model organisms.

5.2 Discussion of key findings

5.2.1 Staging series

One of the main steps in establishing this species as a model organism involved providing the research community with a means of quickly monitoring and affirming developmental stages of the embryos at hand. Indeed, asynchronies in embryonic development are known to occur even in eggs within a single clutch (Kimmel et al., 1995), and such delays can be intensified by the dynamics of internal self-fertilisation and oviposition at various stages (intraparental development) in *K. marmoratus*. These events make it difficult to know when the embryo was fertilised, thus embryonic development expressed as time post-fertilisation offers a very approximate idea of an

embryo's current developmental stage. Consequently, depicting the formation of apparent morphological landmarks, both visually and descriptively, is an essential requirement for the staging of *K. marmoratus* embryos.

Indeed, such staging series is an essential tool for precise embryology, ensuring repeatability and consistency, not only within but also across different laboratories. For instance, the fundamental role in developmental biology of a staging series can be seen with the zebrafish staging paper from Kimmel et al. (1995), and the little over 3780 references of it. Thus, I reassessed and established a developmental staging series of *K. marmoratus*, providing comprehensive photographic images of developmental stages in this species, with detailed descriptions of the embryonic landmarks associated with each stage (Mourabit et al., 2011). Using this series, anyone can quickly determine the stage of an embryo and the developmental landmarks associated with it. It's a common occurrence when collecting mangrove killifish embryos to find a range of stages in the eggs at hand. The description and the clear visual representations of each stages allows of efficient identification, thus helping with the consistency of replication between batches and experiments, but also within and between laboratories.

5.2.2 Embryological manipulation

Alongside this staging series, I established tools and experimental approaches to facilitate efficient manipulation of *K. marmoratus* embryo (Mourabit et al., 2011; Mourabit and Kudoh, 2012). I achieved this by examining several techniques for embryonic handling and for imaging that can be used in an array of experimental designs. In that effect I created a detailed infrastructure for *K. marmoratus* researchers throughout the world to work on, by establishing techniques to examine live embryonic development in depth. One of the main hurdles I encountered was the chorion. This protective layer surrounding the developing embryo is extremely tough in *K.*

marmoratus, even more so than medaka and zebrafish. In the latter species for example, it is possible for a researcher to manually peel off the chorion without harming the embryo or breaking the yolk, at virtually any stage of development (aside early cleavage perhaps). On a large scale, hundreds of zebrafish embryos can be dechorionated with pronase (a protease), quickly and efficiently. On the other hand, in the mangrove killifish, the thick chorion and the small perivitelline space makes manual dechorionation a tricky endeavour. The chorion of older embryos, close to the hatching stage, can be easily peeled off with a pair of sharp forceps, but younger embryos are a different story. We have been able to party resolve this issue by establishing a protocol based on protease treatment, which improves on the Kanamori et al. (2006) protocol which took two days to dechorionate. Using hatching enzyme, as seen in medaka, we have shortened this time to roughly 4 hours. Despite my improvements, I believe this time can be shortened even more, and further research on the chorion and its removal is required in *K. marmoratus* in order to improve manipulation of these embryos.

Another issue that we have faced is the heavy pigmentation of embryos during late stages of development. Unfortunately, due to the relatively young age of this species as a model organism, there are currently no transparent mutants available. I was able to overcome this problem, and achieved transparency in the embryos by means of PTU treatment (Fig. 4 & 5 in Mourabit et al., 2011, chapter 2). Indeed, despite the presence of erythrophores, the orange/red pigments of the embryos, I achieved a good level of transparency, which helped examining internal structures in older embryos. Unfortunately, achieving transparency by chemical treatment is not ideal for any work that compares development in normal *versus* treated embryos for example, or more generally in experiments that involve addition of exogenous or endogenous substances, as one would first have to determine if PTU alters the reactions involved. All things in due time, however, as this issue will be solved with the advent of a *casper*-like mutant

(White et al., 2008). In addition, PTU treatments alongside our retinoic acid trials have demonstrated that *K. marmoratus* embryos are amenable to chemical manipulation despite the hard chorion that we previously discussed (Fig. 6 in Mourabit et al., 2011, chapter 2). An embryo being sensitive to chemical treatment is essential for modifying specific signalling pathways, as shown by the head formation and tail elongation suppression associated with overexposure to retinoic acid.

Another important tool established in this work was microinjection of *K. marmoratus* embryos, an essential technique for manipulation of embryonic development (e.g. by DNA or RNA injection). I tested this technique by monitoring the formation and development of the YSL. The needles were shown to penetrate the thick chorion relatively easily, and microinjection was efficient (Fig. 2 in Mourabit and Kudoh, 2012, chapter 3). This technique can take some time to be performed correctly, as the angle of injection is crucial to avoid breaking the needle. Luckily, *K. marmoratus* embryos develop slower than zebrafish, thus one can take more time injecting accurately, rather than rush to avoid the next cell cleavage.

During these experiments we were able to point out interesting features in *K. marmoratus* embryonic development such as its unusual cleavage patterns, distinct from other model species such as medaka and *F. heteroclitus*. In mangrove killifish, at the 16-cell stage some blastomeres were already separated from the yolk, as the blastodisc was arrange into two tiers of cells. This separation of blastomeres from the yolk only occurs at the 32-cell stage in the other killifish models (*Fundulus heteroclitus* and medaka), demonstrating a variation of cleavage patterns within closely related killifish species (Mourabit et al., 2011). Another interesting aspect of *K. marmoratus* embryology was the large yolk surface of the embryos providing an excellent imaging platform for studying cell morphology and behaviour during early development, as the few cell types present moved on the same Z-plane and were easily traceable without

labelling (Fig. 3 & 4 in Mourabit et al., 2012, chapter 3). Interestingly, we observed that melanophores on the yolk dynamically change their shape during development, to cover the vitelline vessel network. The differential patterns of embryonic development discussed here, compared to other teleost model organisms, may provide a good model to study the evolution of embryonic morphogenesis in closely related species.

5.2.3 Applying the model: Bmp signalling

Having established fundamental tools for *K. marmoratus* developmental research, I went on to apply this knowledge and examine an important signalling pathway for body patterning of the developing embryo. Indeed, despite the conserved nature of Bmp in vertebrates, its inhibition can result in varying consequences depending on morphological and genetic characteristics of the species at hand. Through this research, we established *in situ* hybridisation in *K. marmoratus*, an essential tool for the studying gene expression and associated tissue localisation during embryonic development. We found that Bmp played an important role in epiboly, YSL movements and AP axis formation, and discovered a new phenotype of Bmp inhibition: "splitbody" (Fig. 3 in Mourabit et al. in preparation, chapter 4). This distinct phenotype, of a tiny embryo with a single head but two body axes may be the result of laterally derived structures of the blastoderm margin failing to merge at the end of gastrulation due to delays in epiboly and YSL movements. These delays may occur due to the size of *K. marmoratus* eggs, as these are roughly two times bigger than a zebrafish. The blastoderm is under extreme tension to move over the yolk during epiboly and the larger yolk in mangrove killifish embryos may increase the stretch required for the sheet of deep cells to reach the mid-gastrula point, thus enhancing epiboly defects. In addition, if *K. marmoratus* possess a different number of genes involved in Bmp signalling, Bmp receptors or Bmp

antagonists, they may be more sensitive to dorsomorphin (i.e. Bmp signalling inhibition) than zebrafish, thus producing the more severe phenotype that is “splitbody”.

Our data demonstrated how suppressing Bmp signalling could have significant species-specific differences in vertebrates, highlighting the importance of studying known signalling pathways in unknown organisms. Indeed, although well established model organisms such as zebrafish and medaka already exist, it is essential to transfer the technical knowledge gained from these big models onto new species. Assembling such information for other species can help improve our comprehension of evolutionary differences and similarities of embryogenesis between organisms, and thus our understanding of key and fundamental developmental processes.

5.2.4 Limitations

One of the main problems encountered when working with *K. marmoratus* embryos comes from the lack of a photoperiod and intra-parental development. Due to this, it can be difficult to collect eggs of a very early stage (e.g. one-cell stage), as oviposition can occur throughout the day and the eggs can be at different stages. This egg laying strategy can make it difficult to work on a great number of eggs of the same stage at the same time, but also to conduct injection experiments during early cleavage. Increasing the number of fish kept can help getting around this limitation, as it provides more opportunities for egg collection every day.

Another key difficulty arising from *K. marmoratus* research is the chorionic membrane of its embryos. The latter is hard to remove manually without damaging the developing embryo, and takes up to 4 hours to remove by enzymatic treatment. This protease treatment requires hatching enzyme, which can take time to gather and may have varying potencies between batches. The protocol that I have provided in this thesis is already an improvement but further research is required on hatching enzyme

collection and production to further enhance the manipulation of *K. marmoratus* embryos. In addition, there are still important tools missing due to the relatively young age of this species as a model for developmental biology, such as mutant stocks and a genome project – although the production of these is currently ongoing.

5.3 Concluding thoughts

K. marmoratus represents a very unique genetic model system due to its self-fertilising nature, established and available clonal lines, and its androdioecious reproductive system that allows mixing and production of new strains. Laboratory use of *K. marmoratus* has already been suggested in a variety of fields such as carcinogenicity testing (Koening and Chasar, 1984) and aquatic toxicology studies (Lee et al., 2008), and both genome project and mutant screen are currently ongoing (Kelley et al., 2012; Moore et al., 2012). Alongside, the data in this thesis demonstrates that with its easily obtainable and manipulated embryos, *K. marmoratus* can be used for embryological research in the same light as other model organisms such as zebrafish or medaka. The rising amount of information on *K. marmoratus* will help further take advantage of this unique and intriguing species, and supports the use of this hermaphroditic vertebrate as a strong comparative model in developmental biology.

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