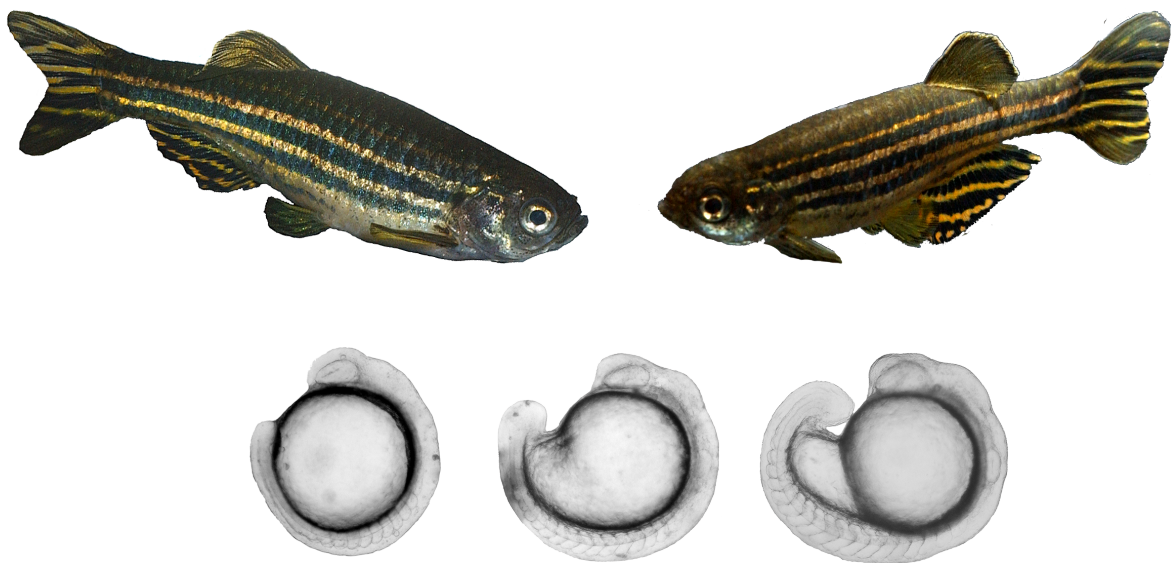# The Phylotypic Stage of Zebrafish

Looking Beyond the Hourglass – About the Underlying Mechanisms Responsible for the Conservation of the Phylotypic Stage

Dissertation zur Erlangung des naturwissenschaftlichen Doktorgrades an der Fakultät für Biologie der Ludwig-Maximilians-Universität München

vorgelegt von
Kai Schmidt

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1. Gutachter: PD Dr. Mario Wullimann

2. Gutachter: Prof. Dr. Gerhard Haszprunar

Tag der mündlichen Prüfung:

Freitag, 24. Juli 2015, 11:00 Uhr

Reprint of the three articles from the Journal of Experimental Zoology Part B: Molecular And Developmental Evolution:

- **Irmler, Schmidt and Starck: Developmental Variability During Early Embryonic Development of Zebrafish**

- **Schmidt and Starck: Developmental Plasticity, Modularity, and Heterochrony During the Phylotypic Stage of the Zebrafish**

- **Schmidt and Starck: Testing Evolutionary Hypotheses About the Phylotypic Stage of Zebrafish**

Eidesstattliche Erklärung
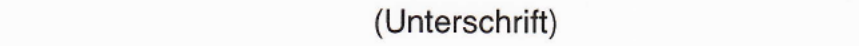
Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

München, den.

(Unterschrift)

Erklärung

Hiermit erkläre ich,

- dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist.

- dass ich mich anderweitig einer Doktorprüfung ohne Erfolg **nicht** unterzogen habe.

München, den.

(Unterschrift)

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Declaration of Contribution / Beitrag der Autoren zu den Einzelnen Artikeln

Kai Schmidt:

1.Artikel:
Vermessung von acht morphometrischen Merkmalen.
Erhebung der Statistik (Übernahme bzw. Neuberechnung des Werts der Variationskoeffizienten für die 5 von Dr. Ingo Irmler vermessenen Merkmale).
Artikelerstellung in enger Zusammenarbeit mit Prof. Dr. J. Matthias Starck.
2. und 3. Artikel:
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Artikelerstellung in enger Zusammenarbeit mit Prof. Dr. J. Matthias Starck.

Prof. Dr. J. Matthias Starck hat folgenden Beitrag zu den 3 Artikeln geleistet:

Entwicklung der Forschungshypothese und des Konzepts, das der Dissertation zu Grunde liegt.
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Summary

The phylotypic stage, as part of the embryonic period, is the stage where embryos of different species of a phylum show a high degree of similarity.

Johann Friedrich Meckel, Karl Ernst von Baer and Ernst Haeckel already described it for vertebrates in the 19th century. They observed that vertebrate embryos pass through a period of morphological similarity. Since then, scientists have researched the field of the phylotypic stage and it was subject of many controversial discussions. The name "phylotypic stage" was coined by Klaus Sander in 1983 and describes not only the stage of the highest similarity but also the stage, typical (characteristic) for a phylum.

The following study examines the phylotypic stage of zebrafish (*Danio rerio*). Looking at different conserving mechanisms like internal constrains and stabilizing selection, different hypothesis and concepts by several researchers were tested.

To test if the phylotypic stage is accessible to selection (although it generally is considered a conserved evolutionary stage) I have studied patterns of variation during embryogenesis. I have looked at the phenotypic variance and the number of significant correlations among embryonic traits and described the phylotypic stage as a period characterized by a high number of internal correlations and declining phenotypic variance.

Then, I tested if changes in the raising conditions could elicit phenotypic changes. Therefore, zebrafish embryos have been raised under different experimental conditions to see if developmental plasticity can be induced during the early developmental period and if clearly defined modules can be identified. Eggs of zebrafish were raised in: (1) different temperatures; (2) different salinities; and (3) different levels of oxygen concentration. Up to 14 characters of individual embryos were measured during early development, encompassing the phylotypic stage. In particular I found a considerable degree of heterochrony and modularity. Embryos grew slower at lower temperatures and lower oxygen levels. Plasticity was detected in the overall size of the embryo and the size of somites in the oxygen and temperature experiment. The development of the eye and otic vesicle was shifted to a later

stage under severe hypoxia. Thus, eye and otic vesicle could be identified as modules, which can be dissociated from other characters of the developing embryo (heterochrony). Changes in raising condition affect early development of the zebrafish on three levels: (1) developmental rate (2) size and shape, and (3) dissociation of modules. Thus, plasticity and modularity are effective during early embryonic development.

Finally I studied the heritability of embryonic traits to examine how inheritance contributes to the stabilization of the phylotypic stage in variable environments. Following the heritabilities of certain traits reveals that the phylotypic stage is not characterized by a certain pattern of decreased heritability and thus decreased additive genetic variance.

The results suggest that the phylotypic stage of zebrafish is constrained by multiple internal correlations when embryos are developing in standard conditions. However, under marginal developmental conditions so far ineffective modules become effective and buffer the embryo against disruptive effects of the environment. Patterns of family resemblance are present, indicating an inherited genetic portion of the phylotypic stage. However, under strong environmental influence it is dominated by variation associated with phenotypic plasticity. My general conclusion is that the phylotypic stage is not established because additive genetic variance is exhausted during the early period of vertebrate development but that it is under environmental and genetic influence, thus is accessible to selection. Internal constraints could be identified to stabilize morphology during the phylotypic stage, but a certain degree of phenotypic variation can be observed.

Zusammenfassung

Das phylotypische Stadium ist das Embryonalstadium, in dem sich die Embryonen eines Phylums sehr ähnlich sind; ähnlicher als in jedem anderen Embryonalstadium. Es wurde in der Embryonalentwicklung der Wirbeltiere bereits von Johann Friedrich Meckel, Karl Ernst von Baer und Ernst Haeckel im 19. Jahrhundert beschrieben und ist seitdem Grundlage kontroverser Diskussionen in der Wissenschaft. Sie beobachteten, dass die Wirbeltierembryonen eine Periode in ihrer Entwicklung durchlaufen, in der sie sich sehr ähnlich sehen. Der Name "Phylotypisches Stadium" wurde 1983 von Klaus Sander geprägt und beschreibt neben dem Stadium mit der größten Ähnlichkeit auch das Embryonalstadium, das für einen Tierstamm (Phylum) typisch ist.

Die folgende Studie untersucht das phylotypische Stadium anhand der Embryonen der Zebrabärblinge (*Danio rerio*). Unterschiedliche Hypothesen für die Ausbildung und Konservierung dieses Stadiums werden aufgrund von empirischen Daten getestet. Zunächst wird der genaue Zeitraum festgelegt, in dem das phylotypische Stadium bei den Zebrabärblingen auftritt. Hierfür wurde die phänotypische Varianz und die Anzahl signifikanter Korrelationen von embryonalen Merkmalen bestimmt. Das phylotypische Stadium bei Zebrabärblingen zeichnet sich als eine Entwicklungsperiode, definiert durch das Auftreten einer größeren Anzahl signifikanter Korrelationen zwischen den einzelnen Merkmalen und einer sich verringernden phänotypischen Varianz, aus.

Als nächstes habe ich getestet, ob sich Änderungen in den Aufzuchtsbedingungen auf den Phänotyp auswirken. Die Embryonen wurden unter unterschiedlichen experimentellen Bedingungen aufgezogen, um zu sehen ob in der Embryonalentwicklung Plastizität beobachtet werden kann und ob klar definierte Entwicklungsmodule (Modularität) identifiziert werden können. Dafür wurden die Eier der Zebrabärblinge unterschiedlichen Temperaturen, Salinitäten und Sauerstoffkonzentrationen ausgesetzt. Bis zu 14 embryonale Strukturen wurden an den einzelnen Individuen während der Frühentwicklung (das phylotypische Stadium eingeschlossen) vermessen. Insbesondere konnte Heterochronie und Modularität nachgewiesen werden. Des Weiteren war zu beobachten, dass Embryos bei niedrigeren Temperaturen und bei

niedrigerer Sauerstoffkonzentration langsamer wuchsen. Merkmale wie die Größe des Embryos und Somitengröße zeigten ein größeres Maß an Plastizität in den Temperatur- und Sauerstoffkonzentrationversuchen. Bei einer reduzierten Sauerstoffkonzentration wurde die Entwicklung der Anlagen für das Auge und die Ohranlage im Vergleich zu anderen embryonalen Merkmalen erst zu einem späteren Zeitpunkt realisiert. Somit konnten Auge und Ohranlage als abgegrenzte Module identifiziert werden, die sich unabhängig von anderen embryonalen Strukturen entwickeln und in ihrer Entwicklungslaufbahn unter bestimmten Bedingungen verschoben werden können.

Veränderungen der Aufzuchtsbedingungen beeinflussen die Entwicklung der Embryonen auf drei Ebenen: (1) Entwicklungsgeschwindigkeit, (2) Größe und Gestalt und (3) Verschiebung von Modulen in der Entwicklungslaufbahn (Heterochronie). Daraus folgt, dass Plastizität, Modularität und Heterochronie bereits in der embryonalen Frühentwicklung und somit auch während des phylotypischen Stadiums nachgewiesen werden können.

Als letztes wurden die Heritabilitäten von embryonalen Merkmalen untersucht, um zu bestimmen inwieweit Vererbung für die Konservierung des phylotypischen Stadiums, auch unter variierenden Umwelteinflüssen, verantwortlich ist. Die Heritabilitäten einzelner Merkmale ergaben, dass sich das phylotypsche Stadium nicht durch ein spezifisches Muster von sich verringernden Heritabilitäten und somit auch nicht durch verringernder additiver genetischer Varianz beschreiben lässt.

Die Gesamtheit der Resultate ergibt, dass das phylotypische Stadium durch entwicklungsbedingte Zusammenhänge einzelner Strukturen unter standardisierten Bedingungen konserviert wird. Jedoch unter grenzwertigen Aufzuchtsbedingungen werden einzelne Module sichtbar und puffern wahrscheinlich die Embryonalentwicklung gegen die Effekte widriger Umwelteinflüsse. Muster von familiären Ähnlichkeiten sind vorhanden und weisen auf einen genetischen Einfluss auf das phylotypische Stadium hin. Allerdings sind unter starken Umwelteinflüssen auch im phylotypischen Stadium Variationen zu finden, die auf Plastizität zurückzuführen sind. Zusammenfassend ist zu sagen, dass während des phylotypischen Stadiums die additive genetische Varianz keines Falls erschöpft ist, sondern dass das phylotypische Stadium unter einem genetischen und umweltbedingten Einfluss steht, und somit auch den

selektiven Kräften unterliegt. Interne Zwänge konnten als der Hauptgrund für die Konservierung des phylotypischen Stadiums identifiziert werden, aber ein gewisser Grad an phänotypischer Varianz bleibt erhalten.

1 Introduction

1.1 The "Phylotypic Stage"

The phylotypic stage is a developmental stage during embryonic development of animals and is marked as a key concept in evolution and development (Richardson 2012).

The concept of the phylotypic stage describes the great similarity of embryos of the same phylum during mid-embryonic development and goes back to the observations of Johann Friedrich Meckel (1811, 1821), Karl Ernst von Baer (1828) and Ernst Haeckel (1866). Meckel was one of the first to describe the similarity among vertebrate embryos and later von Baer recognized by an incident as well the great resemblance of early vertebrate embryos. He had two unlabelled embryo species in his collection and he recognized that even for the trained eye it would be almost impossible to tell them apart and say to which species they belong. This led him to the formulation of four laws, later referred to as von Baer's laws of development.

Von Baer's laws[1](Hall 1997):

1. The more general characters of the group to which the embryo belongs appear before the more specialized characters.
2. Less general structures form after more general structures until finally the most specialized structures appear.
3. During development, embryos progressively diverge from embryos of other groups.
4. Embryos of higher animals resemble embryos and not adults of other animals.

His third law describes the early resemblance of vertebrate embryos, and is still discussed in recent history (Sander and Schmidt-Ott 2004)[2].

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[1] Baersche Regel: 1. Daß das Gemeinsame einer größeren Thiergruppe sich früher im Embryo bildet, als das Besondere. 2. Aus dem Allgemeinsten der Formverhältnisse bildet sich das weniger Allgemeine und so fort, bis endlich das Speciellste auftritt. 3. Jeder Embryo einer bestimmten Thierform, anstatt die anderen bestimmten Formen zu durchlaufen, scheidet sich vielmehr von ihnen. 4. Im Grunde ist also nie der Embryo einer höhern Thierform einer andern Thierform gleich, sondern nur seinem Embryo.

Later, Haeckel conceptualized the similarity of vertebrate embryos, using his famous drawings (Fig. 1), in the biogenetic law. The central statement was that ontogeny recapitulates phylogeny. This means there is a relationship between phylogeny and ontogeny (Haeckel 1874). Also, according to Haeckel, the ontogeny of an organism is explained or created by its phylogeny, in a way that advanced organisms ad new stages at the end of the developmental sequences of its ancestor (for a detailed discussion of the biogenetic law see Richardson and Keuck 2002 , Sander 2002, Sander and Schmidt-Ott 2004 and chapter 2.4). But since Garstang (1922) and de Beer (1940) this view has changed and all modern concepts agree that ontogeny creates phylogeny (Gould 1977, Raff 1996, Hall 1999).

Until today there have been many names for the developmental point or period at which all vertebrate embryos look most similar (see chapter 3.1). I will use here phylotypic stage, coined by Klaus Sander (1983), as it is still the most common name, widely used and even adopted for other fields (Mueller, Vernier et al. 2006). Phylotypic stages are described for many phyla of the animal kingdom (Seidel 1960, Cohen 1977, Sander 1983, Slack, Holland et al. 1993, Galis and Metz 2001) and recently even for plants (Quint, Drost et al. 2012). As indicated in the title I will focus on the phylotypic stage in vertebrates and only touch lightly other phyla in the following chapters.

The current study examines the phylotypic stage of zebrafish (*Danio rerio*), considering the history of the phylotypic stage and its interpretation by several researchers of the last 200 years in the context of comparative evolutionary developmental biology.

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[2] The third law is controversially discussed in recent articles, as it seems to contradict a newer model postulating that development starts rather divergently, converging toward the phylotypic stage and diverging from there on. However, reading carefully von Baer's articles as Sander did, one will recognize that von Baer never considered the divergent part in the beginning as part of what he considers as embryonic development. For him, it actually starts with the formed embryo after gastrulation/neurulation (e.g., the beginning of the phylotypic stage) (Sander and Schmidt-Ott 2004). Thus, his third law does not contradict modern concepts such as the hourglass model, as many articles still assume.

1.2 The Evolution of Development

Many people have researched the field of the embryonic development including the phylotypic stage since the 19th century, such as Meckel, von Baer, Haeckel and de Beer or Goldschmidt.

In the following chapter, the field of evolutionary developmental biology (evo-devo) is introduced as well as some key scientists involved in embryology and evolutionary biology, which laid the foundation for this rather new field.

1.2.1   Evo-Devo

Evo-Devo is a synonym for "evolutionary developmental biology" and is a synthesis of evolutionary and developmental biology seeking answers to questions, which cannot be answered by either one of the fields alone (Hall 2000). Evo-Devo is a young field in biology and emerged in the 1980s. According to Hall (2003), evo-devo attempts to find out how new characteristics come up during evolution and how they will be influenced by genes (Hall 2003, Neukamm 2007). In other words, how the evolution of the ontogeny of organisms have affected the phylogeny of organisms. Hall (2003) summarizes the following topics, which are incorporated in the field of evo-devo:

-   the origin and evolution of embryonic development;

-   how modification of development and developmental processes lead to the production of novel features;

-   the adaptive plasticity of development in life-history evolution;

-   how ecology impacts on development to modulate evolutionary change; and

-   the developmental basis of homoplasy and homology.

In the end, evo-devo is a fusion of experimental and comparative embryology, evolutionary biology, molecular biology and developmental genetics (Love and Raff 2003).

1.2.2   J.F. Meckel (1781-1833)

1.2.2.1   Empirical Basis and Interpretation

The empirical basis of Meckel's pre-Darwinian concept was the similarity of the morphology of embryonic stages of higher animal species and the end stage of lower animal species (Osche 1982). He postulated that every animal species passes through every developmental stage of the animal species during ontogeny. Only lower animal species rest on lower developmental stages without developing into higher stages (Osche 1982). Despite the fact that Meckel was greatly influenced by the Lamarckian concept of evolution, he regarded the similarity of developing embryos as a part of a "Schöpfungsidee" and not due to the idea of common descent[3] (Bromley 1971, Neukamm 2007).

Based on the similarity of embryonic stages of different animal species, Meckel used the concept of stepladder (Neukamm 2007), which was known as a part of natural philosophy.

1.2.2.2   Conclusion and Implications for the Phylotypic Stage

The concept of stepladder by Meckel was a precursor in research of ontogeny and phylogeny and laid the ground stone for Haeckel's biogenic law.

The new aspect in the deliberations of Meckel consists in his combination of ontogeny and phylogeny, while studying the morphology of embryonic stages in animal species. This leads to a new typology of animal species as a development from lower to higher animal species with the human on top of the stepladder.

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[3]Already towards 1750, Pierre Louis Maupertuis developed a concept of common descent. For the first time, long before Darwin, an evolutionary explanation was given to illustrate how all living organisms have developed by differentiation from common ancestors (Bromley 1972 but see also Mayr 1981).

1.2.3   K.E. von Baer (1792-1876)

1.2.3.1   Empirical Basis and Interpretation

Von Baer is regarded as the founder of the comparative embryology (Gould 1977). He drafted his laws based on the similarity of embryos (see chapter 1.1). He concluded from his observation that the similarities of embryos of vertebrates increase when going backwards in the development of vertebrates (Neukamm 2007). He did not believe the theory of recapitulation, thus von Baer did not accept the concept of stepladder by Meckel.

1.2.3.2   Conclusion and Implications for the Phylotypic Stage

Just like Meckel, von Baer rejected the idea of common descent (Sander 2002). And as Meckel, von Baer recognized similarities in embryonic development of different animal species, but in contradiction to Meckel he emphasized that the early embryos of different species display similarities and not that the embryos of higher animals are similar with the adult stages of lower animals (von Baer 1828). Therefore he rejected the stepladder concept. From this perspective von Baer's third and fourth law are describing what we know today as the phylotypic stage.

1.2.4   E. Haeckel (1834-1919)

1.2.4.1   Empirical Basis and Interpretation

Haeckel presented a coherent evolutionary theory based on the knowledge of his time (Bininda-Emonds, Jeffery et al. 2002). Darwin's books and Lamarckian explanations of adaptation influenced his ideas of evolution. Haeckel combined the concept of the stepladder by Meckel with the concepts of Lamarck to construct his biogenetic law (Neukamm 2002, Neukamm 2007) containing this central paradigm:

 "The ontogeny is the short and fast recapitulation of the phylogeny caused by physiological functions of heredity and adaptation"[4] (Haeckel 1866, p. 300). According to Haeckel, phylogeny was a mechanical cause of ontogeny. The biogenetic law was a tool for Haeckel for phylogeny reconstruction (Richardson and Keuck 2002).

Looking at the developmental sequence, he differentiated into palingenetics and caenogenetics to explain which characteristics are preserved characteristics (Palingenesen) and which are newly developed (Känogenesen).

Haeckel shifted embryology towards the centre of evolutionary argumentation (Sander and Schmidt-Ott 2004).

1.2.4.2   Conclusion and Implications for the Phylotypic Stage

Haeckels biogenetic law was controversially discussed since it was published. This went from totally rejection (Gilbert 2013) towards an acceptation of some of the ideas included. The complete rejection seems to be partly based on misunderstandings. Logical flaws in Haeckel's writings and over-interpretations or misconceptions of his ideas evoked these misunderstandings, e.g. that recapitulation is not the same as embryonic resemblance. Recapitulation for certain characters is widely accepted today, though not for entire stages. And it is not clear, if Haeckel was talking about stages or characters in his work (Richardson and Keuck 2002). Heavily criticized was the idea that higher animals run through the adult stages of their ancestors as suggested by Meckel, so that evolution was only possible by adding new stages at the final stage of ancestors. This process is called terminal addition. Some authors are convinced that this reduction to the ideas presented by Meckel is a misconception (Müller 2005). Richardson and Keuck (2002) demonstrated that Haeckel was aware of other mechanism of evolutionary change and not limited to terminal addition but included heterochrony and change of characters in the developmental sequence.

According to Sander, Haeckel's original biogenetic law constitutes the base of evo-devo and embryology (Sander 2004).

In the end, Haeckel's drawings of embryos (Fig. 1) and his ideas about recapitulation inspired contemporary researchers to reinvestigate developmental mecha-

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[4]Die Ontogenesis ist die kurze und schnelle Recapitulation der Phylogenesis, bedingt durch die physiologischen Functionen der Vererbung (Fortpflanzung) und Anpassung (Ernährung).

nisms and the role of (recapitulated) conserved characters such as found in the phylotypic stage (Sander 1983, Hall 1996, Galis and Metz 2001).

1.2.5   R. Goldschmidt (1877-1935)

1.2.5.1   Empirical Basis and Interpretation

Goldschmidt was convinced that macroevolution that leads to changes within phylums is caused by macromutations. He thought that an accumulation of micromutation could not explain macroevolution. He developed the concept of hopeful monsters. Monsters are the outcome of disastrous macromutations but once in a while a macromutation will produce a viable new phenotype, a hopeful monster (Gould 1980).

According to Goldschmidt, macroevolution could not (always) be explained by the mechanism of microevolution.

1.2.5.2   Conclusion and Implications for the Phylotypic Stage

Goldschmidt recognized that minor changes during embryonic development could have huge effects on the adult organism. (Goldschmidt 1940, Gould 1980).

Richardson is elaborating on the ideas of Goldschmidt's hopeful monsters. Richardson suggests that even small changes around a critical developmental point, as the occurrence of the phylotypic stage, might cause viable phenotypic change in the adult and such is important for the evolution (Richardson 1999, Richardson 2012). This is coherent with one of Goldschmidt's concepts for macroevolution, namely, that mutations in rate genes or controlling genes change early development and have large effects on adult phenotype (Goldschmidt 1940, Gould 1980).

1.2.6   G.R. De Beer (1899-1972)

1.2.6.1   Empirical Basis and Interpretation

De Beer followed Garstang's ideas about heterochrony and tried to separate hetero-chrony from recapitulation. He constructed a classification of eight different types of heterochronies and ascribed heterochrony a central role in evolution. According to de Beer changes in the timing of events and the occurrence of new characters can take place at any point of its development and are not limited to the endstages of embry-onic development (Smith 2001, Brigandt 2006). Summarizing the essence of de Beer's work in a sentence, following Garstang, it would be that ontogeny is not a re-capitulation of phylogeny but it rather creates it (de Beer 1940, Brigandt 2006).

1.2.6.2   Conclusion and Implications for the Phylotypic Stage

As a forerunner in modern evolutionary biology, de Beer brought development in the evolutionary syntheses of the 20th century using heterochrony to link developmental and evolutionary mechanisms. De Beer separated heterochrony from Haeckelian re-capitulation and defined it more broadly in a modern way that heterochrony includes any change in timing of developmental events relative to other events (Smith 2001, Brigandt 2006). Looking at heterochronies, like characters that appear at different times in relation to each other, was widely applied in the research of the phylotypic stage (Richardson 1995, Smith 2001, Bininda-Emonds, Jeffery et al. 2002, Jeffery, Bininda-Emonds et al. 2002, Bininda-Emonds, Jeffery et al. 2003, Bininda-Emonds, Jeffrey et al. 2003, Poe and Wake 2004, Jeffery, Bininda-Emonds et al. 2005)

1.3 Terminology and Controversial History of the Phylotypic Stage

A stage of great similarity during embryonic development is mentioned by (Meckel 1811), von Baer (1828), and later conceptionalized by Haeckel at the end of the 19th century. A controversy about the validity of the concept followed at the dawn of the 21st century (Myers 2003). The actual term "phylotypic stage" was introduced by Sander in 1983 (Galis, Wagner et al. 2003). I will present the different ideas behind the phylotypic stage and the different names given to it by several authors.

1.3.1 New Names and Definition for an Old Observation

In 1954 Medawar picked up on Haeckel's (1874) ideas about the great resemblance of early vertebrate embryos. He was the first to draft a model symbolizing the divergence at the beginning of embryonic development, converging to a state of similarity in mid-embryonic development and diverging from there on again (Fig. 2a). He attributed the divergence being before the phylotypic stage to inductive interactions. He argues that inductive interactions allow for more freedom in development, which would be denied if developmental steps such as organ formation would have been determined in respect of their ultimate fates (Medawar 1954). Thus, he lays the groundstone for the developmental hourglass or egg-timer, by dividing the embryonic development in a convergent period and a divergent period encompassing the neurula stage. According to Medawar, the neurula is the stage with the highest similarity of all vertebrate embryos. It is the stage that will later be called the phylotypic stage.

Seidel (1960) reckoned that insects also pass through a universal stage during development and called this "Körpergrundgestalt". Cohen (1963) introduced the term "phyletic stage" to describe the stage with the highest similarity within a phylum. In regard to vertebrates, he also refers to the neurula, as Medawar already did in 1954. Sander (1983) suggested using the phylotypic stage instead of the phyletic stage, as phyletic refers to phylogeny rather than to the typical characters defining a certain stage of individual phyla. The characters in vertebrates are, according to

Sander (1983), the chorda dorsalis, neural tube, somites, gill clefts and tubular heart. His general definition of the phylotypic stage is that it is the stage of greatest similarity between forms, which during evolution have specialized differently in their modes of adult life and with respect to the earliest stages of ontogeny strongly influenced by spatial modes of reproduction. Analysing mechanisms conserving the phylotypic stage, he takes the phylotypic stage of insect, which is the germband stage, for example, which has been conserved for over 200 million years. He agrees with Medawar (1954) that inductive interaction must play a major role, although with a different interpretational approach. He describes ontogeny as a network with an interdependent function. Changes in one function will thus affect the whole developing system and lead to a collapse. Thus, the reliability of development might be in danger and this leads to the conservation of the phylotypic stage. An example is the uniform formation of sequent appendices in insects, which occur during later development after the phylotypic stage get fused and distorted in a huge diversification of mouthparts. A second explanation according to Sander could be that, from a genetic point of view, conserving the germband stage is more economic in terms of coding capacity.

Slack, Holland et al. (1993) presented a new definition for an animal, referring to the phylotypic stage. They introduced the term zootype. It is the stage, where a certain spatial pattern of gene expression can be observed. This is coherent with the positional information model (Wolpert 1969), which assumes that during early stages, positional values are encoded by the same universal positional field in all embryos, and diversification starts later on, due to evolutionary changes in downstream mechanisms. Each organism displaying this pattern of gene expression is, according to Slack et al., an animal. Interestingly, this pattern can be observed around the phylotypic stage for each individual taxon. Thus, an animal is indirectly defined by the possession of a phylotypic stage during its development. Duboule (1994) dwells on the topic of a zootype brought up by Slack, Holland et al. (1993). He describes the phylotypic stage as the crucial stage at which hox genes are sequentially activated. What makes this article so consequential in the history of the phylotypic stage are two significant facts. First, the introduction of the concept called the "Phylotypic Egg-Timer", which is later on referred to as the "Hourglass Model" (Fig. 2c). Second, Du-

boule is the first author who considered the phylotypic stage, not as one certain stage during development but rather a succession of stages, which he calls phylotypic progression. Duboule hypothesized that the activation of the hox genes during the phylotypic stage makes this period so invariant and thus is the reason for the high degree of similarity. His egg-timer/hourglass model has a striking resemblance with the model Medawar drafted in his article (Fig. 2a). Furthermore, the idea that hox genes play a major role in the conservation of the phylotypic stage are not in contradiction with the ideas by Medawar (1954) and Waddington (1956). Medawar explains the lower divergent part of the hourglass by inductive interactions. These interactions between constituent parts of the embryo allow for the greater diversification at the start of development. Two years later, Waddington argues, that any alteration at such a crucial point (the phylotypic stage) where the basic structure of the animal are laid down would throw everything in confusion. This is an important notion, which got picked up later by Raff (1996) and Galis and colleagues (Galis and Metz 2001, Galis and Sinervo 2002, Galis, Van Dooren et al. 2002). Duboule's hox genes theory is also supported by Waddington's idea. Hox genes have a key role in the organization of the body plan. Missing hox gene products will result in the transformation of a given structure in a similar but not identical one.

So far, until the beginning of the 90s, there is a serious debate about the model of a phylotypic stage. Almost all scientific articles agree on the existence of the phylotypic stage and theorize about mechanisms conserving it (but see also Sedgwick 1894, Lillie 1919, de Beer 1940), which are not mutually exclusive or, as in the case of Slack, Holland et al. (1993), using the phylotypic stage to define an animal. Others have tried to identify the exact stage at which the phylotypic stage appears. For Medawar (1954) and Cohen (1963) it is the neurula, Ballard (1981) identifies it at the pharyngula stage. Wolpert (1991) speaks of an "early somite embryo" and Slack, Holland et al. (1993) use the tailbud stage to define their zootype. Collins (1995) claimed that embryos pass through the phylotypic stage when they are the same size. This disagreement about the right stage marking the phylotypic stage in the mid-90s led to a controversial discussion of the phylotypic stage. Some authors even disputed the existence of a highly conserved stage.

1.3.2   Is There a Highly Conserved Stage in Vertebrates?

Richardson's critical paper (1995) was a part of the scientific debate about the phylotypic stage and introduced the new term phylotypic period, followed by a paper by Richardson and colleagues (1997) with the title: "There is no highly conserved stage in vertebrates: implications for current theories of evolution and development."

According to Richardson, looking at the concept of heterochrony, heterochronic shifts throughout development make it impossible to find one certain stage for all vertebrates, which can define the phylotypic stage. He proves Haeckel's drawings, which gave rise to the theory of a highly conserved stage, are over simplistic and thus inaccurate. Richardson is not denying that there is a developmental period where the main organ primordia appear and that a conserved pattern of gene expression can be found as postulated by Slack, Holland et al. (1993). However, he disagrees on a conserved morphology as structures appear at different times relative to each other. In the article from 1997, Richardson and colleagues describe even more sequence heterochronies in the embryonic stages of vertebrates. They investigated the tailbud stage, which equals the stage Haeckel used in his drawings to show the similarity of early embryos and the one Slack, Holland et al. (1993) used for the zootype. Using a large variety of vertebrate embryos, they found, beside heterochronic shifts, differences in body size and body plan, such as changes in the number of units in repeating series and allometry. They conclude, it is true that differences between species become more apparent at later stages, but they reject the idea that vertebrate embryos are virtually identical at earlier stages as implied by Haeckel's drawings. Thus, not all developmental mechanisms are highly conserved such as the zootype.

These articles by Richardson and colleagues gave new fuel to the debate about Haeckel's ideas and drawings and to the question if the concept of the phylotypic stage is valid per se. Therefore, three different things – Haeckel's ideas, his drawings and his concept of the phylotypic stage – were a main part of the discussion. In the following years opponents such as Richardson and colleagues and proponents such as Sander, Raff, Hall and Galis produced a variety of articles to support their ideas.

The first answer for Richardson and colleagues comes from Hall (1997). In his article, "Phylotypic stage or phantom: is there a highly conserved stage in vertebrates?", Hall agrees there are heterochronic shifts. These temporal, but not morphological shifts in evolution do not justify abandoning the concept of a phylotypic stage. Moreover, he argues that it would be premature to abandon the phylotypic stage for one subphylum (vertebrates), as there are abundant evidences for phylotypic stages in invertebrates. He concludes the phylotypic stage is a constrained stage maintained by stabilizing selection due to the epigenetic processes that lead to the phylotypic stage. In this article, he stresses that it is still not known how developmental processes are constrained, despite the variability described by Richardson in the underlying processes. Raff (1996) suggested that a web of intense interaction among organ primordia, caused by a lack of modularity, is responsible for the conservation. Kirschner and Gerhart (1998) proposed that the phylotypic stage was conserved by stabilizing selection with constraints acting on a lower, subordinated level to maintain the evolvability, thus considering the phylotypic stage as a platform from which diversification begins.

In 1998, there was a direct answer from Richardson and colleagues (Richardson, Minelli et al. 1998) to Hall's article. Here they stress again that heterochrony makes it impossible to define one stage as the phylotypic stage for all vertebrates. The main argument is that variation in adult morphology is mostly generated during embryologic development and is not limited to late-embryonic development but can also occur at mid-embryonic development (e.g., the phylotypic stage). Evidence from limb development is used to reinforce the idea that evolutionary changes occur during all stages of development. Richardson is not denying that the hourglass model reflects the phenotypic differences during development. Nevertheless, he does not see that reduced phenotypic variation at the phylotypic stage also means the phylotypic stage is resistant to selection. He argues that changes of developmental mechanisms during the phylotypic stage will manifest in phenotypic changes at later stages, and that even little changes at the phylotypic stage might have major effects in adult morphology as they get amplified throughout development. Richardson also acknowledges the period of the phylotypic stage is a rather vulnerable phase toward perturbance from the outside such as terratogenes. He interprets this not as the evo-

lutionary bottleneck as visualized by the hourglass model, but rather as the perfect time for evolutionary change. The fact that this period is so sensitive toward modification indicates that, if an embryo survives, even little changes can have a huge effect through amplification on the adult organism. This sounds similar to Goldschmidt's theory of hopeful monsters. Goldschmidt recognized that minor changes during embryonic development could have huge effects on the adult organism. So making way for macro evolutionary changes ending up in a hopeful monster (Goldschmidt 1940, Gould 1980). Richardson's articles followed more studies challenging the phylotypic stage. Chipman et al. (2000) identified heterochronies in 12 anuran species, most notably of the ear and the eye. Calloza (2000) also identified variation in anuran species during the phylotypic stage, describing interspecific differences at the development of the neural crest. He concluded, in unison with Richardson, that the concept of the phylotypic stage needed to be reformed.

Although these recent approaches concentrated on the variation during the phylotypic stage and the validity of the concept itself, Galis and Metz (2001) picked up the idea of the vulnerability of the phylotypic stage as a proof for the evolutionary conservation. This is the same idea, which was rejected by Richardson in his article from 1999. Galis and Metz (2001) regarded strong stabilizing selection as the reason for the conserved stage. A second possible explanation model, the lack of genetic variation, could be excluded by proofs from the literature. To illustrate the underlying mechanism, explaining the stabilizing selection, they follow Raff's model, predicting there is a lack of modularity during the phylotypic stage, which leads to pleiotropic effects as proposed by Sander (1983). To prove this hypothesis, they conducted a literature review and analysed studies in which developing embryos were exposed to teratogens. Embryo mortality always had a peak if the teratogen was applied at the phylotypic stage. Their conclusion was that if artificially induced changes had such severe and lethal effects, naturally occurring mutations would also end in a disaster at the phylotypic stage.

The outcome that the phylotypic stage is a rather sensitive and vulnerable stage is not in contrast with Richardson, but their interpretation is. They do not see the possibility that mutations during the phylotypic stage can produce a viable embryo with evolving adult structures. Moreover, they also doubt Duboule's theory that the

precise regulation of hox genes at the phylotypic stage is conserving the stage, but the conserved phylotypic stage, by the lack of modularity and pleiotropic effects, is responsible for the conservation of the hox genes organization. Interestingly, they never mention the heterochronic changes described by the opponents of the phylotypic stage directly in this article. Most likely they do not see a conflict with their concept of the phylotypic stage. Additionally, by defining the phylotypic stage to last from neurulation until most somites have been formed, they acknowledge that the phylotypic stage is observed over an entire period during embryonic development, rather than an occurrence at a certain point. Thus, the question of terminology about stage or period becomes redundant for Galis and Metz. The phylotypic stage is the stage in which vertebrate embryos most resemble each other. These most look alike embryos cannot be found at the same time point in development for all species (taking Richardson's heterochrony critic into account) but within the ascribed timeframe where embryos react highly sensitive to any perturbance. Galis and Sinervo (2002) and Galis, Van Dooren et al. (2002) also addresses the problem of the lately described variation during the phylotypic stage. They elaborate on the theory of the vulnerability of the phylotypic stage and Raff's theory of a web of intense interaction among organ primordia within the phylotypic stage. Galis and Sinervo are not denying that there is variation at the phylotypic stage, but what matters to them is that there is less variation than before or after the phylotypic stage. Thus they are coming back to the hourglass model with the phylotypic stage in the middle at the bottleneck, describing a stage of highest similarity within vertebrates. The reason for the conservation is still seen in mutations having pleiotropic effects, which gets amplified during development and always leads to detrimental effects. This is attributed to the lack of modularity or low effective modularity at the phylotypic stage (Sander 1983, Raff 1996, Galis and Metz 2001). An alternative hypothesis by Von Dassow and colleagues (Von Dassow and Munro 1999, Von Dassow, Meir et al. 2000), that the phylotypic stage in insects is conserved by robustness of the underlying gene network, is discussed and neglected. An evaluation of empirical evidences was supporting the pleiotropic hypothesis. Galis and colleagues came to the conclusion that stabilizing selection against mutation is conserving the phylotypic stage on a long-term scale (robustness would only work on a short-term scale). The underlying mechanisms are

constrained by low effective modularity. Another hint for the conserved nature of the phylotypic stage is presented by Galis et al. (2003). The authors argue that one reason for limb regeneration in amphibians is, that limb development got heterochronically shifted. Limb regeneration is now setting in after the phylotypic stage compared to most other amniotes where regeneration is not possible. Now the limb development can act as semi-independent module and is not constrained by pleiotropic interactions in the phylotypic stage and thus regeneration is possible. Again Galis and colleagues show that they accept variation in the phylotypic stage caused by sequence heterochronies and that they don't see the concept of the phylotypic stage weakened by sequence heterochrony. Just in the contrary, they use heterochrony to support their pleiotropic hypothesis.

1.3.3   Quantitative Approaches to Test the Phylotypic Stage

Around the same time, Galis and colleagues published the pleiotropic hypothesis; Richardson and colleagues concentrate their scientific work on a quantitative model to investigate sequence heterochronies during development including the phylotypic stage (Richardson, Jeffery et al. 2001, Bininda-Emonds, Jeffery et al. 2002, Jeffery, Bininda-Emonds et al. 2002, Bininda-Emonds, Jeffery et al. 2003). The first articles concentrate on a model for a phylogenetic methodology to analyse embryonic characters to determine if they are universal. To avoid the comparison of species according to stages, they concentrated on the concept of event pairing (Smith 1996, Smith 1997). With their model, they are able to quantitatively prove the existence of heterochronic shifts in the sequence of developmental events. Finally, in the article "Inverting the hourglass: quantitative evidence against the phylotypic stage in vertebrate development" in the year 2003, Bininda-Emonds and colleagues use the model for a first quantitative approach to attack the concept of the phylotypic stage. By looking at sequence heterochronies, Bininda-Emonds and colleagues find the highest degree of variation (highest amount of sequence heterochronies) in mid-embryonic development, the phylotypic stage. Thus, they are inverting the hourglass model by substituting phenotypic diversity with the number of observed heterochronies (Bininda-Emonds, Jeffery et al. 2003). Further on, these results lead them to hypoth-

esize that numerous modules exist during the phylotypic stage, which in the end give rise to the diversification of vertebrates. This article is in straight contradiction to the ideas of Sander, Raff, Hall and Galis. Although, as mentioned above, Galis et al. (2003) elegantly showed that sequence heterochronies are not in direct contradiction to the pleiotropic hypothesis. In 2003 the controversy about the phylotypic stage reached a peak with proponents clearly holding on to the concept of the phylotypic stage and opponents not only criticizing, but neglecting the phylotypic stage.

Poe and Wake (2004) modified the method of Bininda-Emonds et al. to reanalyse the data. Their results are in contradiction to both the hourglass model and the inverted hourglass model. They found that sequence heterochronies occur throughout the entire embryonic development without displaying maxima or minima at certain stages. Although they admit they are only considering sequence heterochronies for their model and their approach has several limitations, they conclude their results are supporting the opponents of the phylotypic stage. However, the hourglass model was never meant to regard sequence heterochronies alone but rather phenotypic diversity. Interestingly, an article by Hazkani-Covo, Wool et al. (2005) supports the hourglass model and van Baer's third law coming from the molecular side. Both of these concepts have been questioned by Poe and Wake (2004) and Poe (2006). Hazkani-Covo, Wool et al. (2005) also present a quantitative approach to test the hourglass model and thus the phylotypic stage. They argue that if the hourglass model is a valid concept, the expression of ortholog genes should be more similar around the phylotypic stage, compared to later or earlier stages. Their results support the hourglass model from a molecular perspective. They admit their results are not as strong as optimistically expected, but still are a good support for the concept of the phylotypic stage.

One of the main critics of the opponents of the phylotypic stage has been that the entire phylotypic stage discussion was based on qualitative studies, which never have been underpinned by hard empirical data in a quantitative approach. Now in the beginning of the 21st century looking at the first three quantitative approaches to the problem (excluding here my own studies) one finds that these studies present three different and contradictory results. Thus, despite the hopes put in these approaches, they could not help to solve the conflict either, yet. Nevertheless, a combi-

nation of qualitative and quantitative studies is the right direction for future studies. Quantitative studies alone such as the studies of Hazkani-Covo or Yassin and colleagues (Hazkani-Covo, Wool et al. 2005, Yassin, Lienau et al. 2010) lead to results about optimal tests or the forming of phenotypes in developmental stages such as the phylotypic stage, but there is no statement about the phylotypic stage and their influences between different animal species to create generally concepts.

In the meantime, Sander, who coined the term 'phylotypic stage', tried to mediate between opponents and proponents (Sander and Schmidt-Ott 2004). They offer a discussion of the pro- and anti-phylotypic stage attitudes, addressing the critical questions as if it is a stage or period, which characters define the phylotypic stage and how much heterochrony can be accommodated within a conserved stage. They conclude that it is still a rather subjective question depending on either if one is looking for similarities or concentrating on the differences. Both can be studied and found. The trick would be to include both in one concept instead of building concepts from either one or the other perspective. The authors emphasized that in the future molecular data will help to solve the controversy if a maximum of similarity is conserved in the phylotypic stage. But they also stress that there is no doubt that the phylotypic stage reveals common descent as postulated by Haeckel.

1.3.4   Molecular Approaches to Test the Phylotypic Stage

Hazkani-Covo, Wool et al. (2005) have been the pioneers presenting molecular evidences for the hourglass model followed by an article of Irie and Sehara-Fujisawa (2007). Both established novel tests to analyse the phylotypic stage in different animal species (for example, a mouse by Irie) and their dependence by time and chromosomes (Hazkani-Covo, Wool et al. 2005, Irie and Sehara-Fujisawa 2007). They also argued that if the phylotypic stage exists, conserved genes among vertebrates would be highly expressed at this stage. For their analyses they used expressed sequence tag data and gene orthologies. Their results also support the phylotypic stage. They have been able to show that the mouse embryo passes through a highly constrained stage, which is most likely, the most developmentally conserved stage of vertebrates.

Furthermore, they identified a second conserved stage for all bilaterians, which they called the "bilateriotypic stage". This stage occurs within the cleavage to gastrula period in mouse embryogenesis. Another molecular approach by Roux and Robinson-Rechavi (2008) is in contradiction with Irie and Sehara-Fujisawa (2007) and Hazkani-Covo, Wool et al. (2005) (see the following page). They are looking at gene expression data in zebrafish and mice and, for both species, their results support constraints on early stages and not on mid-embryonic stages as supported by the hourglass model/phylotypic stage. But they stress, they are looking at processes and not patterns as the studies of heterochrony do. Moreover, they discuss the vulnerability of vertebrate embryos described by Galis and Metz (2001). They only find support for this theory in mice and it is the only exception in their analyses, which does not support their early conservation model. Nevertheless, they think the studies by Galis and Metz explain the potential for a stage to produce abnormalities rather than display the constraints on ontogeny. In a later article from 2010, the same authors elaborate on their ideas and present a new explanation for the phylotypic stage (Compte, Roux et al. 2010). They investigate the lack of the modularity theory by looking into the protein-protein interaction, gene expression and character of genes expressed before and within the phylotypic stage in zebrafish and mice. Protein interaction is highest in early development up to the neurula stage. In contrast to their earlier study (Roux and Robinson-Rechavi 2008), the expression of genes involved in signalling and regulating displayed evolutionary conserved peaks during development. However, most of the peaks did not correspond to the phylotypic stage, and if peaks where observed within the phylotypic stage, they extended far over this period until late development. Early development shows a significant pattern pointing toward a conserved stage in accordance with the description of Irie and Sehara-Fujisawa (2007) for what they call the bilateriotypic stage. Comte et al. conclude the observed similarity on a morphological level at the phylotypic stage is a consequence of the early genetic conservation.

A phylogenomic study on the insect phylotypic stage, also from 2010, found support for the hourglass model in insects (Yassin, Lienau et al. 2010) and comes with an explanation for the controversial results reported from the molecular approaches of Hazkani-Covo, Wool et al. (2005), Irie and Sehara-Fujisawa (2007) ver-

sus Roux and Robinson-Rechavi (2008). They propose that differences in the rate of evolution among developmental stages are responsible for the conflicting results in metazoans and that, at higher taxonomic levels, the identification of ortholog genes is more difficult. Yassin and colleagues analysed 51 body segmentation genes in 12 species of drosophila to research the influence of evolution of the development on phylogeny by different tests (Yassin, Lienau et al. 2010). For drosophila, they also report conflicting results looking at gene expression; they argue the findings depend on a high degree of taxonomic levels under investigation. Yassin, Lienau et al. (2010) concluded that pairwise phonetic comparisons are not optimal for studying the macroevolutionary support to the hourglass model of the developmental constraints on genome evolution. Hazkani-Covo and colleagues postulated in 2005 that "A possible "translation" of the hourglass model into molecular terminology would suggest that orthologs expressed in stages, described by the tapered part of the hourglass, should resemble one another more than orthologs expressed in the expansive parts that precede or succeed the phylotypic stage" (Hazkani-Covo et al. 2005, p.150f.) and analysed the thesis using 1585 mice genes expressed during 26 embryonic stages (Hazkani-Covo, Wool et al. 2005). They confirm the existence of a phylotypic stage, which temporal location agreed with the morphologically defined phylotypic stage (Hazkani-Covo, Wool et al. 2005). In this context, Irie and Sehara-Fujisawa established a new method for evaluating the ancestral nature of mice embryonic stages by analysing day 8.0 to 8.5, where a highly conserved embryonic period exists in mice. This is similar to the developmental time of pharyngeal arch and somites. At the end, Irie and Sehara-Fujisawa concluded the mid-embryonic stage of the mice is highly constrained and is evidence for the existence of the phylotypic stage (Irie and Sehara-Fujisawa 2007).

Another supportive paper on a molecular base for the morphological similarity in mid-embryonic development is presented by Elinson and Kezmoh (2010). They postulate that looking at gene expression patterns, one finds a greater similarity than even Haeckel might have imagined. They circumvent the problem of heterochrony by looking at the tissues and organs of the embryo, instead of the morphology of the whole embryo. They create a generic embryo and place regulatory molecules on it. As their approach is extremely different from the other molecular approaches men-

tioned above, one could not use the results of this article to either support one or the other hypotheses from other molecular approaches, because the analysis is not comparable with conventional tests and is not transferable.

Domazet-Loso and Tautz (2010) were testing the hourglass-model of the phylotypic stage by looking at evolutionary age of transcriptomes at given ontogenetic stages in zebrafish. The oldest transcriptomesets have been found around the phylotypic stage, with younger sets before and after the occurrence of the phylotypic stage. This pattern of the age distribution of transcriptomesets is a reflection of the hourglass-model. Irie and Kuratani (2011) supported those findings by looking at the transcriptomes of several model vertebrate embryos. Moreover, a study about drosophila development unveiled similar results (Kalinka, Varga et al. 2010). Here, conserved patterns of gene expression reflect as well the hourglass model. The authors attribute the phylotypic stage in drosophila development to stabilizing selection with maximized selective constraints during the phylotypic stage. Whereas Domazet-Loso and Tautz (2010) consider constraints as one possible option besides the possibility that selection is inactive due to a lack of adaptation. Newman (2011) agrees with the aforementioned authors about the bottleneck of the hourglass model but gives a new explanation for the lower part of the hourglass. He considers that eggs are evolutionary novelties, which arose independently in different animal bodyplans/classes. In his opinion, this is the explanation for the great variety of eggs forming the lower part of the hourglass. Physical constraints in self-organizing cellclusters lead from this variety to the bottleneck of the hourglass (i.e., the phylotypic stage). In his view, there is no need to ascribe the conserved stage to the stabilizing effects of natural selection, but to the expression of the most ancient and conserved developmental genes.

Recent studies, looking at the expression of transcription factors, expression of transcriptional regulators, cis-regulatory elements in non-coding DNA during embryo development and embryonic gene expression in turtles, support the idea of the phylotypic stage and the hourglass model (Bogdanović, Fernandez-Miñán et al. 2012, Armant, März et al. 2013, Nelson and Wardle 2013, Schep and Adryan 2013, Wang, Pascual-Anaya et al. 2013). Richardson (2012) elaborates on the findings of Levin, Hashimshony et al. (2012), describing a phylotypic stage for nematodes. They

suggest a model, based on transcriptome analyses, which explains the differences in the Baupläne of large animal groups (vertebrates, flies, etc.) that arise through modification of the phylotypic stage (Groeters and Shaw 1992, Raff 1996). Richardson sees support for his idea that the phylotypic stage is a key target for the evolution and the question if the phylotypic stage represents an evolutionary lockdown or a powerhouse of speciation is still unanswered (Richardson 2012).

1.4 Evolutionary Mechanisms and Concepts

1.4.1   Modularity

With the rise of evo-devo as a new field in biology, the concept of Modularity came in the focus of evo-devo scientists, as it was a rallying point for all the different disciplines, which got merged into the new field of evo-devo (Bolker 2000, Wagner, Pavlicev et al. 2007). Meanwhile it is accepted that developing organisms are modular organised (Raff 1996). A complex but modularized organism should favour the evolvability. Modules can be altered and then selected without bringing havoc to the whole developing system. Thus mutation within a module will only affect this module and not necessarily other modules, trade-offs for evolutionary change will be reduced (Schlosser and Wagner 2004). The developmental modules, which are of interest for this study, can be defined as a part of an embryo that is quasi-autonomous with respect to pattern formation and differentiation. Quasi-autonomous means that the elements of a module are highly interconnected but separated from other modules (Wagner, Pavlicev et al. 2007). Schlosser and Wagner (2004) suggest that this separation is not necessarily complete, but that modularity rather comes in degrees, where some subunits have higher independence from their surroundings than others. The independency of modules allows dissociability of modules (Needham 1950) and opens the door for heterochrony (see next chapter).

A concept of low effective modularity or lack of modularity is discussed by several authors as an explanation for the conservation of the phylotypic stage (Sander 1983, Raff 1996, Galis and Sinervo 2002, Galis, Van Dooren et al. 2002).

1.4.2   Heterochrony

Heterochrony is one important concept of evo-devo (Brigandt 2006). Nevertheless it is almost as controversially discussed as the phylotypic stage itself. Here I can only

touch this topic on the surface and discuss it on the terms important for this study. The term heterochrony was introduced by Haeckel to explain exceptions in his bio-genetic law: Compared to ancestors in the phylogeny, organs are formed earlier or later during development in relation to others organs (Richardson and Keuck 2002). Haeckel laid the ground stone for future research that stressed the importance of heterochrony for evolution. Gould (1977) argued for a central role of heterochrony in macroevolution and McNamara (1997) went as far as stating that, without hetero-chrony evolution would not happen.

Raff (1996), based on the studies of Garstang, de Beer, Gould and Alberch, offered a straightforward definition for heterochrony: An evolutionary change in the timing of developmental events.

Heterochrony always requires that the timing of developmental structures or events can be separated from each other independently. Needham (1950) called this dissociability. This is possible if the organism is modularized, so that independent modules can be dissociated and appear at a different time in the developmental tra-jectory (see chapter 4.1). Heterochrony as an agent for evolution is a main research target in the recent history of the phylotypic stage (Richardson 1995, Richardson, Hanken et al. 1997, Richardson 1999, Collazo 2000, Bininda-Emonds, Jeffery et al. 2002, Sander 2002, Bininda-Emonds, Jeffery et al. 2003, Bininda-Emonds, Jeffrey et al. 2003, Poe 2006)

1.4.3   Phenotypic Plasticity

Phenotypic plasticity describes the ability of an organism (genotype) to produce dif-ferent phenotypes as a reaction to environmental cues (Pigliucci 2001). Environmen-tal cues encompass abiotic and biotic factors (Fordyce 2006). This can be tempera-ture, light and salinity as examples for abiotic factors or population density or the presence of a predator as a biotic factor.

Phenotypic Plasticity is not only a heritable, evolvable trait on its own (Scheiner 2002) which can be a target of selection (Pigliucci, Murren et al. 2006), but

as well a source for variation on which selection can act. Novel traits in evolution cannot only occur by mutation but as well by plasticity (Pigliucci 2001, West-Eberhard 2005). And West-Eberhard (2005) suggested that environmental initiated novelties may have greater evolutionary potential than mutationally induced ones. This makes phenotypic plasticity another important agent for selection and evolvability. Thus if the phylotypic stage also displays phenotypic plasticity, it can be assumed that selection can act upon the phenotypic variation of the phylotypic stage.

1.4.4   Stabilising Selection

Stabilizing selection is the most frequent form of selection having a favourable effect of species, by eliminating extreme phenotypes at the ends of the general distribution of phenotypic characteristics (Sauermost 1994). Therefore, the average individuals of a population are favoured and the genetic and phenotypic variance will be reduced (Stearns and Hoekstra 2001). Nearby factors such as environmental factors, lack of food, illnesses or enemies occur as selective factors. Important to consider is, that favourable phenotypes will be only conserved as long the aforementioned selective factors remain stable. Based on Darwin's evolution theory, the individual with the best characteristics survives by supporting optimal phenotypes within stabilizing selection. In the recent past stabilizing selection was discussed as one possible mechanism conserving the phylotypic stage by several authors (Hall 1997, Kirschner and Gerhart 1998, Galis and Metz 2001, Galis and Sinervo 2002, Galis, Van Dooren et al. 2002, Kalinka, Varga et al. 2010). This would imply that the selective factors responsible for the conservation of the phylotypic stage have not changed for a very long time.

1.4.5 Developmental Constraints

In the developing embryo, organ systems become functionally connected. Such functional connections impose boundaries on further change (Hall 1999). These boundaries are constraints. In an insightful overview of the topic Maynard Smith, Burian et al. (1985) described it as follows: "a developmental constraint is a bias on the production of variant phenotypes caused by the structure, character, composition, or dynamics of the developmental system." Thus constraints will make it difficult for certain phenotypes to be produced (Williams 1992). This is also discussed as a reason for the conservation of the phylotypic stage; high interconnectivity reduces the phenotypic variance and conserves the phylotypic stage (Raff 1996).

1.5 Aims of the Thesis

The following questions were investigated in the study of the development of zebrafish embryos:

Concerning the phylotypic stage in general

- Do zebra fish embryos pass through a phylotypic stage and at which time and stage does it occur? (1st article) – My study will test, if zebrafish pass through a phylotypic stage and defining the timeframe of the phylotypic stage.

Concerning the degree of conservation of the phylotypic stage

- How much plasticity can be detected during the development and especially during the phylotypic stage? (2nd article) – Plasticity is one of three important agents for evolution under investigation. If I can detect plasticity, evolution can also be expected to act on the plastic response.

- Is modularity already present during the phylotypic stage or is there a lack of modularity? (2nd article) – Modularity is another important agent for evolution and thus the evolvability. If there is no modularity (during a specific evolutionary stage), there is also not much room for evolution.

- Can heterochrony be observed during the phylotypic stage? (2nd article) – Heterochrony is the third agent of evolution under observation.

Looking at these 3 agents, I should be able to make predictions, if the phylotypic stage is evolutionary conserved, or if evolution can act as well during the phylotypic stage.

Concerning the mechanisms of the conservation of the phylotipic stage

- Are there differences in the heritability and thus in the additive genetic variance at different developmental stages? (, 3rd article) – Heritability is a measure for the additive gentic variance, thus the genetic contribution to the phenotype.

- If I can define a phylotypic stage for zebra fish, what are the mechanisms, which are conserving the phylotypic stage? (1st, 2nd and 3rd article) – Taking all my previous results into account, I will test different hypotheses about the mechanisms conserving the phylotypic stage.

1.5.1 A Morphological Quantitative Approach in Three Steps to Test the Phylotypic Stage

There are many hypotheses and theories aiming at an evolutionary explanation of the phylotypic stage as laid out in the previous chapter. Here I present a quantitative approach based on an empirical dataset to test different hypothesis about the phylotypic stage.

The data was acquired by morphometrical methods in an experimental set-up to test ideas about the phylotypic stage in vertebrates. The zebrafish as model organism for research was chosen for several reasons:

- Zebrafish eggs and early embryos are transparent and offer the possibility of repeated observation throughout their development without disturbing or even killing the embryo.
- Development takes place outside the womb.
- Egg laying can be triggered by light, thus the start of development can be determined with an accuracy of about a couple of minutes.

- The developmental period is short, from egg laying to hatching, it takes approximately 72h.

- Zebrafish have a short generation period. After only 3 months they reach maturity.

- Zebrafish are suitable for both genetic studies and developmental physiological experiments.

In the first step (1st article), I analysed if I can detect phenotypic variance within the time period beginning with neurulation until almost all somites have been formed. This is the developmental period in which the typical landmarks defining the phylotypic stage occur.

I have analysed patterns of variation and co-variation. Therefore, I calculated the phenotypic variance and the number of significant correlations among embryonic traits. This data enabled me to define a timeframe for the phylotypic stage for the zebrafish.

According to certain patterns of the parameters under investigation for different evolutionary processes, I made the following predictions about the phylotypic stage:

(1) Directional selection will result in reduced phenotypic variance and no covariation among traits during the phylotypic stage; (2) Internal constraints should be detected by reduced phenotypic variance and increased covariation among traits during the phylotypic stage; (3) Stabilizing selection should be revealed by a pattern of reduced phenotypic variance and same amount of covariation among traits during the phylotypic stage; (4) Lack of modularity will result in increased phenotypic variance and a higher number of covariation among traits during the phylotypic stage.

In the second step (2nd article), I studied if changes in raising conditions could evoke a plastic reaction during the phylotypic stage, which is considered as conserved and thus immune to evolutionary changes. Plasticity is a strong agent for evolutionary changes (Waddington 1942, Waddington 1953, Hall 2003, West-Eberhard 2003). Therefore, I analysed embryonic trajectories. I compared trajectories over

time as well as over stages (number of somites where used as staging criterion) with the experimental setup of my study. The combined results led to different predictions about plasticity, modularity and heterochrony during early embryonic development. The conditions I used were:

-        standard condition

-        high and low temperature

-        high salinity

-        low oxygen concentration

With this approach I tested the following prediction:

(1) No Plasticity – The phylotypic stage is conserved and selection cannot act on a plastic response. (2) Acceleration or deceleration of growth and development – Acceleration and deceleration describe the development in accordance to time. For example, environmental factors could lead to faster development (acceleration) or slower development (deceleration). If the developing embryo is affected equally in all of its parts, I will observe different developmental stages at a given time (meaning that an identical stage occurs earlier or later), but no changes if I compare embryos by stages. Thus, embryos are flexible but not necessarily modularized. (3) Change in size and shape - I expect to observe changes of the morphometry when the embryos are compared over time and over stages. This could indicate that modules are present and allow phenotypic changes in discrete traits of the embryo. (4) Developmental dissociation (heterochrony) - I expect to observe changes in the developmental sequence resulting in a dissociation of developmental characters, observed over time and stages. Modules exist and react independently on the environmental cue.

In the third step (3rd article), I looked at the heritabilities and additive genetic and phenotypic variance of embryonic traits. I investigated how they developed/changed over time — before, within and after the phylotypic stage. Heritability measures the impact of the genotype on the phenotypes.

Based on this approach, I have refined the predictions from our first experiment, according to the new parameters we could measure. Combining the results of all studies, I came up with the following three evolutionary hypotheses, which aim at explaining the phylotypic stage: (1) stabilizing selection, (2) random, and (3) epigenetic effects in the sense of Waddington (Table1).

Each of the hypotheses above makes specific predictions about the phenotypic and genetic variances and correlations among traits during the phylotypic stage, which I wanted to test with the described approach.

Hypothesis	Predictions		
	Additive Genetic Variance	**Phenotypic Variance**	**Correlations Among Phenotypic Traits**
Stabilizing selection	decreasing	decreasing	no change
random	no change or increasing	increasing	no change or increasing
Epigenetic effects	no change or increasing	decreasing	maximized during phylotypic period

Table 1. Predictions about changes in phenotypic and genetic variances during the phylotypic period

1: Stabilizing selection tends to reduce the additive genetic variance of a trait, and a reduced additive genetic variance may result in stable phenotypes of early vertebrate embryos.

With a reduced genetic variance I also expect a decreased phenotypic variance and no significant correlation among traits. Therefore, I predict that if stabilizing selection acts on the phylotypic stage, I expect the phenotypic variance and the additive genetic variance to decrease and no changes in correlation among traits as compared to before and after the phylotypic stage.

2: According to ideas presented by Williams (1992) and Newman (2011) which suggested that the phylotypic stage emerges as a by-product of directional and/or diverging selection on later developmental stages, I drafted the "random-

hypothesis". In this hypothesis, the similarity of early embryos is neither selected nor constrained, and the phylotypic stage is simply the residual of what is not selected. The prediction from that hypothesis is that phenotypic variance increases with the increasing number of degrees of freedom; for instance phenotypic variances increase with increasing complexity of the developing embryo. Additive genetic variance would be stable or increase with the increasing number of gene interactions and thus correlation among traits is expected to be stable or also steadily increase.

3: the epigenetic hypothesis predicts that the phylotypic stage results from constraints during early embryogenesis, in other words, multiple inductive interactions among cells, tissues, and developing organs prevent selection on the phylotypic stage. All stages of development are potentially open to evolutionary change, but, as the epigenetic consequences (for example, of eye development or neurulation) are so fundamental, the early embryonic stages are evolutionary stable. Predictions from this hypothesis are that phenotypic variance declines during the phylotypic stage and that genetic variance remains constant or increases with the increasing number of gene interactions and a peak of correlations among traits at the phylotypic stage.
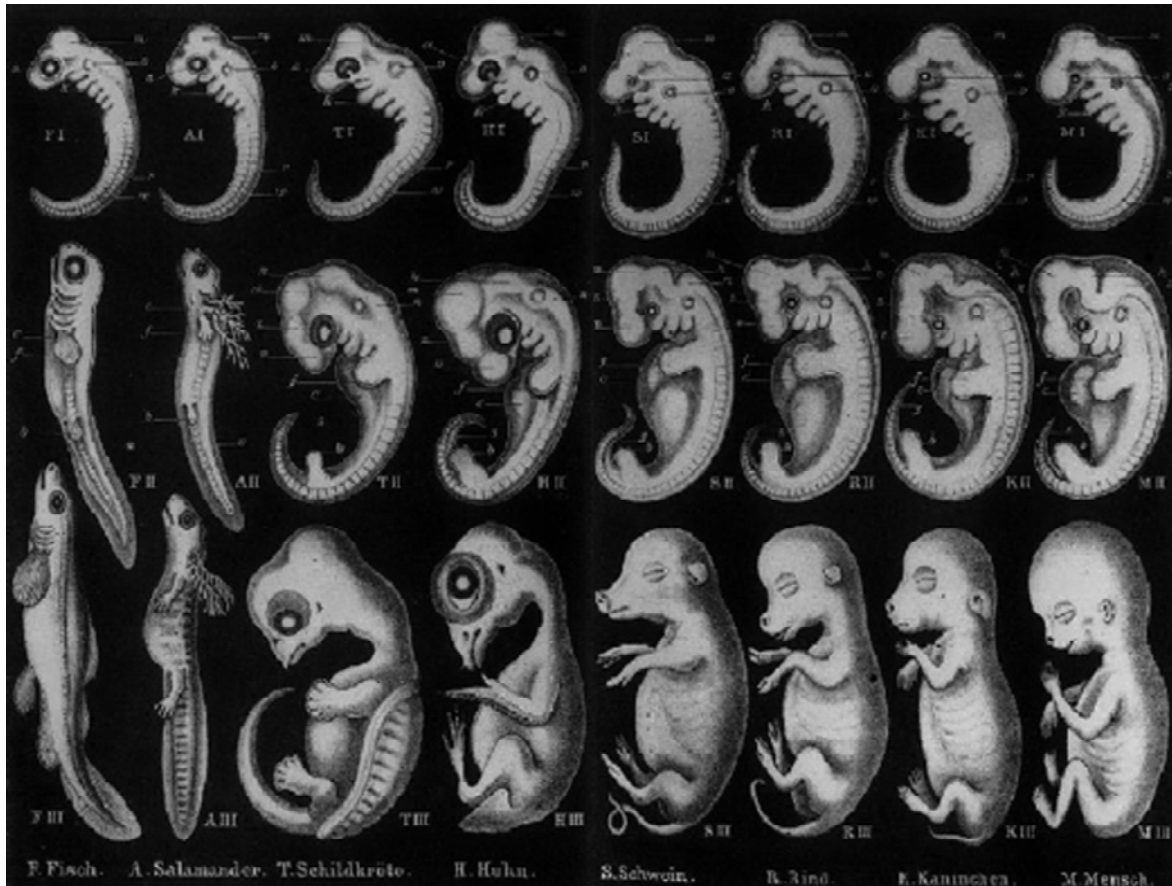
Fig. 1. Successive stages in the development of each species read from top to bottom. The top row shows the stage at which the highest degree of similarity is presented. Taken from Haeckel 1874. Anthropogenie, oder Entwicklungsgeschichte des Menschen.
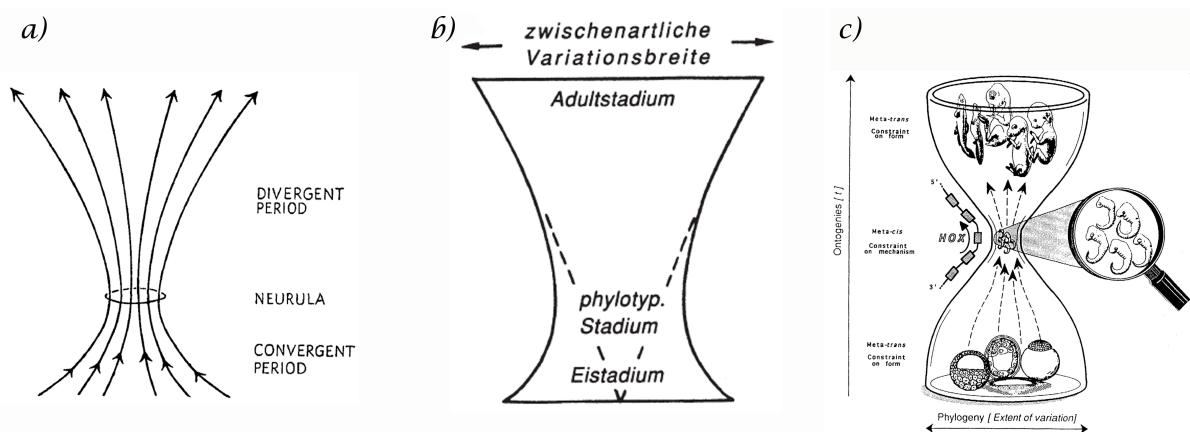

Fig 2. These three sketches display the evolution of the hourglass model. Images taken from: a) Medawar 1954. J Embryol. Exp. Morph. 2. 172-174 b) Sander 1994. Spuren der Evolution in den Mechanismen der Ontogenese – neue Facetten eines zeitlosen Themas 297-319 c) Duboule 1994. Dev Suppl. 135-142.

2 Results

2.1 Developmental Variability During Early Embryonic Development of Zebrafish

Developmental Variability During Early Embryonic Development of Zebra Fish, *Danio rerio*

KAI SCHMIDT AND J. MATTHIAS STARCK*
*Department of Biology II, University of Munich (LMU),
D-82152 Planegg-Martinsried, Germany*

ABSTRACT   Early vertebrate embryos pass through a period of remarkable morphological similarity. Possible causes for such similarity of early embryos include modularity, developmental constraints, stabilizing selection, canalization, and exhausted genetic variability. Supposedly, each process creates different patterns of variation and covariation of embryonic traits. We study the patterns of variation of the embryonic phenotype to test ideas about possible evolutionary mechanisms shaping the early embryonic development. We use the zebra fish, *Danio rerio*, as a model organism and apply repeated measures of individual embryos to study temporal changes of phenotypic variability during development. In particular, we are looking at the embryonic development from 12 hours post fertilization until 27 hours post fertilization. During this time period, the development of individual embryos is documented at hourly intervals. We measured maximum diameter of the eye, length of embryo, number of somites, inclination of somites, and the yolk size (as a maternal effect). The coefficient of variation (CV) was used as a measure of variability that was independent of size. We used a principal component analysis for analysis of morphological integration. The experimental setup kept environment × genotype interactions constant. Nongenetic parental contributions had no significant effects on interindividual variability. Thus all observed phenotypic variation was based on additive genetic variance and error variance. The average CV declined from 14% to 7.7%. The decline of the CV was in particular expressed during 15–19 h post fertilization and occurred in association with multiple correlations among embryonic traits and a relatively high degree of morphological integration. We suggest that internal constraints determine the patterns of variability during early embryonic development of zebra fish. *J. Exp. Zool. (Mol. Dev. Evol.) 302B:446–457, 2004.*   © 2004 Wiley-Liss, Inc.

INTRODUCTION

Studies in evolution of development have used large scale comparisons of diverging taxa in a known phylogenetic context to uncover the evolution of developmental pathways. This macroevolutionary approach reveals major patterns of the history of evolution of development but does not necessarily inform us about processes underlying the evolutionary diversification. As a pattern, the close similarity of early vertebrate embryos was recognized more than 150 years ago (e.g., von Baer, 1828; Haeckel, 1866, ’03). Haeckel’s (’03) biogenetic law was a first attempt to find a causal explanation for the resemblance of early vertebrate embryos. Assuming that evolution proceeds by addition of new elements to an adult phenotype (terminal addition/anagenesis), he conjectured that each developing organism had to pass through the evolutionary stages of its ancestors (recapitulation), i.e., phylogeny creates ontogeny.

This view has changed since Garstang (’22) and De Beer (’40, ’51, ’58). All modern concepts in evolution and development suppose that ontogeny creates phylogeny (e.g., Gould, ’77; Raff, ’96; Hall, ’99), but, the paradigmatic character of Haeckel’s biogenetic law has persisted until recent years as found in terms like ‘Körpergrundgestalt’ (Seidel, ’60), ‘phyletic stage’ (Cohen, ’77), or the ‘phylotypic stage’ (Sander, ’83). The phylotypic stage was defined as ‘‘… the stage of greatest similarity between forms which, during evolution, differently specialized both in their modes of adult life and with respect to the earliest stages of

ontogenesis …'' (Sander, '83). The germ band characterizes the phylotypic stage of arthropods, while in vertebrates the phylotypic stage comprises a broader range of embryonic morphologies. Ballard ('81) described the pharyngula or tail-bud stage as the phylotypic stage. Slack et al. ('93) referred to the synchronized expression of hox-genes during early embryogenesis as the 'zootype.' In their view, the basal patterns of hox-gene expression characterize the phylotypic stage. The embryonic morphologies of the phylotypic stage, e.g., pharyngula or tail-bud stage, were guidelines to detect the otherwise invisible 'zootype.' Patterns of morphological diversity during embryonic development may be more diverse as the early embryos of related species may differ widely. Consequently, the hourglass model (Raff et al., '91; Raff, '96) and the 'phylotypic egg-timer' model (Duboule, '94) were introduced to characterize the phylotypic stage as a period of reduced morphological variability if compared to earlier and later developmental stages of related taxa. Recently, Galis and Metz (2001) re-defined the phylotypic stage as the period from the beginning of neurulation to the complete formation of most of the somites. However, others have questioned the existence of a phylotypic stage (Richardson, '95, '99; Richardson et al., '97, 2001) and even inverted the hourglass model (Bininda-Emonds et al., 2003) because comparative analyses of a broad spectrum of vertebrate taxa revealed considerable heterochronic shifts, developmental differences, increased phenotypic diversity during mid embryonic stages, and stage incompatibilities. However, Richardson ('95) acknowledged the general similarity of embryos during their early embryogenesis but suggested 'phylotypic period' as a more appropriate term to describe it.

The debate about patterns and processes in early embryogenesis and their evolutionary interpretation has persisted for more than 100 years, and although the phylotypic stage has become a central paradigm in developmental biology (Hall, '97) it has only rarely been tested. Raff ('94, '96) explained the similarity of embryos during the phylotypic stage by suggesting that early embryogenesis were dominate by axial information systems allowing for a considerable degree of variability. Middle development exhibited a high degree of interconnectivity between elements which later separate into modules. This interconnectivity would limit variability of development. During late development the embryo were modularized, thus allowing for considerable developmental variability. Kirschner and Gerhart ('98) proposed that the phylotypic stage was conserved by stabilizing selection in order to maintain the evolvability of the organisms. In their view, the phylotypic stage serves as a platform from which diversification starts but developmental constraints act on a lower level so that they are subordinated to stabilizing selection. Galis and Metz (2001) and Galis and Sinervo (2002) explained the similarity of early vertebrate embryos as resulting from a variety of developmental constraints. Galis and Metz (2001) showed that teratogens have different effects during different phases of embryogenesis with generally strongest adverse effects during the phylotypic stage. Recurring on Raff's ('94, '96) ideas, they suggested that early vertebrate embryos are so vulnerable because they lack modularity and because developmental constraints prohibit evolutionary change. In other words, a plethora of inductive interactions within the embryo makes it vulnerable to fatal developmental aberrations due to external disturbances. Vulnerability is supposed to decline when modularity compartmentalizes the developing organism into quasi-independent developing units. Galis and Sinervo (2002) elaborated on that view assuming that modularity might be present during the phylotypic stage but that it is not effective because there are too many interactions between the different modules, i.e., low effective modularity. They proposed that, due to the interacting modules, mutational changes will have adverse pleiotropic effects and therefore selection acts against them, thus conserving body plans of early vertebrate embryos (Galis and Sinervo, 2002; Galis et al., 2003). The high interdependence between the different developmental processes is hypothesized to be a constraint that limits the action of selection. However, modularity occurs already in gastrulating *Xenopus* (Bolker, 2000), where the dorsal marginal zone acts as a module. Another well described module is the limb bud, which contains submodules of yet unclear level of independence (Raff, '96; Richardson, '99). Appearance of limb buds can heterochronically shift throughout the phylotypic stage (Richardson, '95), or even to later developmental stages to the end of metamorphosis in amphibians (Galis et al., 2003). Therefore, it was suggested that patterns of heterochrony indicate modularity of early embryos because developmental connectivity would prohibit the change of the sequence of organogenesis and ontogenetic repatterning would not be possible

without modularity (Roth and Wake, '85; Wake, '89; Chipman et al., 2000; Richardson and Oelschläger, 2002; Bininda-Emonds et al., 2003).

Although the difference between proponents of the phylotypic stage and those who oppose it appears to be fundamental, these concepts are not necessarily mutually exclusive. Rather, the divergence seems to root in different phylogenetic approaches and interpretations. Those trained in phylogenetic systematics look at derived characters of a clade (i.e., apomorphic traits) and tend to leave aside shared characters (i.e., phyletic homologies, plesiomorphies). Of course, they see the differences rather than the common features. In contrast, those who are interested in the major patterns of development and evolution tend to look at phyletic homologies and, by focusing on similarities, are likely to ignore the 'little' differences. Also, it seems that the proponents of the phylotypic stage are primarily interested in general architectonic features or organizational characters than quantitative variation. Recently, Sander and Schmidt-Ott (2004) paraphrased the conceptual divergence around the phylotypic stage/period as "…some people are interested in the forest whereas others prefer to look at each single tree and thus have more stringent criteria in defining a global stage for all vertebrates." In face of these contrasting ideas it is still more or less unclear today which evolutionary processes led to the striking similarity of early vertebrate embryos. Also, a yet open question is whether the phylotypic stage/period makes any prediction about quantitative variation of embryonic characters.

Here, we analyze patterns of embryonic variability to test ideas about evolutionary processes that may have resulted in the similarity of early vertebrate embryos. Supposedly, different evolutionary processes, i.e., exhausted genetic variance, stabilizing selection, constraints, and lack of modularity, result in different pattern of variation and covariation of embryonic traits. Variability can be quantified as phenotypic variance of (embryonic) characters and the total phenotypic variance can be partitioned into several components, i.e., additive genetic variance, environment induced variance, error variance, environment $\times$ gene covariation, epistasis, and nongenetic parental effects (e.g., Falconer and Mackay, '96; Lynch and Walsh, '98). We kept the environmental component and the environment $\times$ gene covariation constant by raising embryos under identical conditions. We used measurements of the size of

the yolk sac to control for nongenetic parental (maternal) effects on embryonic development. Thus, observed phenotypic variation is due to genetic effects (additive genetic variance and epistasis), and error while the constant environmental conditions supposedly do not contribute to variation of the observed phenotype. We predict the following patterns of variance and covariance among traits resulting from different evolutionary processes: (1) strong directional selection will lead to exhausted additive genetic variance and, consequently, will result in fixed phenotypes and zero genetic variance. Then, the observed phenotypic variance equals error variance and we expect no covariance between the traits. Because error variance is not directional traits should not covary. If strong directional selection has shaped the phylotypic stage we expect the total phenotypic variance to be significantly reduced during the phylotypic period if compared with earlier or later stages. (2) Internal constraints emerge from multiple (functional) interactions among traits (Raff, '96; Kirschner and Gerhart, '98; Schwenk 2001, Schwenk and Wagner, 2004, 2003) and, supposedly, result in significant covariance among phenotypic characters (Williams, '92). Internal constraints reduce the phenotypic variance because the coupling of traits reduces the degrees of freedom. Presumably, error variances remain unchanged because constraints do not necessarily require more precise development. If, however, traits covary in anatomically discrete pattern, it will be difficult to differentiate between internal constraints and modules (see below). (3) Stabilizing selection and canalization cannot be separated with our approach, as both will result in the same pattern of phenotypic variance and covariance. Stabilizing selection/canalization reduce genetic variance and possibly error variances by improved developmental processing. With the experimental setup suggested here, the observed total phenotypic variance will be reduced if compared to periods without stabilizing selection/canalization. Assuming that stabilizing selection/canalization does not increase the level of connectivity among traits we expect no change in covariance among characters. (4) When a developing embryo lacks modularity (as suggested by Galis and Metz [2001] and Galis and Sinervo [2002]) a higher number of interactions and mutual inductions is theoretically possible than in modular organisms. Because each interaction has its own error variances and because variances are additive, we predict that periods of zero modularity can be recognized by

the increased phenotypic variance. Also, a high number of inductive interactions (lack of modularity) may result in covariation among traits across the entire embryo, i.e., not in a topographically discrete patterns. In contrast, modularity compartmentalizes the embryo into subunits/modules. In modular embryos, inductive interactions are reduced to characters within the module resulting in reduced trait variability and morphologically discrete patterns of covariation. Our predictions are summarized in Table 1. However, the experimental setup of our study is controlled for constant environmental effects but does not allow to differentiate between the remaining components of variance. Therefore, we use the observed phenotypic variance a simplified measure of patterns of variation and covariation as outlined in Table 2. The predictions of patterns of variation and covariation associated with the four evolutionary processes are distinct. This makes it possible to distinguish the four processes discussed.

To test the above predictions, we have studied phenotypic variability of zebra fish embryos. We have chosen the developmental period between 12h post fertilization (end of neurulation) through the tail bud stage (12h–24h) until 27h post fertilization (pharyngula stage; Kimmel et al., '95). This 16h period encompasses all possible definitions of the phylotypic stage. We intended to produce a robust data set of repeated measures of

phenotypic variability of individual fish embryos (N=100) over a period of 16 hours. The coefficient of variation (standard deviation divided by mean $*$ 100) is a size independent measure of the variability of any given trait that allows comparisons among a variety of different characters. The measurements of embryonic characters were necessarily restricted to external characters. Although we acknowledge that we do not cover all morphologically distinct characters of zebra fish embryos, we think that the chosen characters (see below) enable us to capture major patterns of variability during the early development of zebra fish. We measured the size of the yolk sac as potential nongenetic parental effect that may lead to increased variability between individuals.

MATERIALS AND METHODS

Animals

Zebra fish (*Danio rerio*) were obtained from different pet shops in Jena, Germany, and from breeding stocks of the Max-Planck-Institute for Developmental Biology in Tübingen and the Zoological Institute of the University of Jena. We are aware of the fact that the genetic variation in our breeding population may be reduced as compared to natural populations. However, a potentially depressed genetic variability provides a conservative experimental background for our

TABLE 1. *Predictions about variance components emerging from different evo devo processes during the phylotypic stage*

	Phenotypic variance	Genetic variance	Error variance	Covariation among traits
Additive genetic variance exhausted	equal error variance	zero	unchanged	zero
Internal constraints	declined	reduced	unchanged	significant covariation
Stabilizing selection and canalization	declined	reduced	reduced	unchanged
Lack of modularity	increased	unchanged	increased	no topographically discrete patterns of covariations/ higher number of covariations

TABLE 2. *Predictions from the experimental setup of this study. We measure phenotypic variance and covariation among traits. These two measures characterize unequivocally the four possible evo devo processes underlying the observed patterns of variation*

	Observed variance	Observed covariation
Additive genetic variance exhausted	low	zero
Internal constraints	declined	significant covariation
Stabilizing selection and canalization	declined	unchanged
Lack of modularity/pleiotropy	increased	no topographically discrete patterns of covariations but higher number of covariations

study. Animals were kept in 80 l tanks at 27.4 ± 0.1 °C in standardized water (60 µg NaCl l ^{1}, pH 5.5, electrical conductivity 136.8 µS/cm, O_2 concentration 6.2 mg/l). For the breeding experiments, we kept pairs of individually known fish in 12 l tanks.

Raising conditions

Egg laying and fertilization occurred immediately after switching the light on. We observed egg laying and collected eggs immediately after fertilization. From each clutch we collected 5–10 eggs and placed them into small glass containers (20 ml volume). Fertilized eggs were raised individually under conditions given above. Because the egg membrane swells immediately after fertilization, it was possible to determine the time of fertilization at a precision of <1 min. All ages are given in hours (± 1 min.) after fertilization.

Microscopy and documentation

Developing eggs were observed with an inverted microscope (Zeis, Axiovert S 100) and photographs were taken with a spot digital camera (Diagnostic Instruments). The container for microscopic observation of embryos had a controlled temperature of 27°C. After observation, embryos were placed back into their individual containers. The left side of the embryo was photographed, to exclude variation in measurements due to asymmetrical growth.

The development of 100 individual embryos was documented between 12h and 27h of embryonic development (Fig. 1). Between 12h and 19h, digital photographs were taken in hourly intervals from 100 individual embryos. At an age of 20h post fertilization and older, embryos were bending within the egg and therefore had to be removed from the egg. Of course, those could not be measured repeatedly. Approximately 1700 images were recorded and analyzed.

Measurements

In early zebra fish embryos, only a limited set of morphological landmarks is accessible for repeated noninvasive measurements. We measured the following traits (Fig. 2A, B): (1) maximum diameter of the eye, (2) total length of embryo, i.e., the length from snout to tip of tail as seen in lateral view; (3) length and height of the fifth somite, (4) number of somites, (5) the angles between the fourth and fifths and fifth and sixth myoseptum, (6) diameter of the yolk sac (as maternal component) at 12h post fertilization.

Morphometry and statistics

We used SigmaScanPro (version 4.0, Jandel Scientific, SPSS Inc., Chicago, USA) for image analysis and morphometric data acquisition. Measurement error was calculated from repeated measurements of same structures. On average, we are dealing with measurement error of less than 1% of the measured value. Values are given as means $\pm$ S.D. (N=sample size). Comparisons of phenotypic variability of different traits were made by calculating the coefficient of variation (CV) which is a size independent measure of the overall variability of a trait. To test for developmental constraints, correlations after Pearson and covariances have been calculated for all measured embryonic traits. To amend these tests by a more complex measure of 'morphological integration' we run principle component analyses for all variables and compared the eigenvalues of PC1 over developmental time. Possible parental effects were tested by calculating for each individual fish the regression lines of the measured trait over time (i.e., 12h to 27h). Then, we plotted the slopes of the resulting regression lines as the dependent parameter of the diameter of yolk sac at 12h as the independent parameter. The size of the yolk sac at 12h pf was chosen because at this time the least yolk was absorbed. The $P < 0.05$ level was considered significant. All statistical procedures were performed using SPSS version 10.07.

RESULTS

Length of the embryo

Between 12h and 27h, the growth of the embryo is linear (Fig. 3). The CV of the length of 100 embryos at a given time ranges between 6.25% at 12h and 4.26% at 27h. A linear regression of the CV over time (Fig. 4A) showed a slight but significant decline (slope=–0.121; intercept=6.98; ANOVA: df 1,14; F=20.46; $P < 0.001$; R^2=0.59). All observed values were within the prediction limits. The size of the yolk sac at 12h pf had no significant effect on the length increase of the embryo (N=100, slope=0.045; intercept=$-$0.372; ANOVA: df 1,98; F=0.062; P=0.803; R^2=0.025).

Number of somites at a given time

First somites occur between 11h and 12h. At 15h post fertilization, 100% of the embryos had at least one or more somites. For characters that develop in discrete units, like somites, it is not appropriate to calculate a CV. Therefore, we have
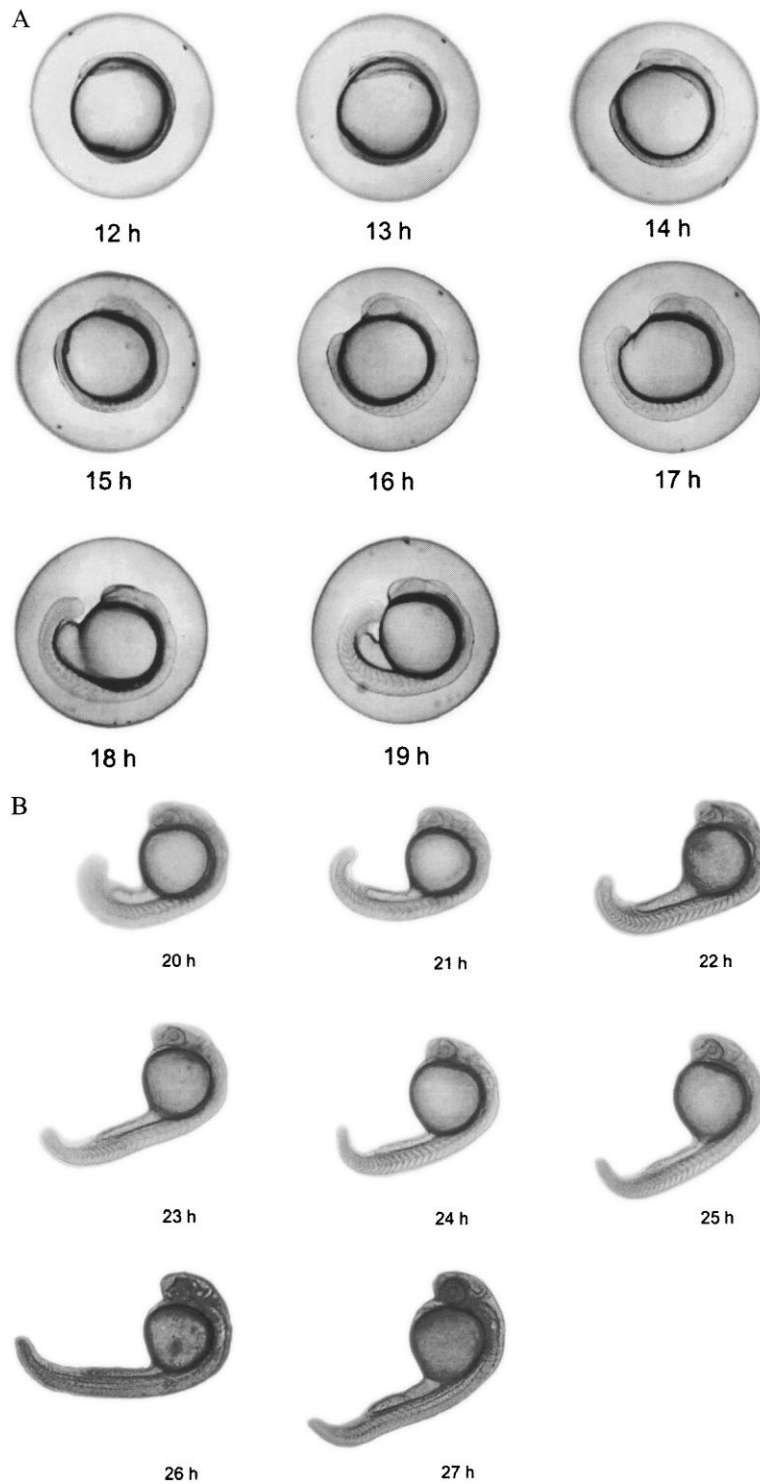
Fig. 1. Embryonic stages of zebra fish; (A) between 12h and 19h post fertilization. One hundred embryos were kept in individually marked containers and measured repeatedly, i.e., every hour between 12 19h. (B) Zebra fish embryos between 20h and 27h pf. These embryos were removed from the egg thus could not be measured repeatedly.
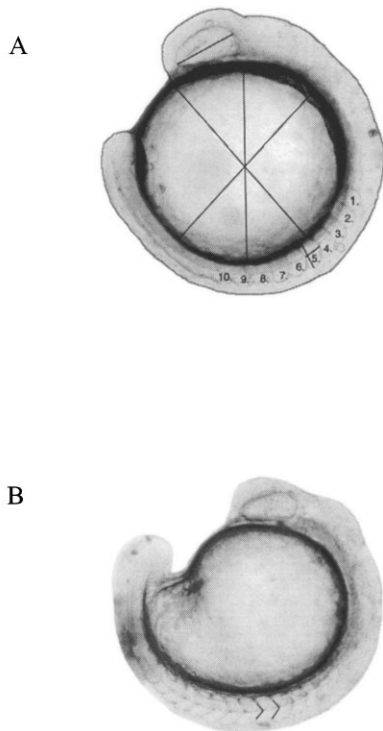
Fig. 2. (A) Position of morphometric measurement taken from embryos. (B) Position of measurement of the angle of 5th somite.

calculated the rate of somitogenesis for each individual (=number of newly formed somites per hour). Between 12h and 19h new somites appear at a constant rate of 2.4 somites per hour, thereafter the rate of somitogenesis declines. The CV of the rate of somitogenesis is 5.9%. Somitogenesis was not affected by the size of the yolk sac (N=98, slope=0.003; intercept=20.16; ANOVA: df 1,96; F=0.114; P=0.736; R^2=0.001).

Size of the 5th somite

Somites develop from anterior to posterior. New somites have a rectangular shape which changes after a few hours to the typical bent shape of a later somite. Between 12h and 17h the fifth somite shortens in length (Fig. 4B) and increases in height (Fig. 4C). After that age, its length increases. The CV of the length of the 5th somite ranges between 7.2% and 13.1%. A linear regression of the CV of somite length over time showed a significant increase (slope=0.28, intercept=4.57, ANOVA: df 1,13; F=8.17; P=0.013; R^2=0.39). All observed values were within the prediction limits of that regression. The length of the 5th somite was not affected by size of the yolk
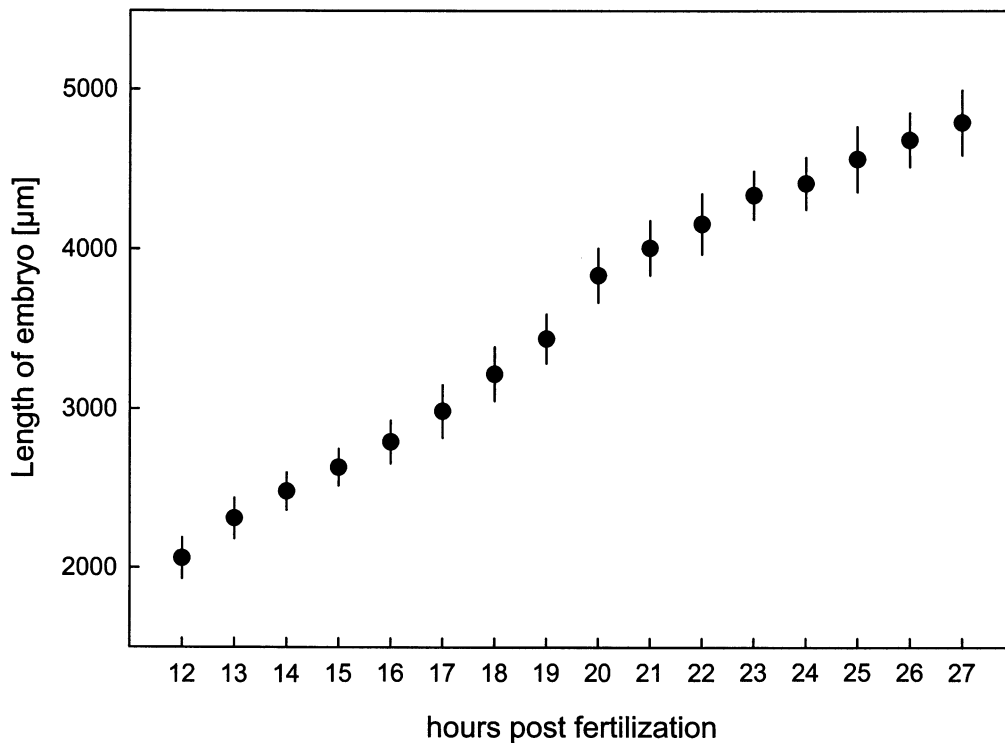

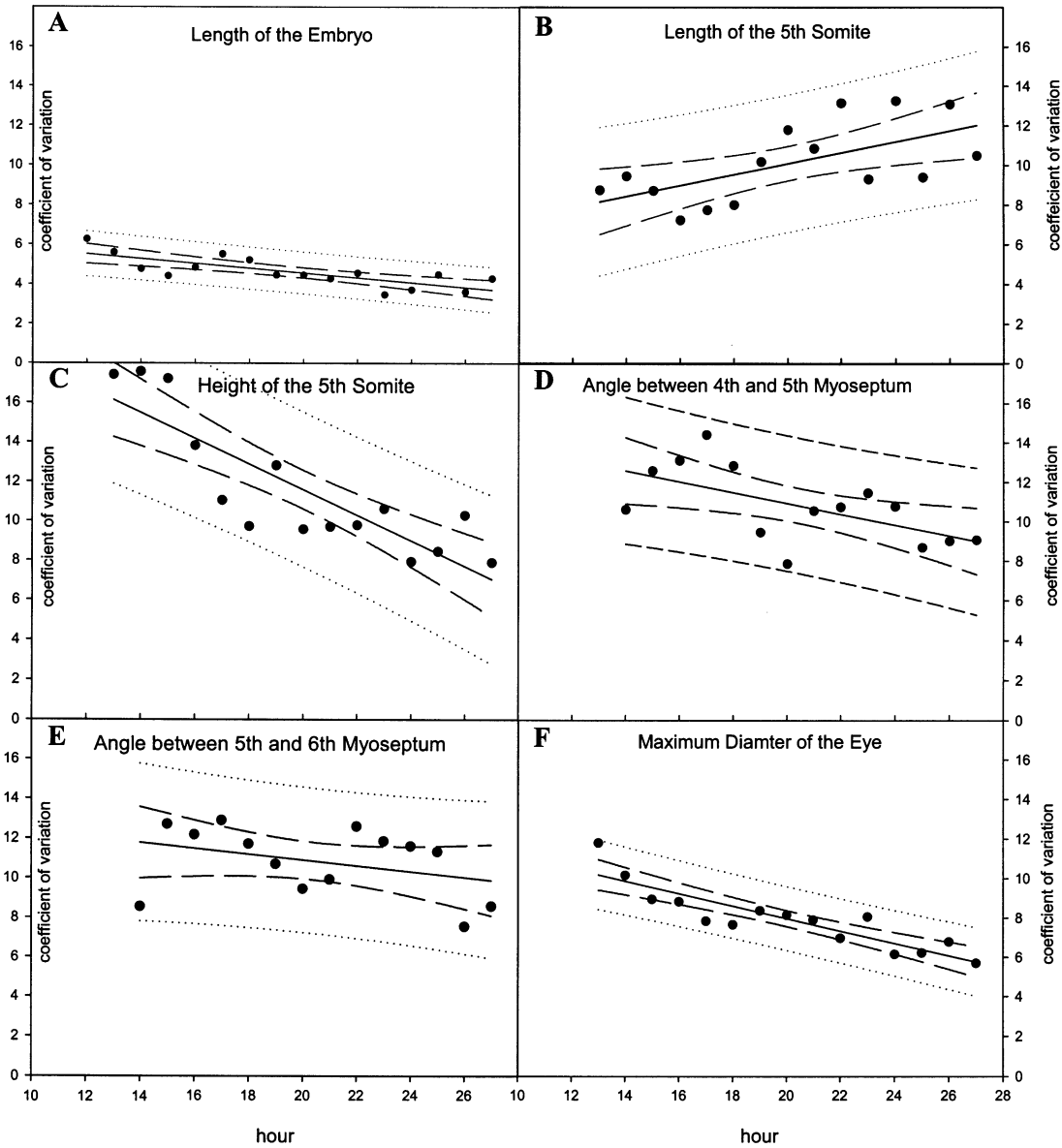Fig. 3.   Growth of zebra fish embryos between 12h and 27h pf.

Fig. 4. Changes of the coefficient of variation (CV) for all traits over time. A linear regression was used to estimate slope and intercept and an ANOVA was used to test if the slope was significantly different from zero. (A) Change of the CV of the length of the embryo during the observation period (ANOVA, slope 0.34, df 1,14, F 60.23, $P<0.001$, R^2 0.82). (B) Change of the CV of the length of the 5th somite during the observation period (ANOVA, slope 0.29, df 1,13, F 8.17, P 0.013, R^2 0.39). (C) Change of the CV of the height of the 5th somite during the observation period (ANOVA, slope 0.65, df 1,13, F 35.39, $P<0.001$, R^2 0.73). (D) Change of the CV of the angle between 4th and 5th somite during the observation period (ANOVA, slope 0.28, df 1,12, F 6.83, P 0.023, R^2 0.36). (E) Change of the CV of the angle between 5th and 6th somite during the observation period (ANOVA, slope 0.15, df 1,12, F 1.70, P 0.022, R^2 0.12). (F) Change of the CV of the maximum diameter of the eye during the observation period (ANOVA, slope 0.34, df 1,13, F 60.23, $P<0.001$, R^2 0.82).

sac (N=100, slope=−0.009; intercept= 3.21; ANOVA: df 1,98; F=3.561; P=0.062; R^2=0.035).

The height of the 5th somite more than doubled between 12h and 19h and the CV ranged between 17.6% and 9.7%. A linear regression of the CV of height of the 5th somite over time showed a significant decline (slope=−0.65, intercept= 24.60, ANOVA: df 1,13; F=35.39; $P<0.001$; R^2=0.73). The height of the 5th somite was not affected by the size of the yolk sac (N=97, slope=0.007; intercept=5.061; ANOVA: df 1,99; F=0.458; P=0.5; R^2=0.005). Angle of the fifth

somite: The angles can be measured only after the somites have developed their typical shape. For the observation period, in which the angles can be measured, a slight decline of the coefficient of variation was observed (Fig. 4D). The coefficient of variation varied between 7.5% and 14.4% (angle between 4th and 5th myoseptum: slope=-0.275; intercept=16.431; ANOVA: df 1,12; F=6.825; P=0.023; R^2=0.363; angle between 5th and 6th myoseptum: slope=-0.147; intercept=13.832; ANOVA: df 1,12; F=1.703; P=0.0216; R^2=0.124; Fig. 4E).

Maximum diameter of the eye

Development of the eye begins between 12h and 13h post fertilization. At 12h pf, the primordia of the eye are present in 15% of all individuals; at 13h pf in 75% of the embryos (Fig. 4F). During the development of the embryo, the form of the eye changes from elliptical and to round. In early embryos, the maximum diameter of the eye extends from rostral to caudal in a horizontal plane through the eye. It declines between 13h and 16h pf but increases again thereafter until it has obtained a round shape at 22h pf. Between 13h and 15h, the coefficient of variation of the maximum diameter of the eye peaks at 12%; it ranges from 7.7% to 9% between 15h and 19h, and from 8.1 to 5.7% between 20h and 27h. As a general trend, the coefficient of variation declined during the early embryonic period. All observed fluctuations of the CV were within the prediction limits (Fig. 4F) of a linear regression

(slope=-0.34; intercept=14.64; ANOVA: df 1,13; F=60.2; $P<0.001$; R^2=0.82). The diameter of the eye was not affected by the size of the yolk sac (N=100, slope =-0.0449; intercept=143.549; ANOVA: df 1,98; F=0.564; P=0.454; R^2=0.006).

Size of yolk

12h after fertilization the yolk ball has a circular shape (Fig. 2A); its diameter is on average 659.55 ± 21.87 μm and the coefficient of variation between individuals is 3.32 %.

Correlations and morphological integration

We tested for correlations among traits at each hour of development to find out about changing functional relationship within the developing embryos. To visualize changing relationships among embryonic traits we plotted the number of significant correlations as a function of developmental time (Table 3). The highest number of significant correlations was found between 15h and 18h post fertilization. The outstanding correlations were total length of embryo and the height of the 5th somite at each hour of the observed time period. Also, the maximum diameter of the eye was correlated with the length of the embryo, except for the measurements at 14h pf. Not surprisingly, the angles between the fourth and fifth and fifth and sixth myoseptum displayed a significant correlation for the entire observation period except for 12h pf and 13h pf, when the sample size was too small (N<2).

TABLE 3. *Change of the coefficient of variation and the number of correlations over developmental time*

Age	Phenotypic variance (coeff. of variation)		Number of significant correlations		Eigenvalues of PC1		Conclusion
12	14.06		1				
13	10.88		5				
14	10.18		3		3.32		
15	10.76		4		3.72		Internal constraints
16	10.00		9		3.10		Internal constraints
17	9.91		9		2.94		Internal constraints
18	9.19		5		3.10		Internal constraints
19	9.33		5		1.94		Internal constraints
20	8.53		2		2.09		
21	8.86		1		1.86		
22	9.63		4		2.36		
23	9.12		3		1.93		
24	8.89		1		2.35		
25	8.08		2		2.44		
26	8.38		2		2.61		
27	7.66		2				
	8 10 12 14		0 2 4 6 8 10		2 3 4		

Developmental variability

We performed a principal component analysis for each hour of development to obtain a measure of overall morphological integration of embryonic characters. The eigenvalues of the first principal component were used as a measure of morphological integration. A high eigenvalue stands for a high number of original variables contributing to PC1, thus indicates high morphological integration. The analysis had to be truncated because the data matrix was incomplete for some traits during the early hours of our study, e.g., number of somites. The eigenvalues changed in accordance to our pairwise correlations as shown in the previous paragraph. Highest eigenvalues were found around 15 h pf, after which they declined (Table 3).

DISCUSSION

In the zebra fish, the phylotypic stage extends over a considerable period if determined by morphological landmarks. From the end of neurulation (about 10h post fertilization) to the pharyngula stage (24h–30h) the developing zebra fish embryo shows traits that are characteristic of the phylotypic stage. Those traits may also be found in embryos of other vertebrate species of similar developmental stage.

In Tables 1 and 2 we predicted how different evolutionary processes may shape the pattern of variance and covariance of embryonic traits. Table 3 summarizes the pattern of variance and covariance during early embryogenesis of zebra fish. In all but one trait, the coefficient of variation declined linearly during the early embryonic period. Only the CV of the length of the 5th somite increased. The covariation among traits showed a clear peak between 15h and 19h pf, i.e., during a period when phenotypic variability declined for all traits. According to our predictions (Tables 1 and 2), significant covariance among phenotypic characters associated with declining phenotypic variance emerges from internal constraints, i.e., multiple (functional) interactions among traits. Phenotypic variance is reduced as compared to earlier developmental stages because the coupling of traits reduces the degrees of freedom and thus reduces the phenotypic variance. Thus, for the zebra fish embryos between 15h and 19h pf we recognize a period of restricted variation of the phenotype due to internal developmental constraints. These observations are paralleled by a period of high morphological integration (Table 3) as recognized by a principal component analysis (Chernoff and Magwene, '99). We can rule out directional selection (exhausted additive genetic variance), modularity, and canalization/stabilizing selection because the patterns of variance and covariance we observed do not match predictions from those processes. Thus, for the zebra fish we suggest that the phylotypic stage emerges because of internal constraints rather than as a result of natural selection. This result supports the idea of Raff ('94, '96) that during middle embryogenesis high interconnectivity of embryonic traits limits developmental variability, during later development, the embryo is compartmentalized into independent modules which allow for more variability. A lack of modularity as favored by Galis and Metz (2001), Galis et al. (2002, 2003) can also be excluded because total phenotypic variances were clearly declining whilst lack of modularity predicted increasing phenotypic variances. However, we acknowledge that there is a certain degree of conceptual fuzziness which makes it difficult to differentiate between constraints as suggested here, interconnectivity (Raff, '94, '96), and low effective modularity (Galis and Sinervo, 2002; Galis et al., 2003). When constraints are anatomically restricted or when modularity increases gradually during development a gradual model will be required to test for different degrees of modularity. Today, no such scale is available that would allow us to gauge different degrees of modularity. Therefore, we prefer a conservative interpretation of our results and suggest that during 15h–19h of embryogenesis of zebra fish developmental variability is limited by internal constrained and high morphological integration rather than low (effective) modularity (Galis and Sinervo, 2002; Galis et al., 2003).

Comparative data are not available and therefore generalizations are necessarily characterized by a certain degree of uncertainty. However, our results appear not to be in conflict with ideas presented by Galis and Metz (2001), Galis et al. (2002, 2003) who found increased mortality of mice if they were exposed to teratogenes during the phylotypic stage. Galis and coworkers suggested that lack of modularity during the phylotypic stage permitted fatal effects of teratogenes, while the increasingly modular architecture of later embryos would prevent teratogenic effects to spread through the embryo. However, Galis and coworkers had no tools to differentiate between different evo-devo processes. We think that

the results from our experiments and their interpretation as internal constraints are not in contrast to the results obtained by Galis and coworkers. If the 'phylotypic period' evolved because of developmental constraints or a high degree of morphological integration, then a disruption of such interactions may result in increased teratogenesis and ultimately increased mortality and as described by Galis and Metz (2001) for mice. Lethal effects of teratogenes do not allow to differentiate between different evo-devo processes because interrupted functional correlations and spreading of detrimental effects through the embryo may both result in increased (later) mortality.

Our results also agree with Richardson ('95) who showed that there is no highly conserved embryonic stage in vertebrate embryos but rather a period of general similiarity. The morphological landmarks of the 'phylotypic stage' occur during a 16h hour period (=23% of embryonic development of zebra fish; Galis and Metz, 2001). In this species, phenotypic trait variation during the early embryogenesis is restrained by functional/developmental interactions among traits, thus, this period between 12h and 28h pf must to be relatively resistant to selection because changing any trait will affect all other that are functionally linked. Developmental constraints may be different in diverging taxa, explaining why the period of embryonic similarity is rather diffusely defined and not by (a low number of a) few clearly recognizable stage(s). However, at the present state of knowledge and with a considerable lack of comparative data, we have gained insight into zebra fish development and recognize that the phylotypic period of this particular species is caused by internal constraints. Our study remains descriptive in as far as we have no ideas about the proximate functional interactions among embryonic traits that affect the phylotypic period and we must remain open about possible causes of the phylotypic stage in other vertebrate species. However, we think that an analysis of patterns of variance and covariance of embryonic traits as conducted in this study is a helpful tool to direct research in evolution of development towards those embryonic periods that are resistant to selection.

ACKNOWLEDGEMENTS

LITERATURE CITED

Alberch P. 1985. Problems with the interpretation of developmental sequences. Syst Zool Mobile 34:46 58.

Ballard WB. 1981. Morphogenetic movements and fate maps of vertebrates. Am. Zool. 21:391 199.

Bininda Emonds ORP, Jeffery JE, and Richardson MK. 2003. Inverting the hourglass: quantitative evidence against the phylotypic stage in vertebrate development. Proc R Soc London 270:341 346.

Bolker JA. 2000. Modularity in development and why it matters to evo devo. Amer Zool 40:770 776.

Chernoff B and Magwene PM, 1999. Morphological integration: forty years later. In: Morphological Integration. Olson EC and Miller RL. University of Chicago Press, Chicago. 319 353.

Chipman AD, Haas A, Tchernov E, and Khaner O. 2000. Variation in anuran embryogenesis: differences in sequence and timing of early developmental events. J Exp Zool (Mol Dev Evol) 288:352 365.

Cockburn A. 1995. Evolutions Oekologie. (Fischer G, Cohen J, editors). Reproduction. Butterworth, London.

De Beer GR. 1958. Embryos and Ancestors. Clarendon Press, Oxford UK.

Duboule D. 1994. Temporal colinearity and the phylotypic progression: a basis for the stability of a vertebrate Bauplan and the evolution of morphologies through heterochrony. Dev Suppl 1994:135 142.

Falconer DS and Mackay TFC. 1996. Introduction to Quantitative Genetics. 4th Ed. Longman Sci. and Tech., Harlow, UK.

Galis F and Metz JA. 2001. Testing the vulnerability of the phylotypic stage: on modularity and evolutionary conservation. J Exp Zool (Mol Dev Evol) 291:195 204.

Galis F, van Dooren TJM and Metz JA. 2002. Conservation of the segmented germband stage: robustness or pleiotropy? Trends Genetics 18:504 509.

Galis F and Sinervo B. 2002. Divergence and convergence in early embryonic stages of metazoans. Contributions to Zoology 71(1/3):101 113.

Galis F, Wagner GP, and Jockush E. 2003. Why is limb regeneration possible in amphibians but not in reptiles, birds, and mammals? Evolution and Development 52:1 13.

Garstang W. 1922. The theory of recapitulation: A critical restatement of the biogenetic law. J Linn Soc Zool 35: 81 101.

Gould SJ. 1977. Ontogeny and Phylogeny. Belknap Press of Harvard University Press, Cambridge MA.

Haeckel E. 1866. Generelle Morphologie. 2 Vols., G. Reimer, Berlin.

Haeckel E. 1903. Anthropogenie oder Entwicklungsgeschichte des Menschen. Leipzig, Engelmann. Hall BK. 1997. Phylotypic stage or phantom: is there a highly conserved

embryonic stage in vertebrates? Trends Ecol Evol 12: 461 463.

Hall BK. 1999. Evolutionary Developmental Biology. 2nd edition, Kluwer Academic Press, Dordrecht.

Hernandez LP, Barresi JF, and Devoto SH. 2002. Functional morphology and developmental biology of zebra fish: reciprocal illumination from an unlikely couple. Integ Comp Biol 42:222 231.

Horder TJ. 1983. Embryological bases of evolution. Develop ment and Evolution. (Goodwin BC, Holder N, Wylie CG, editors), Cambridge University Press, Cambridge, UK: 315 352.

Irmler I. 2001. Variabilität im phylotypischen Stadium bei Zebrafischen. Unpublished Diploma thesis, Department of Biology, University of Jena.

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, and Schilling TF. 1995. Stages of embryonic development of the zebra fish. Dev Dyn 203:253 310.

Klingenberg CP. and Ekau W. 1996. Combined morphometric and phylogenetic analysis of an ecomorphological trend: pelagization in Antarctic fishes (Perciformes: Notothenii dae). Biol J Linn Soc 59:143 177.

Kirschner M. and Gerhart J. 1998. Evolvability. Proc Natl Acad Sci USA 95:8420 8427.

Lynch M and Walsh B. 1998. Genetics and Analyses of Quantitative Traits. Sinauer, Sunderland, USA.

Raff RA. 1994. Developmental mechanism in the evolution of animal form: origins and evolvability of body plans. In: Early Life on Earth (Bengston S, editor). Columbia University Press, New York. 489 500.

Raff RA. 1996. The Shape of Life. Genes, Development, and the Evolution of Animal Form. University of Chicago Press, Chicago.

Raff RA, Wray GA, and Henry JJ. 1991. Implications of radical evolutionary changes in early development for concepts of developmental constraint. In: New Perspectives on Evolu tion. (Warren L and Koprowski H, editors). Wiley Liss. New York. 189 207.

Rice SH. 1983. The evolution of canalization and the breaking of von Baer's laws: modelling the evolution of development with epistasis. Evolution 52:647 656.

Richardson MK. 1995. Heterochrony and the phylotypic period. Dev Biol 172:412 421.

Richardson MK. 1999. Vertebrate Evolution: the develop mental origins of adult variation. BioEssays 21:604 613.

Richardson MK, Hanken J, Gooneratne ML, Pieau C, Raynaud A, Selwood L, and Wright GM. 1997. There is no highly conserved embryonic stage in the vertebrates: implications for current theories of evolution and develop ment. Anat Embryol 196:91 106.

Richardson MK, Jeffery JE, Coates MI, Bininda Emonds OR. 2001. Comperative methods in development. Zoology 104:278 283.

Richardson MK, and Oelschläger HHA. 2002. Time, pattern, and heterochrony: a study of hyperphalangy in the dolphin embryo flipper. Evolution and Development 4:435 444.

Roth G and Wake DB. 1985. Trends in the functional morphology and sensorimotor control of feeding behavior in salamanders: an example of the role of internal dynamics in evolution. Acta Biotheoretica Leiden 34:175 192.

Sander K. 1983. The evolution of patterning mechanisms: gleanings from insect embryogenesis and spermatogenesis. In: Development and Evolution (Goodwin BC, Holder N, Wylie CC, editors), Cambridge University Press, Cambridge. 123 159.

Sander K and Schmidt Ott U. 2004. Evo devo aspects of classical and molecular data in a historical perspective. J Exp Zool (Mol Dev Evol) 302b:69 91.

Schwenk K. 2001. Functional units and their evolution. In: The Character Concept in Evolutionary Biology. (Wagner GP, editor). Academic Press, San Diego. 165 198.

Schwenk K and Wagner GP. 2003. Constraint. In: Key Words and Concepts in Evolutionary Developmental Biology. (Hall BK, Olson WM, editors). Harvard University Press, Cambridge. 52 61.

Schwenk K and Wagner GP. 2004. The relativism of constraints on phenotypic evolution. In: Phenotypic Inte gration: Studying the Ecology and Evolution of Complex Phenotypes. (Pigliucci M and Preston K, editors). Oxford Univ. Press, Oxford. 390 408.

Seidel F. 1960. Körpergrundgestalt und Keimstruktur. Eine Erörterung über die Grundlagen der vergleichenden und experimentellen Embryologie und deren Gültigkeit bei phylogenetischen Überlegungen. Zool Anz 164:245 305.

Slack JMW, Holland PWH, and Graham CF. 1993. The zootype and the phylotypic stage. Nature 361:490 492.

von Baer KE. 1828. Entwicklungsgeschichte der Tiere. Beobachtungen und Reflexionen. Königsberg: Bornträger, 264 pp.

Wagner GP. 1988. Developmental constraints. Population, Genetics and Evolution. (de Jong G, editor) Springer Verlag, Berlin, Heidelberg: 222 229.

Wagner GP and Altenberg L. 1996. Perspective: Complex Adaptations and the Evolution of Evolvability. Evolution 50:967 976.

Wake D. 1989. Phylogenetic implications of ontogenetic data. Geobios, memoire special 12:369 378.

Williams GC. 1991. Natural Selection. Domains, Levels and Challenges. Oxford Series in Ecology and Evolution. Oxford University Press, New York.

2.1.1   Erratum

ERRATUM

Irmler I, Schmidt K, Starck JM. 2004. In developmental variability during early embryonic development of zebra fish, *Danio rerio*. J. Exp. Zool. (Mol. Dev. Evol.) 302B:446–457.

In the above-referenced article, the first author, Dr. Ingo Irmler, was inadvertently omitted from the published article. The citation above has been corrected and the three authors are listed in the correct order. The first two authors, Dr. Irmler and Dr. Schmidt, contributed equally to this paper, and Dr. Starck is the corresponding author.

The authors regret this error.

2.2 Developmental Variability, **Modularity, and Heterochrony During the Phylotypic Stage of the Zebrafish**

I

Developmental Plasticity, Modularity, and Heterochrony During the Phylotypic Stage of the Zebra Fish, *Danio rerio*

KAI SCHMIDT* AND J. MATTHIAS STARCK
Department of Biology II, University of Munich (LMU), Planegg-Martinsried, Germany

ABSTRACT

We studied early embryonic development of zebra fish and tested if changes in the external raising conditions could elicit phenotypic changes during the phylotypic stage which, classically, is considered as a conserved embryonic stage. In particular, we tested for internal constraints, plasticity, and heterochrony during the early embryonic development. Our tested hypotheses predict (i) no change associated with developmental stability/internal constraints, (ii) change of the rate of development associated with developmental flexibility, and (iii) heterochronic disruption of developmental pattern associated with a modular organization of the embryo. We measured 14 traits of embryos raised in different conditions (temperature, salinity, oxygen concentration). The results of our study show that zebra fish embryos respond flexibly to changes in external parameters even during the conserved "phylotypic stage." It also showed that internal constraints canalize early development when exposed to moderate external challenges. Hypoxic conditions, however, elicited a heterochronic delay of the onset of the development of the Anlagen of the eye and the otic vesicle from the remaining embryo. Therefore, we concluded that the eye and the otic vesicle are modules that may develop, to a certain degree, independently of the rest of the embryo. Because these modules become recognizable only under specific raising conditions, we suggest that the modularization acts as buffering mechanism against extreme developmental deviations. Our results provide support to the idea that modularity is present during the phylotypic stage, but it is not effective under normal conditions. *J. Exp. Zool. (Mol. Dev. Ecol.) 314B:166–178, 2010.* © 2009 Wiley-Liss, Inc.

Heterochrony and modularity are two tightly connected concepts to explain the evolution of development (Raff and Raff, 2000; Raff and Sly, 2000; Olson and Rosell, 2006; Goswami, 2007). Modularity suggests that developing organisms are composed of more or less independent units, the modules. Individual modules can evolve without necessarily affecting other modules of the organism. Therefore, it was suggested that a high degree of modularity of an embryo facilitates evolutionary change of development (Von Dassow and Munro, '99; Bolker, 2000; Schlosser, 2002; Hansen, 2003; Schlosser and Wagner, 2004; Altenberg, 2005; Franz Odendaal and Hall, 2006; Tokita et al., 2007; Wagner et al., 2007; Pigliucci, 2008; Sauro, 2008). In contrast, a low degree of modularity would conserve a phenotype because the many interactions between the traits of an organism

would impair its evolvability (Raff, '96; Galis and Sinervo, 2002; Galis et al., 2002).

Heterochrony has been discussed intensively and with considerably controversy as a developmental mechanism (Gould, '77; Alberch et al., '79; Klingenberg, '98; Zelditch et al., '98;

McNamara, 2002; McNamara and McKinney, 2005). According to Gould ('77, 2000), heterochrony is the divergence between developmental timing of the germ line and the somatic cell line (clock model). In evolutionary terms, heterochrony may result in diverging developmental pathways and ultimately in the emergence of new species. Raff ('96) suggested that "hetero chrony is the dissociation of the relative timing of events in development between ancestral and descendant ontogenies." Here, we extend the definition of heterochrony as suggested by Raff ('96), McNamara ('97), and Smith (2001) by comparing ontogenies of different individuals of the same species exposed to different external conditions, that is, extend it to develop mental plasticity.

However, a certain period of the early embryogenesis of vertebrate development appears to be conserved among clades of vertebrates, that is, the phylotypic stage (Sander, '83; Galis and Metz, 2001; Sander and Schmidt Ott, 2004). As Haeckel ('03) postulated that anagenesis and recapitulation may cause the morphological similarity during the phylotypic stage, the mechanisms preventing the evolution of development during the phylotypic stage have been a subject of a controversial discussion (Duboule, '94; Hall, '97; Richardson et al., '97; Galis and Metz, 2001; Galis and Sinervo, 2002; Galis et al., 2002; Bininda Emonds et al., 2003; Irmler et al., 2004; Poe and Wake, 2004). Raff ('96) suggested that because the embryos were not yet modularized, multiple interactions among developing traits would make the entire embryo susceptible even to minor changes in just one trait. Only when modules had formed during later development, the embryo would become more stabilized because changes in one module would not affect traits in the other modules. The lack of modularity during the phylotypic stage would therefore constrain developmental variation and the evolvability of the embryo, resulting in a conserved period. Galis et al. (2002) and Galis and Sinervo (2002) elaborated on Raff's ('96) idea and hypothesized that the phylotypic stage might well be modularized but modules were not effective, thus the phylotypic stage would result from constraints because of "low effective modularity."

In an earlier study, we analyzed correlations among multiple traits in early embryos of zebra fish, *Danio rerio*, and showed that the phylotypic period is indeed characterized by a high number of correlations between morphological traits across the entire embryo (Irmler et al., 2004). That study supported Raff's ('96) idea that multiple interactions among embryonic traits constrain phenotypic variation. The temporarily increased number of morphological correlations across the entire embryo was interpreted as internal constraints restraining developmental variation and thus resulting in a period that has classically been described as the "phylotypic stage."

The idea of low effective modularity (Galis and Sinervo, 2002; Galis et al., 2002; Schlosser and Wagner, 2004) is intriguing, but, modularity can be recognized only if developmental traits can be dissociated from other traits along a developmental trajectory (Olson and Rosell, 2006). If, as outlined above, modularity and heterochrony are associated, we may be able to gauge the degree of modularity as heterochronic shift of associated traits along a developmental trajectory. Also, during periods of "low effective modularity," modularity may simply not be expressed. Conse quently, we may hypothesize that increasing external stressors might elicit increasing modularity thus stabilizing development in an increasingly stressful environment. Therefore, experiments in developmental plasticity may contribute to the analysis of heterochrony and modularity of organisms.

Finally, one may keep in mind that developmental plasticity may act as another mechanism that allows for evolutionary change of development (Hall, 2003). Developmental plasticity is the ability of an embryo to express different phenotypes when raised in different external conditions. If these different phenotypes later become fixed by genetic assimilation, plasticity has acted as a pace maker of evolution of development (Waddington, '42, '53; West Eberhard, 2003).

Most studies on evolution of development present a broad scale phylogenetic comparisons. We take a different approach by exposing a group of genetically similar individuals (sibling zebra fish) to different experimental conditions to elicit deviations in their developmental pathway (elicited developmental plasticity). We hope to potentially detect modules that were not discovered earlier because they were not effective under standard conditions. In this study, we ask if variation during the phylotypic stage is constrained or if morphological variation can be induced by exposing developing embryos to different external conditions. If the embryo during the phylotypic stage is not (yet) modularized and the phylotypic stage arises because of multiple interactions within the developing embryo, we expect relatively little induced variation of the entire embryo. If, however, the embryo is modularized we expect discrete packages of traits to be affected or shifted heterochronically along the developmental trajectory.

We use changes in temperature, salinity, and oxygen content of the water to induce changes in the pattern of development. Any expected change in development would either occur as changes in the rate of growth (acceleration/deceleration), as a change in shape (sensu Gould, '77), or related to age (resulting in the different domains of heterochrony as summarized in Gould ('77)). Our predictions deviate from Gould's concept of hetero chrony because we do not use the developing germ line of an ancestral species as reference for timing, but standardized normal development.

Temperature, oxygen, and salinity are known to affect fish development. In particular, increased temperature may result in accelerated development in many fish species (Blaxter, '88; Pepin, '91). In some salmonid and clupeid species, increasing temperature results in faster development but hatchlings tend to weigh less than those raised under standard conditions (Blaxter,

'88). Also, the number of serial structures such as vertebrae, fin rays, scales may be affected by temperature (Taning, '52; Barlow, '61; Blaxter, '69). For zebra fish, the temperature range for development is between 18 and 31°C, with an optimum at 27°C; above or below in which embryo mortality increases (Westerfield, '94; Kimmel et al., '95; Froese and Daniel, 2004). Water salinity also affects fish development and growth. Generally, freshwater fish grow faster at higher salinity (Bunn et al., 2000; Bœuf and Payan, 2001). Finally, low oxygen concentration of the water may results in delayed development (Rombough, '88; Bunn et al., 2000) and premature hatching (Hempel, '79; DiMichele and Taylor, '80; DiMichele and Powers, '82), it results in morphological changes, and physiological adjustments (Rombough, '88).

By exposing embryos during the phylotypic period to different raising conditions, we intend to elicit developmental changes and thus shall be able to detect the developmental mechanisms by which the phenotype during the phylotypic stage is maintained. We test the following predictions: (1) exposing the embryo to different external conditions does not elicit any change in the developing phenotype. This indicates a conserved phenotype either because of internal constraints or because of the lack of modularity. Both would be measured as multiple correlations among developmental traits and could not be separated by morphometric methods. (2) Exposing the embryo to different external conditions results in faster or slower growth. This indicates a flexible phenotype resulting in a potentially different adult phenotype. However, if all traits develop faster or slower we would interpret this as flexible but not modularized development. In traditional terminology such an observation would refer to acceleration/progenesis or deceleration/hypermorphosis (sensu Gould, '77). (3) If traits cluster together and develop at a different speed than others traits, we recognize modularity, that is, heterochrony of that particular character.

MATERIAL AND METHODS

Animals
Wild type zebra fish, *Danio rerio* (Hamilton, 1822), were obtained from three different pet shops in Germany. Animals were kept in 80 L tanks at 27.0°C in standardized water (10 mosmol/L salinity, and 100% oxygen saturation) according to Kimmel et al. ('95) and a light:dark cycle of 14:10 hr. For breeding experiments, we kept pairs of individually known fish in 12 L tanks.

Egg Collection
After the dark period, onset of light initiated egg laying and fertilization. To allow for a precise timing of fertilization, pairs were allowed to mate only for 5 min and were then separated. Once initiated, the mating impulse persists for at least 1 hr so that mating and egg collecting could be repeated after 25 min. From each clutch, we collected up to 100 eggs and placed them in small

glass containers (20 mL volume) covered with gaze. Collected eggs within their gaze covered glass containers were cleaned with fresh water and transferred to the raising tanks (see raising condition of embryos). Because we allowed only 5 min for mating, the time of fertilization was known at a precision of 5 min. Embryos were assigned to normal stages according to Kimmel et al. ('95).

Raising Conditions
We conducted three independent experiments in which embryos were exposed to different raising conditions. In each experiment, an equal number of eggs from same clutches were randomly assigned to the experimental and control conditions, so that the genetic distance was minimized. Eggs were transferred to raising conditions immediately after fertilization. Standard condition was 27°C, 10 mosmol/L NaCl, and 100% oxygen concentration. In each experiment we changed only one of the following standard parameters: (1) temperature: embryos were raised at 24, 27, and at 30°C. (2) Salinity: embryos were raised in 10 mosmol/L NaCl and 200 mosmol/L NaCl. (3) Oxygen concentration: embryos were raised in 100% oxygen saturated water (i.e., 8.32 mg O_2/L), 20% oxygen saturated water (i.e., 1.66 mg O_2/L), 15% oxygen saturated water (i.e., 1.25 mg O_2/L), and 10% oxygen saturated water (i.e., 0.83 mg O_2/L).

Temperature in the raising tanks was set to constant temperature and controlled by a Haake C10 immersion circulator (Haake GmbH, Karlsruhe, Germany) with an accuracy of ± 0.04°C. The osmolality was measured with a Knauer Osmometer Automatic (Knaur Gerätebau, Berlin, Germany). The oxygen concentration of the water in raising tanks was controlled with a LoliOxy (Loligo Systems; ApS, Hobro, Denmark) in which an oxygen sensor is connected to a control unit that operates a nitrogen valve and bubbles nitrogen through the water when the oxygen concentration rises above a preset level (accuracy: better than 1% of measured value).

Microscopy and Documentation
Developing eggs were observed with an inverted microscope (Nikon, Eclipse TS100, Nikon GmbH, Düsseldorf, Germany) and photographs were taken with a Nikon Coolpix 990 digital camera mounted on the microscope. The container for microscopic observation of embryos was temperature controlled so that embryos were kept at constant temperature. After documentation, embryos were transferred back into their individual containers. Only the left side of the embryo was photographed, to avoid variation in measurements due to asymmetrical growth.

The development was documented for 1,500 individual embryos between 12 and 24 hr post fertilization. If at the beginning of the observation period, an embryo had no measurable characters developed, these embryos were omitted from the analysis. A total of 4,711 images were recorded and analyzed.

Embryonic Stages and Morphometric Measurements

Irmler et al. (2004) showed that the period between 15 and 19 hr post fertilization represents the phylotypic stage in zebra fish. Therefore, embryos in this study were studied at 12, 15, 18, and 24 hr post fertilization. This period broadly overlaps with the phylotypic stage given by Galis and Metz (2001), who suggested that the phylotypic period of (all) vertebrates extends between the beginning of neurulation until most somites have been formed. Because development in ectothermic animals largely depends on environmental temperature, we used embryonic normal stages as described by Kimmel et al. ('95) to characterize the development of zebra fish embryos under different raising conditions. During the segmentation period the number of somites is used for a detailed staging. However, only a limited set of morphological landmarks is accessible for repeated noninvasive measurements of living zebra fish embryos in an early developmental stage. We measured: (1) the length of the embryo, that is, the outline from snout to tip of tail as seen in lateral view, (2) the maximum diameter of the Anlage of the eye (optic cup), (3) the length of the 5th somite, (4) the height of the 5th somite, (5) the length of the 8th somite, (6) the height of the 8th somite, (7) the maximum diameter of the otic vesicle, (8) the distance between eye and otic vesicle, (9) the number of somites, (10) the angle between the fourth and fifths myoseptum, (11) the angle between the 5th and 6th myoseptum, (12) the angle between the 7th and 8th myoseptum, (13) the angle between the 8th and 9th myoseptum, (14) the diameter of the chorda dorsalis, (15) and the diameter of the yolk sac (Fig. 1). All measurements were conducted by one person (K. U. S.). Measurement error was calculated from repeated measurements of same structures and, on average, it was below 1% of the measured value. Measurements were performed using a Graphic Tablet, Summa Sketch II plus. We used SigmaScanPro (version 4.0, Jandel Scientific, SPSS Inc., Chicago, IL) for image analysis and morphometric data acquisition.
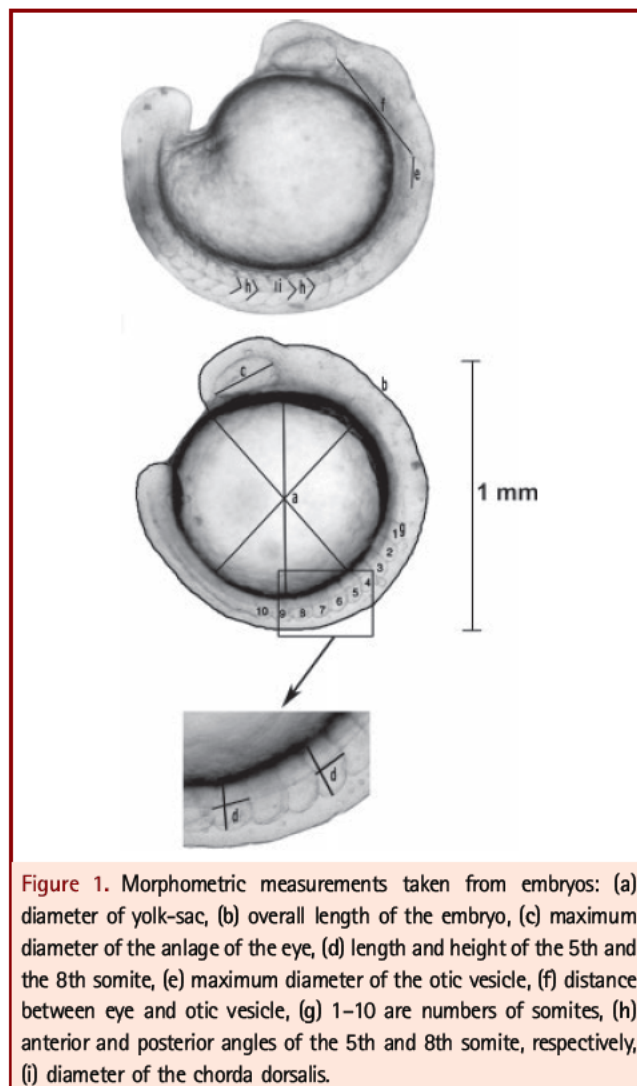

Figure 1. Morphometric measurements taken from embryos: (a) diameter of yolk-sac, (b) overall length of the embryo, (c) maximum diameter of the anlage of the eye, (d) length and height of the 5th and the 8th somite, (e) maximum diameter of the otic vesicle, (f) distance between eye and otic vesicle, (g) 1–10 are numbers of somites, (h) anterior and posterior angles of the 5th and 8th somite, respectively, (i) diameter of the chorda dorsalis.

Statistics

(1) Mortality data were compared by a one way analysis of variance (ANOVA) with temperature, salinity, or oxygen as fixed factor. Mortality of the fertilized eggs in a clutch was calculated between 12 and 18 hr pf. (2) The effects of temperature, salinity, and oxygen on the length of the embryo and the number of somites during different embryonic ages were tested by a multivariate analysis of variance (MANOVA). (3) The effects of the same external factors on multiple traits during different somite stages were tested by a MANOVA.

Because not all embryonic traits were present right from the beginning of the phylotypic period, we merged embryonic stages into four periods within which we could analyze the effects of different raising conditions using a MANOVA. (4) If the general models were significant, we continued by testing between subject effects, that is, between individual embryonic traits. Embryonic traits that differed between subjects have been analyzed further by a univariate analysis of variance (uniANCOVA) with temperature, salinity, and oxygen concentration as fixed factors and somite number as a covariate. If uniANCOVA was significant, we calculated linear regressions for the specific traits and used the slope and the intercept to characterize growth trajectories. All statistical procedures were performed using SPSS version 12.0.1 (SPSS Inc.).

RESULTS

Mortality

The mortality of early embryos raised in standard condition (27°C) was 7% ($N = 44$). When zebra fish embryo were raised at 24°C the mortality was 24% ($N = 27$) and it was 9% at 30°C ($N = 37$). The effect of temperature on mortality was significant (ANOVA: df 2; $F = 12.074$; $P < 0.001$). A Scheffé's post hoc comparison of multiple means showed that the mortality differed
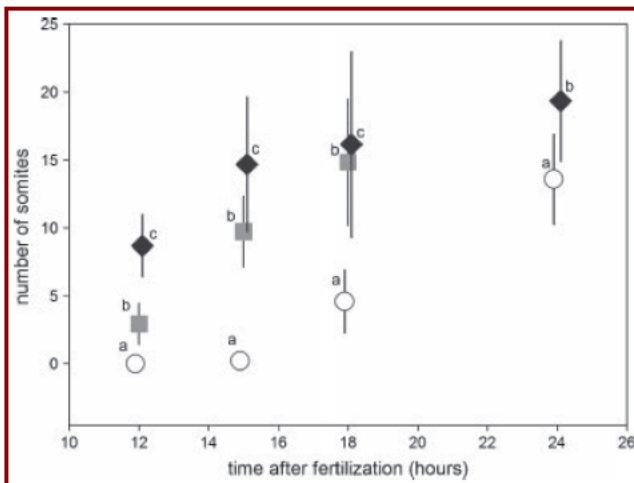
Figure 2. Effect of temperature on the number of somites of zebra fish embryos developing in 24°C (white circles), 27°C (gray squares), and 30°C (black diamonds). For each age, significant differences in somite number between temperature groups are indicated by different letters (mANOVA with Scheffé's test for post-hoc comparison of means; $\alpha = 0.05$).

Figure 3. Effect of temperature on the length of zebra fish embryos developing at 24°C (white circles), 27°C (gray squares), and 30°C (black diamonds). For each age, significant differences in embryo length between temperature groups are indicated by different letters (mANOVA with Scheffé's test for post-hoc comparison of means; $\alpha = 0.05$).

significantly when embryos raised at 24°C were compared with embryos raised at 27 and 30°C, respectively. A significant effect was also observed when embryos were raised in 200 mosmol/L NaCl water. Then the mortality increased from 7% in standard condition to 18% in high salinity ($N = 23$; ANOVA: df 1; $F = 4.827$; $P = 0.032$). Reduced oxygen concentration (20, 15, 10%) did not affect the mortality of developing embryos (ANOVA; df 3; $F = 0.296$; $P = $ n.s.) and individuals could be raised to adult size without showing any abnormalities.

Developmental Time
Temperature had a significant effect on the number of somites and embryo length (supplementary online material: Table 1a). At 12, 15, 18 and 24 hr pf, embryos raised at higher temperature had more somites and the embryos were longer. For a detailed analysis, we continued with a test of between subject effects. When raised at 24°C embryos had no somites after 12 h, when raised at 27°C they had on an average 3 28 ± 1.20 somites, and when raised at 30°C they had on an average 8.69 ± 2.25 somites (Fig. 2). The same was observed for the length of the embryo; when raised at higher temperatures, embryos at a given age were larger than those raised at lower temperatures (Fig. 3). Differences in length seem to disappear during development because embryos raised at 27 and 30°C were not different anymore at 18 hr pf. In 24 hr post fertilization, no differences in embryonic length were found between the different temperature groups (supplementary online material: Table 1b). However, the strong effect of temperature made comparisons between raising groups difficult because some traits found after 12 hr pf at 30°C had not
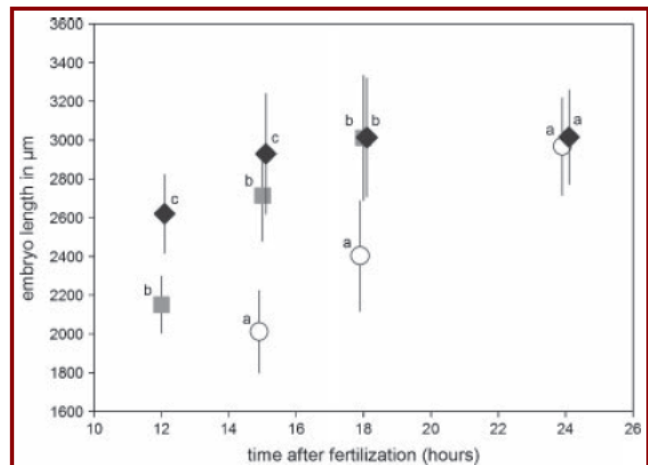
yet developed in embryos raised at 24°C. Therefore, we continued with a comparison by embryonic stages (number of somites; see below).

Effects of Salinity
First, to test if the egg membrane is actually permeable for salt ions we exposed embryos to media concentrations of 10 and 200 mosmol/L for 5½ hr after fertilization, then homogenized the eggs and measured the osmolality of the homogenized eggs. The osmolality of eggs kept in medium containing 10 mosmol/L NaCl was 73 mosmol/L but that of eggs kept in 200 mosmol/L NaCl was 260 mosmol/L. Quite obviously, the egg membrane did not seal the developing embryo from the osmotic environment, which affected the embryo directly.

A MANOVA with salinity as the fixed factor and somite number and embryo length as dependent variables revealed significant differences at 12, 15, and 18 hr pf (supplementary online material: Table 1a). Testing for between subject effects we found that the somite number for embryos raised in higher osmolality differed significantly from standard condition at 12 hr pf (df 1; $F = 6.489$; $P = 0.012$) and 18 hr pf (df 2; $F = 16.068$; $P < 0.001$; Fig. 4). High salinity did not affect the length of embryos at any age (supplementary online material: Table 1b). Thus, the significant effects in the model (above) were exclusively based on differences in somite numbers.

Effects of Oxygen
In normoxic condition, all embryonic traits developed according to normal stages. Embryos developing in hypoxic conditions had
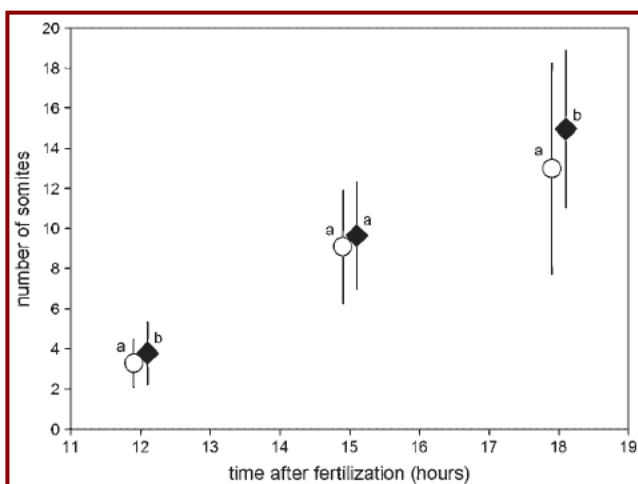
Figure 4. Effect of salinity on the number of somites of zebra fish embryos developing at 10 mosmol/L NaCl (white circles), and 200 mosmol/L NaCl (black diamonds). For each age, significant differences in somite number between salinity groups are indicated by different letters.
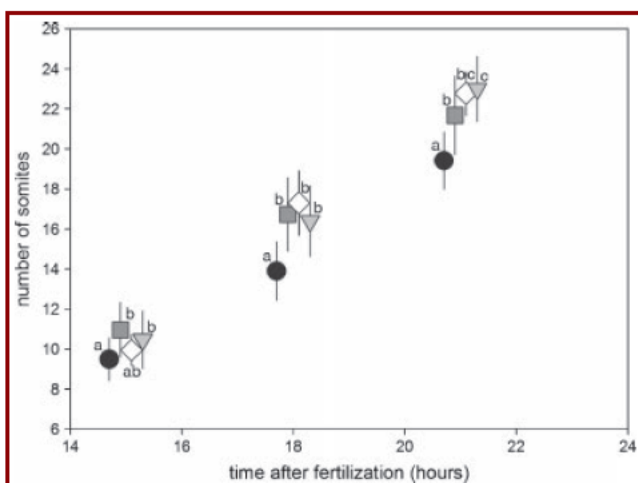

Figure 5. Effect of oxygen concentration on the number of somites of zebra fish embryos developing in 10% oxygen-saturated water (black circles), 15% oxygen-saturated water (gray squares), 20% oxygen-saturated water (white diamonds) and 100% oxygen-saturated water (gray triangles). For each age, significant differences in somite number between oxygen groups are indicated by different letters (mANOVA with Scheffé's test for post-hoc comparison of means; $\alpha = 0.05$).

significantly less somites (Fig. 5) and were significantly shorter (Fig. 6) at 15, 18 and 21 hr post fertilization than embryos developing in normoxic conditions (supplementary online material: Table 1a). Testing for between subject effects showed that low oxygen concentration affected both somite number and
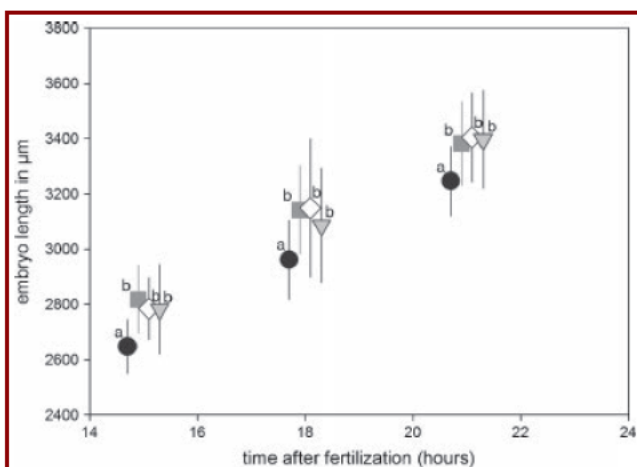

Figure 6. Effect of oxygen concentrastion on the length of zebra fish embryos developing in 10% oxygen-saturated water (black circles), 15% oxygen-saturated water (gray squares), 20% oxygen-saturated water (white diamonds) and 100% oxygen-saturated water (gray triangles). For each age, significant differences in embryo length between oxygen groups are indicated by different letters (mANOVA with Scheffé's test for post-hoc comparison of means; $\alpha = 0.05$).

embryo length (supplementary online material: Table 1b). However, the post hoc pairwise comparisons by Scheffe's test showed that only in the 10% group the somite number was significantly smaller than in all other groups. The number of somites of embryos raised at 15 and 20% oxygen becomes significantly different from normoxic only at 21 hr after fertilization. This indicates that in this group the low oxygen becomes effective somewhat later.

Developmental Stage

Because temperature, salinity, and oxygen had such strong effects on the time of development (above), we eliminated the differences in physical time by comparing embryonic stages defined by the number of somites. This way, we were able to detect dissociations of developmental traits during the phylotypic stage. Because early embryonic stages have different sets of characters than late embryos we created four stage groups (2 5; 6 8; 9 13; and 14 somite stages and higher) in which we tested for effects of temperature, salinity, and oxygen.

Temperature

Four MANOVAs of somite stages 7, 12, 16, and 17 with temperature as the fixed factor and up to 14 measured characters as dependent variables showed significant differences between the embryonic traits developed at the three different rearing temperatures. The model was rejected for stage 16 (supplementary online material: Table 2a). The following tests of between

subject effects did not result in a straightforward pattern. Significant differences were shown for embryo length at stage 7, 12, and 17. Temperature resulted in significantly different measures of the height of the 5th somite at stage 7 and 12. Also the height of the 8th somite was significantly affected at stage 12. The length of the 5th somite at stage 7 and the length of the 8th somite at stage 12 and 17 were significantly different at different raising temperatures. Temperature had a significant effect on the length of the otic vesicle at stage 17 and on the diameter of the eye at stage 12. The diameter of the yolk sac, the distance between eye and otic vesicle, the angles between somites, and the size of the chorda dorsalis were not affected by differences in raising temperature (supplementary online material: Table 2b). For all MANOVAs, the significantly tested traits of the embryos were always smaller at higher temperature, that is, 30°C.

Embryo length and the height of the 5th somite were the characters that showed consistent differences when embryos were raised at different temperatures. We therefore conducted a uniANCOVA over the entire data set with "number of somites" as a covariate. From that we calculated regression lines for 24, 27, and 30°C as temperature specific growth trajectories. In the 30°C group, the size increase was always less than in the other two groups, that is, with higher temperature the embryos remained smaller (although they grew faster, see above). The regression lines for the length of embryos developing at 24°C (slope, 57.3; intercept, 2,209.7) and 30°C (slope, 49.7; intercept, 2,203.0) were significantly different (uniANCOVA: df 1; $F = 13.194$; $P < 0.000$; $R^2 = 0.643$). Significant differences in slope were also found when comparing embryos raised in the 27°C (slope, 59.1; intercept, 2,141.2) with those raised in 30°C (uniANCOVA: df 1; $F = 6.283$; $P = 0.012$; $R^2 = 0.649$). The slopes of the regression of

the 24 and 27°C group were not significantly different but the intercept was significantly affected by temperature resulting in larger embryo size at lower temperature (uniANCOVA: df 1; $F = 4.512$; $P = 0.034$; $R^2 = 0.714$; Fig. 7).

The regression lines of the height of the 5th somite over developmental stages for embryos developing at 24°C (slope, 5.5; intercept, 24.4) and at 30°C (slope, 4.9; intercept, 25.7) differed significantly (uniANCOVA: df 1; $F = 6.101$; $P = 0.014$; $R^2 = 0.795$). The comparison of embryos raised at 24°C with embryos raised at 27°C (slope, 4.8; intercept, 30.2) showed significant differences (uniANCOVA: df 1; $F = 9.114$; $P = 0.003$; $R^2 = 0.779$), but the slopes of the regressions of embryos raised at 27°C and those raised at 30°C did not differ. However, there was a significant temperature effect between both groups (uniANCOVA: df 1; $F = 4.010$; $P = 0.045$; $R^2 = 0.714$; Fig. 8). No significant differences were found for the height of the 8th somite.

Salinity

MANOVAs of somite stages 4, 8, 11, 17, and 18 with salinity as fixed factor and up to 14 measured characters as dependent variables showed that salinity had a significant effect on development at all stages examined (supplementary online material: Table 2a). Testing for between subject effects showed that the yolk sac was significantly larger at lower salinity during the earlier stages. Also, the embryo was significantly larger when raised at 10 mosmol/L but only during stages 4, 8, and 11. The significant differences to the embryos raised in high salinity disappeared during the later stages. The length of the 5th somite was significantly larger in the 10 mosmol/L group at stage 8 and 11. Significant differences between embryos raised in control

Figure 7. Developmental trajectories of the length of zebra fish embryos according to somite stages. Embryos developing at 24°C are indicated by white circles, at 27°C by gray squares, and at 30°C by black diamonds.
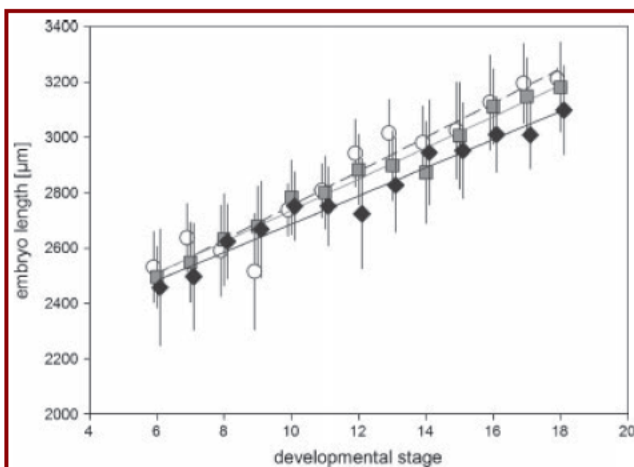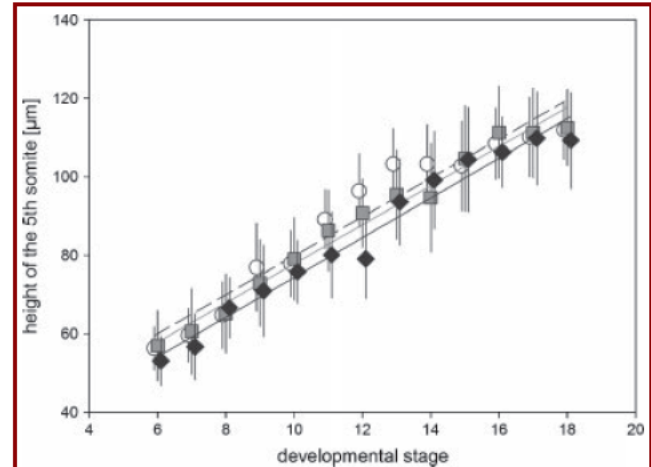

Figure 8. Developmental trajectories for the height of the 5th somite of zebra fish embryos according to somite stages. Embryos developing at 24°C are indicated by white circles, at 27°C by gray squares, and at 30°C by black diamonds.

condition and those raised in high salinity were also found for: the height of the 8th somite which was larger in the 200 mosmol/L group at stage 18; the distance between eye and otic vesicle which was significantly larger at 10 mosmol/L at the 17 and 18 somites stage; the angle between the 8th and the 9th somite which was significantly smaller in the 10 mosmol/L group at stage 18, and the chorda dorsalis which was significantly smaller in the 10 mosmol/L group at stage 17 and 18. No differences were found for the eye, height of the 5th somite, length of the 8th somite, the angle between the 4th and the 5th somite, the angle between the 5th and the 6th somite and the angle between the 7th and the 8th somite (supplementary online material: Table 2b).

When testing traits that were significantly affected by salinity with a uniANCOVA and somite number as covariate for differences in regression lines, the diameter of yolk sac and the embryo length were significantly different between treatment groups. The covariate "number of somites" had a highly significant influence on diameter of yolk sac, embryo length, height, and length of somites and diameter of chorda dorsalis. No influence was found for the distance between eye and otic vesicle. The slope of the regression line was not significantly different between groups for any of the analyzed traits.

Embryos were longer and had bigger yolk sacs when raised at 10 mosmol/L. The intercept of the regression lines for the diameter of yolk sac of embryos developing at 10 mosmol/L (slope, -0.2; intercept, 105.7) and 200 mosmol/L (slope, -0.2; intercept, 93.7) were significantly different (uniANCOVA: df 1; $F = 8.814$; $P = 0.003$; $R^2 = 0.212$; Fig. 9). The intercept of the regression lines for the length of embryos developing at 10 mosmol/L (slope, 59.1; intercept, 2,141.2) and 200 mosmol/L (slope, 60.0; intercept, 2,059.0) were significantly different

(uniANCOVA: df 1; $F = 6.801$; $P = 0.009$; $R^2 = 0.759$; Fig. 10). No effect was found for the height and length of somites, diameter of chorda dorsalis and distance between eye and otic vesicle.

Oxygen

Using MANOVAs to test for the effects of oxygen concentration on up to 14 embryonic traits during the somite stages 8, 9, 13, 18, and 19 showed a significant effect of oxygen concentration for all stages (supplementary online material: Table 2a). The test of between subject effects revealed that oxygen concentration had a significant size effect on the following traits: (i) the yolk sac was significantly smaller in the normoxic group at stage 8; (ii) the eye was significantly larger in the hypoxic group at stage 9 and smaller in the hypoxic group at stage 18 and 19; (iii) the height of the 5th and the 8th somites was significantly smaller in the hypoxic group in all examined stages; (iv) the length of the 5th somite was significantly shorter in the hypoxic group at stage 8 and 13; (v) the length of the 8th somite was significantly shorter in the hypoxic group at stage 9; (vi) the otic vesicle was significantly smaller in the hypoxic group at stage 18 and 19; (vii) the distance between the otic vesicle and the eye was significantly smaller in the normoxic group at stage 19; (viii) at stage 18, the angle between the 4th and the 5th and the angle between the 8th and the 9th somite were larger in the hypoxic group. No differences were found for the embryo size, the chorda dorsalis, the angle between the 5th and the 6th somite and the angle between the 7th and the 8th somite (supplementary online material: Table 2b).

The growth trajectories of the height 5th and the 8th somite and of the distance between Anlage of the eye and the otic vesicle

Figure 9. Developmental trajectories of the diameter of the yolk-sac of zebra fish embryos according to somite stages. Embryos developing in 10 mosmol/L NaCl (white circles), and 200 mosmol/L NaCl (black diamonds).
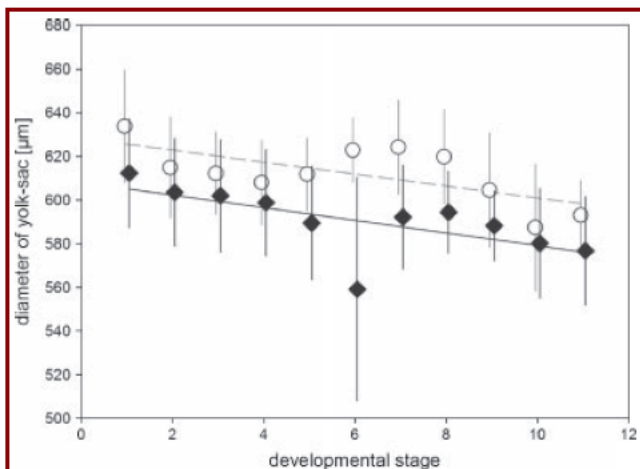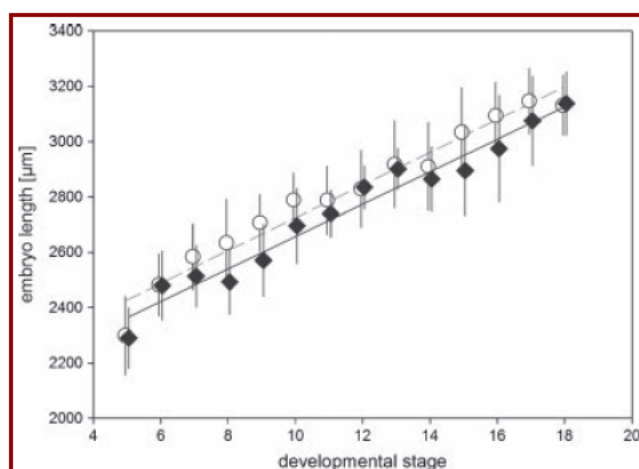

Figure 10. Developmental trajectories of the length of zebra fish embryos developing according to somite stages. Embryos developing in 10 mosmol/L NaCl are indicated by white circles, in 200 mosmol/L NaCl by black diamonds.

were analyzed by a uniANCOVA. In all the stages, the height of the 5th somite was consistently smaller when embryos were raised in low oxygen water as compared with normoxic condition. The slopes of the regression between normoxic and hypoxic condition were not significantly different. The intercept of the regression lines for the height of the 5th somite of embryos developing in low oxygen water (slope, 3.7; intercept, 33.2) and normoxic water (slope, 3,7; intercept, 42.6) was significantly different (uniANCOVA: df 1; $F = 8.814$; $P < 0.000$; $R^2 = 0.711$; Fig. 11).

The slope of growth trajectories of the height of the 8th somite differed between embryos raised under normoxic and hypoxic conditions, respectively (uniANCOVA: df 1; $F = 5.974$; $P = 0.015$; $R^2 = 0.799$). A significant difference in the slopes of growth trajectories was also found for the distance between eye and otic vesicle (uniANCOVA: df 1; $F = 6.285$; $P = 0.012$; $R^2 = 0.336$). The distance between the eye and otic vesicle was always smaller in the normoxic group. The trajectory of this trait had a significantly different slope in the normoxic group (slope, 7.8; intercept, 221.4) compared with the hypoxic group (slope, 3.7; intercept, 319.3), whereas somites have been larger in the normoxic group (slope, 5.1; intercept, 23.4) compared with the hypoxic group (slope, 4.8; intercept, 19.6). No effect of oxygen content on the length of the otic vesicle and on the length of the 5th somite was found.

The development of the eye was more difficult to describe because the diameter of the eye was repeatedly increasing and decreasing during normal development. The eye does not grow linearly, but undergoes changes of the maximum diameter that can be best described by a complicated growth trajectory

(Fig. 12). In normoxic condition, the maximum diameter was reached at somite stage 5 and then declines until somite stage 9. After the 9th somite stage its size increases again until stage 19 (with a short but marked decline during stage 13). After stage 19 it declines until stage 27. However, if the onset of eye development was compared by somite stages it became obvious that in low oxygen concentration the eye development was delayed by 4 somite stages as compared with development in normoxic condition. This peculiar pattern is very distinct and is precisely conserved also when eye development begins later. In hypoxic condition, the development of the eye follows exactly the same pattern but at $n+4$ somite stage. Thus, we could actually match the growth trajectories of the diameter of the eye when embryos were raised in standard or low oxygen condition by simply shifting the low oxygen curve by 4 developmental stages (Fig. 12b).

Figure 13 compares embryos in the 8 somite stage that developed under normoxic and hypoxic condition. The close up of the head shows the different developmental stage of the eye
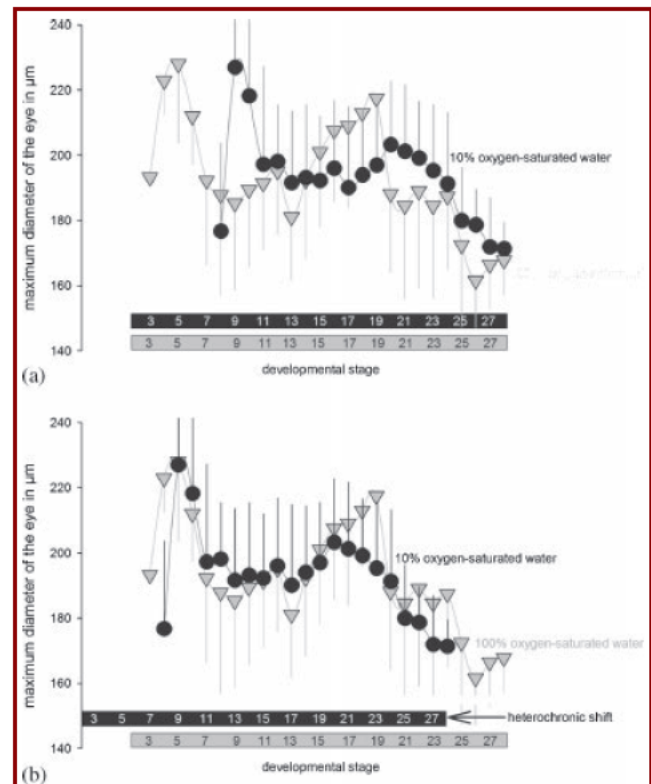
Figure 12. Developmental trajectories for the maximum diameter of the Anlage of the eye. (a) In normoxic (= 100% oxygen-saturated water, gray triangles) and hypoxic conditions (= 10% oxygen-saturated water; black circles). (b) Heterochronic shift of the development of the Anlage of the eye by 4 stages shows an almost perfect match of the complicated growth trajectories.
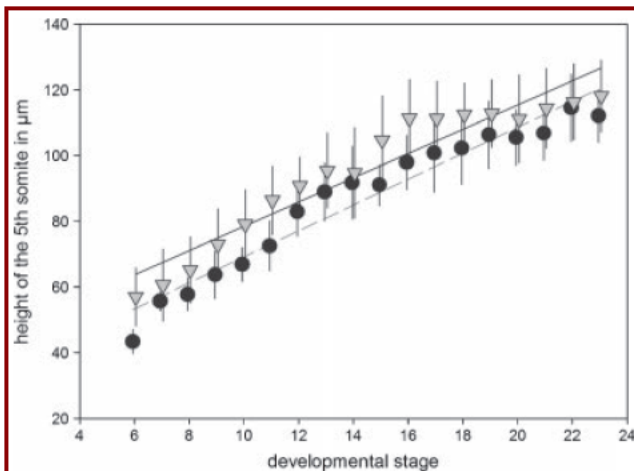

Figure 11. Developmental trajectories of the height of the 5th somite of zebra fish embryos developing according to somite stages. Embryos developing in 10% oxygen-saturated water are indicated by black circles, in 100% oxygen-saturated water by gray triangles.

Figure 13. Zebra fish embryos at the 8-somite stage. (a) Embryo raised in normoxic condition with a well-developed Anlage of the eye (arrow). (b) Embryo raised in hypoxic condition with the Anlage of the eye not yet present (arrow). In (a) and (b) the inset to the right shows the development of 8 somites.

(eye cup stage) in the embryos that grew in normoxic condition, the Anlage of the eye is well developed and clearly differentiated (Fig. 13a). When embryos develop in hypoxic condition there is no eye formed, only a cellular condensation can be recognized which, however, is not differentiated against the surrounding tissue (Fig. 13b). In Figure 13, a close up at the right side of each embryo shows the somite region that has developed to the 8 somite stage under both conditions but somites height is smaller under hypoxic conditions. Thus, we observe that an individual embryonic trait (the development of the eye) becomes uncoupled from the remaining patterns of development and its development begins heterochronically delayed. Although the onset of devel opment is delayed, the development of the eye follows exactly the same growth trajectory as under normal conditions.

The development of the otic vesicle also showed a similar heterochronic shift in the developmental sequence, that is, the onset of development of the otic vesicle was delayed in embryos raised in hypoxic condition as compared with those raised in normoxic condition. When raised in normoxic condition, the otic vesicle appeared first in somite stage 13. When raised in hypoxic condition, the first appearance of the otic vesicle was delayed by 4 somite stages, that is, it first appeared in somite stage 17 (Fig. 14a). The growth trajectory of the otic vesicle does not show such peculiar characteristics like that of the eye, but again, shifting the growth trajectory by 4 somite stages matches the normal and hypoxic development (Fig. 14b). The development of the otic vesicle also shows a distinct delay of the onset of development followed by an unchanged growth trajectory (developmental pathway) of the otic vesicle. At these early stages, the organs are still simple in morphology and have few morphological characters. However, the shift of the growth trajectories of the Anlage of the eye and the otic vesicle appears as very clear heterochronic shifts of developing modules that appear to be fairly independent of the surrounding tissue.

Figure 14. Developmental trajectories for the maximum diameter of the otic vesicle in normoxic and hypoxic conditions (x-axis = normal stages; y-axis = size of the otic vesicle): (a) development of the otic vesicle, (b) development of the otic vesicle with the x-axis for the hypoxic condition shifted by 4 stages to visualize the heterochrony of otic vesicle-development.
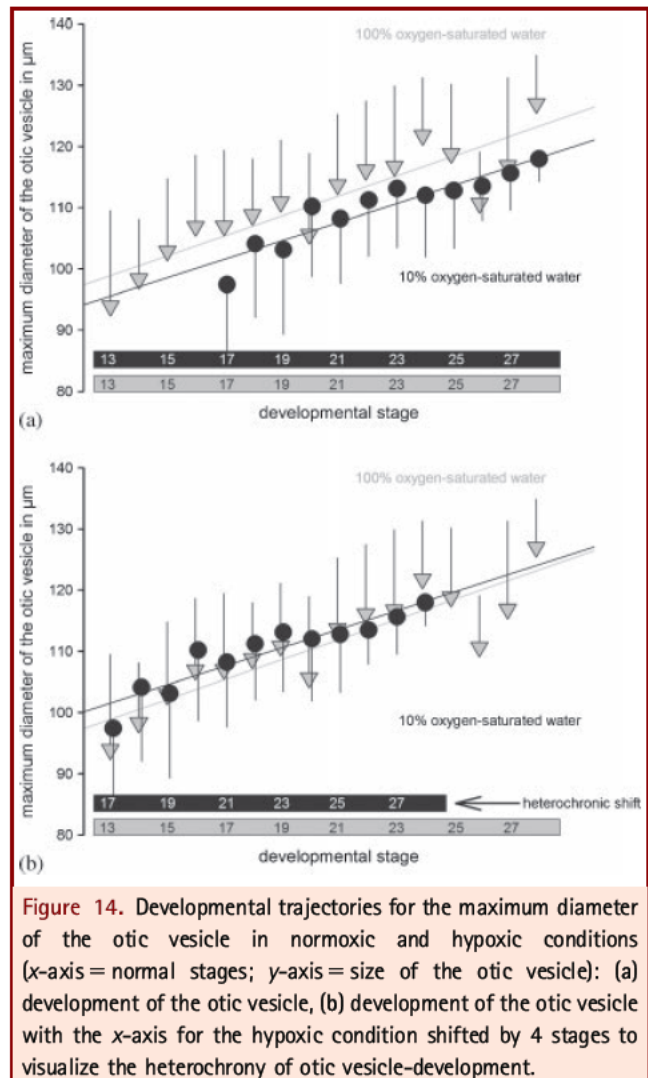
DISCUSSION

Our results show that variation of external parameters can induce phenotypic variation in several embryonic structures during the phylotypic period of the zebra fish. It is not possible, however, to recognize a pattern of response that could be interpreted in functional terms of development. But, we did not aim at understanding functional responses to changes in external parameters. Instead, we intended to find out if changes in external raising condition may induce phenotypic variation during the phylotypic stage, a stage that generally is considered to be conservative and resistant to change. Interestingly, moderate changes of the raising condition elicited different responses in embryonic structures. Of course, some structures were invariable and constrained (e.g., chorda dorsalis), but other showed changes either in (1) the rate of development (e.g., somite number, length of embryo), (2) the shape (e.g., the 5th somite

changes in height but not in length), or (3) the developmental sequence (e.g., delayed onset of development of the Anlage of the eye and the otic vesicle). Different treatments affect different traits of the developing embryo and in some cases the same traits in different directions. For example, raising zebra fish embryo at low temperature or at low oxygen concentration both resulted in a reduced developmental speed, but, at low temperature the size of certain traits increased while it decreased when embryos were raised in low oxygen.

The effect of temperature on fish development has been shown in a plethora of studies (Blaxter, '88; Pepin, '91), but to our knowledge nobody has studied the effect on developmental stages during the phylotypic stage and nobody has tested the effects of temperature on developmental mechanisms. Here, we have shown that temperature affects the rate of embryonic development in a way that increasing the temperature results in an acceleration of development but smaller final size (e.g., somite size). Because of the fairly consistent effect on the entire embryonic phenotype our results reject the first prediction made in the introduction (conserved phenotype) and support the second prediction, that is, a flexible phenotype that is not modularized. Of course, it is also possible that the modularization of the embryo is not effective under these conditions or, in other words, the temperature shifts were not strong enough turning low effective modularity into effective modularity. Similar but less strong effects were found when embryos were raised in elevated salinity. Bunn et al. (2000) reported that salinity has an overall effect on development of fresh water species, but that this effect was much weaker than effects of temperature. Without recognizing a pattern that would allow a functionally meaningful interpretation, the elicited response of accelerated or decelerated growth throughout the phylotypic period and the observed changes in shape showed that the embryos are flexible during the phylotypic stage. However, the flexible response concerns the entire embryo thus indicating a high level of morphological/developmental integration in all parts of the embryo, that is, no modularization. Again, it is possible that the changes in raising conditions were not strong enough to elicit modular and heterochronic pattern of development. However, given the significantly increasing embryonic mortality rates for changes in temperature and salinity, we dare to suggest that no modular response can be elicited by further changing either temperature or salinity.

Oxygen concentration of the raising medium had a profound effect on the timing of development. Our results showed a distinct heterochronic displacement of the onset of the development of the Anlage of the eye and the otic vesicle. Hypoxia is effective only at 10% oxygen saturation. This is coherent with Rombough ('88), who reports that shortly after fertilization oxygen consumption is low thus the animal are possibly tolerant to low oxygen concentrations (Lukina, '73; Kaushik et al., '82). Some tropical freshwater species spawn in virtually anoxic water but published data on their tolerance of hypoxia are more or less absent. Again, the functional considerations were not in the focus of the study. But hypoxic raising conditions apparently resulted in a dissociation of developmental patterns in the zebra fish. This rejects our predictions (1) "no flexibility" and (2) "coherent development but not modularized." Instead the highly specific growth trajectory of the Anlage of the eye shows that although its onset is delayed in hypoxia the development of the eye follows exactly the same pattern and rate of growth as described for standard condition. The same was observed for the otic vesicle. Both findings suggest that under the condition of low oxygen concentration the eye and the otic vesicle act as independent modules that can be decoupled from the development of the rest of the embryo. Thus, heterochronically delayed onset of development of the modules eye and otic vesicle under hypoxic conditions support ideas of low effective modularity. Low effective modularity might be understood in a way that only some external conditions are able to elicit modularization of the embryo while others are not. The interesting and important point is that within one single model organism we have shown that different developmental mechanisms may act to alter develop mental pathways under different conditions. Some traits do not respond at all thus support the "classic" view of internal constrains resulting in a conserved phylotypic stage. Other external factors cause a change in rate, which indicates some flexibility but that the degree of flexibility is constrained by multiple interactions between embryonic traits. The temporary dynamics of an increasing and decreasing number of such internal correlations among embryonic traits was shown by Irmler et al. (2004). This has led us to suggest that the phylotypic stage is constrained by multiple internal correlations among developing traits (Irmler et al., 2004). This view is entirely correct when embryonic development in normal conditions is studied. However, Raff ('96) and Galis et al. (2002) took a somewhat broader approach and suggested the idea that the phylotypic stage was indeed constrained but because modules were not effective ("low effective" modularity). As shown here, these authors suggested a correct concept. The results of our study present empirical support to the idea that modularity is not effective under normal conditions. Only when developmental conditions are pushed to the margins, modules suddenly become effective. What we have observed in this study may be a phenomenon already postulated by Waddington ('40, '56) who said that developmental trajectories are certainly flexible within certain margins. Modularity at the margins of development may act as stabilizing mechanism that ensures an undisturbed development or the embryo by buffering it against disruptive external influences.

LITERATURE CITED

Alberch P, Gould SJ, Oster GF, Wake DB. 1979. Size and shape in ontogeny and phylogeny. Paleobiology 5:296–317.

Altenberg L. 2005. Modularity understanding the development and evolution of natural complex systems. In: Callebaut W, Rasskin-Gutman D, editors. Vienna series in theoretical biology. Cambridge, MA: MIT Press.

Barlow GW. 1961. Causes and significance of morphological variation in fishes. Syst Zool 10:105–117.

Bininda-Emonds ORP, Jeffery JE, Richardson MK. 2003. Inverting the hourglass: quantitative evidence against the phylotypic stage in vertebrate development. Proc R Soc B: Biol Sci 270:341–346.

Blaxter JHS. 1969. Development: eggs and larvae. In: Hoar WS, Randall DJ, editors. Fish physiology, Vol. 3. New York: Academic Press. p 177–252.

Blaxter JHS. 1988. Pattern and variety in development. In: Hoar WS, Randall DJ, editors. Fish physiology, Vol. 11, Part A. London: Academic Press. p 1–58.

Bœuf G, Payan P. 2001. How should salinity influence fish growth? Comp Biochem Physiol—C Toxicol Pharmacol 130:411–423.

Bolker JA. 2000. Modularity in development and why it matters to evo-devo. Am Zool 40:770–776.

Bunn NA, Fox CJ, Webb T. 2000. A literature review of studies on fish egg mortality: implications for the estimation of spawning stock biomass by the annual egg production method. Sci Ser Tech Rep 111:1–37.

DiMichele L, Powers DA. 1982. LDH-B genotype-specific hatching times of Fundulus heteroclitus embryos. Nature 296:563–564.

DiMichele L, Taylor MH. 1980. The environmental control of hatching in Fundulus heteroclitus. J Exp Zool 214:181–187.

Duboule D. 1994. Temporal colinearity and the phylotypic progression: a basis for the stability of a vertebrate Bauplan and the evolution of morphologies through heterochrony. Development 120:135–142.

Franz-Odendaal TA, Hall BK. 2006. Modularity and sense organs in the blind cavefish, Astyanax mexicanus. Evol Dev 8:94–100.

Froese R, Daniel P. 2004. FishBase. In: Froese R, Pauly D, editors. World Wide Web Electronic Publication. www.fishbase.org, version (02/2009).

Galis F, Metz JAJ. 2001. Testing the vulnerability of the phylotypic stage: on modularity and evolutionary conservation. J Exp Zool 291:195–204.

Galis F, Sinervo B. 2002. Divergence and convergence in early embryonic stages of metazoans. Contrib Zool 71:101–113.

Galis F, Van Dooren TJM, Metz JAJ. 2002. Conservation of the segmented germband stage: robustness or pleiotropy? Trends Genet 18:504–509.

Goswami A. 2007. Cranial modularity and sequence heterochrony in mammals. Evol Dev 9:290–298.

Gould SJ. 1977. Ontogeny and phylogeny. Cambridge: Harvard University Press.

Gould SJ. 2000. Of coiled oysters and big brains: how to rescue the terminology of heterochrony, now gone astray. Evol Dev 2:241–248.

Haeckel E. 1903. Anthropogenie oder entwickelungsgeschichte des menschen. Leipzig: Engelmann.

Hall BK. 1997. Phylotypic stage or phantom: is there a highly conserved embryonic stage in vertebrates? Trends Ecol Evol 12:461–463.

Hall BK. 2003. Evo-Devo: evolutionary developmental mechanisms. Int J Dev Biol 47:491–495.

Hansen TF. 2003. Is modularity necessary for evolvability? Remarks on the relationship between pleiotropy and evolvability. BioSystems 69:83–94.

Hempel G. 1979. Early life history of marine fish. Seattle: University of Washington Press.

Irmler I, Schmidt K, Starck JM. 2004. Developmental variability during early embryonic development of zebra fish, Danio rerio. J Exp Zool B: Mol Dev Evol 302:446–457.

Kaushik SJ, Dabrowski K, Luquet P. 1982. Patterns of nitrogen excretion and oxygen consumption during ontogenesis of common carp (Cyprinus carpio). Can J Fish Aquat Sci 39:1095–1105.

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. 1995. Stages of embryonic development of the zebrafish. Dev Dyn 203:253–310.

Klingenberg CP. 1998. Heterochrony and allometry: the analysis of evolutionary change in ontogeny. Biol Rev Camb Philos Soc 73:79–123.

Lukina OV. 1973. Respiratory rate of the North Okhostsk chum salmon (Oncorhynchus keta (Walb.)). J Ichthyol 13:425–430.

McNamara KJ. 1997. Shapes of time: the evolution of growth and development. Baltimore, MD: The John Hopkins University Press.

McNamara KJ. 2002. Changing times, changing places: heterochrony and heterotopy. Paleobiology 28:551–558.

McNamara KJ, McKinney ML. 2005. Heterochrony, disparity, and macroevolution. Paleobiology 31:17–26.

Olson ME, Rosell JA. 2006. Using heterochrony to detect modularity in the evolution of stem diversity in the plant family moringaceae. Evolution 60:724–734.

Pepin P. 1991. Effect of temperature and size on development, mortality, and survival rates of the pelagic early life history stages of marine fish. Can J Fish Aquat Sci 48:503–518.

Pigliucci M. 2008. Is evolvability evolvable? Nat Rev Genet 9:75–82.

Poe S, Wake MH. 2004. Quantitative tests of general models for the evolution of development. Am Nat 164:415–422.

Raff EC, Raff RA. 2000. Dissociability, modularity, evolvability. Evol Dev 2:235–237.

Raff RA. 1996. The shape of life: genes, development, and the evolution of animal form. Chicago: University of Chicago Press.

Raff RA, Sly BJ. 2000. Modularity and dissociation in the evolution of gene expression territories in development. Evol Dev 2:102–113.

Richardson MK, Hanken J, Gooneratne ML, Pieau C, Raynaud A, Selwood L, Wright GM. 1997. There is no highly conserved embryonic stage in the vertebrates: implications for current theories of evolution and development. Anat Embryol 196:91–106.

Rombough PJ. 1988. Respiratory gas exchange, aerobic metabolism, and effects of hypoxia during early life. In: Hoar WS, Randall DJ, editors. Fish physiology, Vol. 11, Part A. London: Academic Press.

Sander K. 1983. The evolution of patterning mechanisms: gleanings from insect embryogenesis and spermatogenesis. Dev Evol 302b:137–159.

Sander K, Schmidt-Ott U. 2004. Evo-devo aspects of classical and molecular data in a historical perspective. J Exp Zool B: Mol Dev Evol 302:69–91.

Sauro HM. 2008. Modularity defined. Mol Syst Biol 4:166.

Schlosser G. 2002. Modularity and the units of evolution. Theory Biosci 121:1–80.

Schlosser G, Wagner GP. 2004. Modularity in development and evolution. Chicago: University of Chicago Press.

Smith KK. 2001. Heterochrony revisited: the evolution of developmental sequences. Biol J Linn Soc 73:169–186.

Taning ÅV. 1952. Experimental study of meristic characters in fishes. Biol Rev 27:169–193.

Tokita M, Kiyoshi T, Armstrong KN. 2007. Evolution of craniofacial novelty in parrots through developmental modularity and heterochrony. Evol Dev 9:590–601.

Von Dassow G, Munro E. 1999. Modularity in animal development and evolution: elements of a conceptual framework for EvoDevo. J Exp Zool 285:307–325.

Waddington CH. 1940. Organizers and genes. Cambridge biological studies. Cambridge, MA: Cambridge University Press.

Waddington CH. 1942. Canalization of development and the inheritance of acquired characters. Nature 150:563–565.

Waddington CH. 1953. Genetic assimilation of an acquired character. Evolution 7:118–126.

Waddington CH. 1956. Principles of embryology. London, UK: George Allen and Unwin Ltd.

Wagner GP, Pavlicev M, Cheverud JM. 2007. The road to modularity. Nat Rev Genet 8:921–931.

West-Eberhard MJ. 2003. Developmental plasticity and evolution. New York: Oxford University Press.

Westerfield M. 1994. The Zebrafish book. A guide for the laboratory use of Zebrafish (Brachydanio rerio). Eugene, Oregon: University of Oregon Press.

Zelditch ML, Fink WL, Swiderski DL, Lundrigan BL. 1998. On applications of geometric morphometrics to studies of ontogeny and phylogeny: a reply to Rohlf. Syst Biol 47:159–167.

2.3 Testing Evolutionary Hypotheses About the Phylotypic Stage of Zebrafish

Testing Evolutionary Hypotheses About the Phylotypic Period of Zebrafish

KAI SCHMIDT* AND J. MATTHIAS STARCK

University of Munich (LMU), Department of Biology II, Planegg-Martinsried, Germany

ABSTRACT

Vertebrate embryos pass through a period of morphological similarity, the phylotypic period. Since Haeckel's biogenetic law of recapitulation, proximate and ultimate evolutionary causes of such similarity of embryos were discussed. We test predictions about changes in phenotypic and genetic variances that were derived from three hypotheses about the evolutionary origin of the phylotypic stage, i.e. random, epigenetic effects, and stabilizing selection. The random hypothesis predicts increasing values for phenotypic variances and stable or increasing values for genetic variances; the epigenetic effects hypothesis predicts declining values for phenotypic variances but stable or increasing values of genetic variances, and the stabilizing selection predicts stable phenotypic variances but decreasing genetic variances. We studied zebrafish as a model species, because it can be bred in large numbers as necessary for a quantitative genetics breeding design. A half-sib breeding scheme provided estimates of additive genetic variances from 11 embryonic characters from 12 through to 24 hr after fertilization, i.e. before, during (15–19 hr), and after the phylotypic period. Because additive genetic variances are size dependent, we calculated narrow-sense heritabilities as a size independent gauge of genetic contributions to the phenotype. The results show declining phenotypic variances and stable heritabilities. In conclusion, we reject the random and the stabilizing selection hypotheses and favor ideas about epigenetic effects that constrain the early embryonic development. Additive genetic variance during the phylotypic stage makes it accessible for evolution, thus explaining in a simple and straightforward way why the phylotypic period differs among vertebrates in timing, duration, and morphologies. *J. Exp. Zool. (Mol. Dev. Evol.) 314B, 2011.* © 2011 Wiley-Liss, Inc.

During early embryogenesis, all vertebrates pass through a period of morphological similarity. Meckel (1811) and von Baer (1828) provided pioneering but fundamentally nonevolutionary comparisons of early vertebrate embryos. These early findings were soon replaced by Haeckel's (1866) evolutionary "biogenetic law" and principle of "recapitulation." Haeckel's (1866) concept of recapitulation fueled the discussion of evolution and development for many decades, and established itself as a mechanistic explanation of evolutionary diversification (reviews: Gould, '77; Richardson and Keuck, 2002; Horder, 2008). Haeckel's (1866) view was based on the assumption that evolution proceeded by terminal' addition', i.e. addition of structures to the final/adult form of a developmental line (see: Arthur, '88; Horder, 2008). Therefore, to reach an "advanced" evolutionary stage, organisms always had to pass through earlier stages of their phylogeny, i.e. recapitulate the phylogeny during their own ontogeny. In the light of 19th century contemporary knowledge, recapitulation

presented a satisfying explanation for the similarity of embryos. However, seeking modern evolutionary and mechanistic explanations for the similarity of vertebrate embryos has initiated a lively debate about pattern, processes, and phylogenies (e.g. Gould, '77; Sander, '83; Hall, '97, 2003; Richardson et al., '97, '98). A diverse terminology emerged highlighting different

aspects of the similarity of early vertebrate embryos: "Körper grundgestalt" (Seidel, '60), phylotypic stage (Sander, '83), phyletic stage (Cohen, '77), "zootype/phylotype" (Slack et al., '93), phylotypic period (Richardson, '95), "hourglass model" (Medawar, '54; Duboule, '94), and recently "molecular Haeckel" (Elinson and Kezmoh, 2010). The work of Richardson and collaborators (Richardson, '95, '99; Richardson et al., '97, '98, 2001) has provided helpful precision to recognize that there is not a single stage but a period during which we observe an overall similarity of vertebrate embryos; hence, the phylotypic period and that this period differs among vertebrate taxa. Today, von Baer and Haeckel have only historical importance and three evolutionary hypotheses aim at explaining the phylotypic period: (1) *random*, (2) *epigenetic effects*, and (3) *stabilizing selection*. Hypotheses (2) and (3) are not mutually exclusive, thus they potentially co occur. Variability in the phenotype is affected by additive genetic variances, environmental effects, and nongenetic components. Thus, the lack of morphological variability during the phylotypic stage has direct implications on phenotypic and genetic variances. Each of the hypotheses above makes specific predictions about the phenotypic and genetic variances during the phylotypic stage. *ad 1*: Williams' ('92) suggested that the similarity of early embryos during the phylotypic period emerges as a byproduct of directional/diverging selection on later developmental stages. In this hypothesis, the similarity of early embryos is neither selected nor constrained, and the phylotypic period is simply the residual of what is not selected; we call this the "random hypothesis." The most parsimonious prediction from that hypothesis is that phenotypic variances increase with the increasing number of degrees of freedom; i.e. phenotypic variances increase with increasing complexity of the devel oping embryo. Additive genetic variance would be stable or increase with the increasing number of gene interactions. The random hypothesis may serve as an evolutionary null hypothesis (Table 1).

ad 2: The idea of epigenetic effects shaping early embryonic development goes back to de Beer ('40), Huxley ('42), and

Waddington ('40, '56, '57). Today, a broad consensus agrees on those epigenetic ideas (Horder, 2006, 2008). In this concept, the phylotypic period results from constraints during early embry ogenesis; i.e. multiple inductive interactions among cells, tissues, and developing organs prevent selection on the phylotypic period, because change of one element might have deleterious consequences on the many integrated structures and functions. All stages of development are potentially open to evolutionary change, but because the epigenetic consequences (e.g. of gastrulation or neurulation) are so fundamental, the early embryonic stages are evolutionary stable (Horder, 2006). Predic tions from this hypothesis are that phenotypic variances decline during the phylotypic stage and that genetic variances remain constant or increase with the increasing number of gene interactions (Table 1). Several recent articles have provided theoretical (Raff, '94, '96; Galis and Metz, 2001; Galis and Sinervo, 2002) and empirical support (Irmler et al., 2004; Schmidt and Starck, 2010) for this idea. In particular, it was shown that the number of constraints during the phylotypic stage increases significantly as compared with earlier or later embryonic stages (recognized as a high number of correlations among characters and a high level of morphological integration (Irmler et al., 2004). It was also suggested that the phylotypic period is characterized by low effective modularity (Raff, '96; Raff and Sly, 2000; Galis and Metz, 2001; Galis and Sinervo, 2002). Low effective modularity is not simply a reciprocal expression for a high number of internal constraints; rather it characterizes a condition when modules are present but their boundaries are relaxed under standard conditions, so that manifold inductive interactions among structures are possible. Our own work has provided evidence that low effective modularity is indeed (partially) responsible for the phylotypic stage and acts in concert with internal constraints. Because the degree of modularization changed with the environmental effects, we suggested that modularization acts as a buffering mechanism against develop mental deviations in extreme environments (Schmidt and Starck, 2010). On the molecular level, several articles have shown that integrated pattern of gene expression occur and might cause developmental constraints (Slack et al., '93; Duboule, '94; Irie and Sehara Fujisawa, 2007; Elinson and Kezmoh, 2010), resulting in the overall similarity of early vertebrate embryo because of similar gene expression pattern in early vertebrate embryos.

ad 3: Stabilizing selection may result in stable phenotypes of early vertebrate embryos. The reasons why stabilizing selection acts on the phylotypic period reach from maintaining common gene expression pattern to morphological integration (Kirschner and Gerhart, '98; Galis and Metz, 2001; Bininda Emonds et al., 2003). Predictions from this hypothesis are straightforward but have not been tested, yet. Stabilizing selection tends to reduce or, in the extreme, exhaust the additive genetic variance of a trait. Therefore, if stabilizing selection acts on the phylotypic period, we expect the phenotypic variances and the additive genetic

Table 1. Predictions about changes in phenotypic and genetic variances during the phylotypic period, according to the three hypotheses discussed.

Hypothesis	Predictions	
	Phenotypic variance	Additive genetic variance
Random	Increasing	No change or increasing
Epigenetic effects	Decreasing	No change or increasing
Stabilizing selection	Decreasing	Decreasing

variance to decrease as compared with before and after the phylotypic period (Table 1). In the extreme, one could speculate that the phenotypic variance equals the error variance, and additive genetic variance would be zero. However, this is purely hypothetical and improbable, because mutation always brings in a small amount of new variation in each generation (Arnold, '92; Lynch and Walsh, '98). Supposing that stabilizing selection acts on early embryonic stages, we realistically would expect the phylotypic period to be characterized as a period of reduced additive genetic variance. We expect phenotypic variances to decrease as compared with stage before or after the phylotypic period.

This study is designed to test the three hypotheses summarized in Table 1. After a number of previous theoretical and empirical studies had shown that epigenetic effects affect the phylotypic stage (in zebrafish), the focus of this article is on the third hypothesis. Because additive genetic variance is a size dependent measure, we will use narrow sense heritability as a gauge of the genetic contributions to the embryonic characters around the phylotypic stage that is independent of size and dimension (Atchley, '84). We attribute changes in heritability to changes in additive genetic variance. Owing to our breeding design (see Material and Methods), we exclude that changes in heritability are caused by changes in nongenetic maternal variance or residual variance. Thus, different characters and stages can be compared directly. Comparing heritabilities among stages of development is a univariate approach that ignores potential polygenic effects on embryonic characters as well as potential genetic correlations (genetic covariances) among characters. An appropriate approach to capture genetic con straints that arise from additive genetic variances and their covariances would be to calculate the G matrices for a sequence of developmental stages (Atchley, '84; Arnold, '92). Such developmental G matrices would show regularities in their elements, thus reflect constraints (Arnold, '92). However, this approach requires that all measured characters are present during all stages of the developmental period. If not, the matrix automatically is reduced to a univariate structure. Unfortunately, this is the case during the early development of zebrafish, before the basic body plan has been established. In each stage, new structures occur or temporary embryonic features disappear. Therefore, we have been restricted to a univariate approach the same problems hold for the phenotypic variances and will use the coefficient of variation as a size independent measure of phenotypic variances (Irmler et al., 2004).

Narrow sense heritability is environment dependent. There fore, we carefully controlled the rearing conditions for the embryos. However, to estimate the effect of the rearing conditions on the heritabilities, we also considered embryos reared in different environments (= rearing conditions). Also, we showed in a previous study (Schmidt and Starck, 2010) that some embryonic structures of zebrafish display a considerable degree of developmental plasticity, i.e. the same genotype develops different phenotypes in different rearing conditions. This can be considered an additional/extended test of the stabilizing selection hypothesis.

We study zebrafish because early embryos are translucent and can be studied repeatedly with the light microscope. The phylotypic stage of zebrafish was recognized by multiple internal constraints between 15 and 19 hr after fertilization (Irmler et al., 2004; Schmidt and Starck, 2010). Also, a quantitative genetic breeding design, as applied here, is laborious and requires large numbers of embryos which are difficult to obtain from other vertebrate species. Certainly, the results will primarily apply to only one species, the zebrafish, and generalizations need to be made cautiously. However, we think that the approach provides so many new exciting insights and a new quality of data with strong statistical support so that the focus on just one model species is justified, even if generalizations are restricted.

MATERIALS AND METHODS

Animals
Wild type zebrafish, *Danio rerio* (Hamilton, 1822), were obtained from different pet shops in Germany. Animals were kept in 80 L tanks at 27°C in standardized water, according to Kimmel et al. ('95). For the breeding experiments, we kept individual fish separated in compartments of the aquaria to allow identification. Pairs of individually known fish were transferred to 12 L tanks for pair wise mating. After successful mating, the pairs were separated and the fish was transferred back to their compartments.

Egg Collection
Mating of zebrafish can be triggered by switching from dark to light. Thus, fish were transferred into mating tanks in low light condition; eggs were laid and fertilized immediately after switching on the light. We observed egg laying and collected eggs directly after fertilization. From each clutch, we collected up to 100 eggs, washed them with clean water, and transferred them into small glass containers (20 mL volume) covered with gauze, which were placed in the rearing tanks (see rearing condition of embryos). After 5 min of mating, fish were separated for 25 min and then joined again for a second mating. Egg collecting procedure was repeated, as described above. Because mating was allowed only for 5 min, the developmental time in this article is given with a precision of 5 min. From a fertilized clutch, individual embryos were selected randomly and each developing embryo was kept in an individually labeled glass container so that it could be recognized for repeated measurements.

Rearing Conditions
Standard condition was: temperature (27°C), salinity (NaCl 0.29 g L^{-1}, pH 7.1 7.5, total osmolarity was 10 mosmol L^{-1}),

and oxygen concentrations ($8.2\,mg\ O_2\,L^{-1}$). Experimentally altered conditions were: (1) altered temperature, i.e. embryos were reared either in 24 or 30°C in an osmolarity of $10\,mosmol\,L^{-1}$ and 100% oxygen concentration, (2) altered salinity, i.e. embryos were reared in $200\,mosmol\,L^{-1}$ (NaCl $5.84\,g\,L^{-1}$, pH 7.17.5, total osmolarity was $200\,mosmol\,L^{-1}$) at 27°C and 100% oxygen concentration, and (3) altered oxygen concentration, i.e. embryos were reared in 10% oxygen saturated water ($0.83\,mg\ O_2\,L^{-1}$) at 27°C and 10 mosmol. For all tanks and conditions, temperature in the rearing tanks was controlled by a Haake C10 immersion circulator (Haake GmbH, Karlsruhe, Germany) with an accuracy of ± 0.04°C. A digital thermometer (P 550, Dostmann Electronic GmbH, Wertheim Reicholzheim, Germany) was used to control the temperature in the rearing tanks. The osmolarity was measured with an osmometer from Knauer (Knauer Osmometer Automatic). The oxygen concentration in the rearing tanks was controlled using a LoliOxy Oxygen Analyzer and Regulator System (Loligo Systems ApS, Hobro, Denmark). When the oxygen concentration rises above a preset level, nitrogen bubbles through a ceramic diffuser in the rearing tank until the preset oxygen concentration is reached.

Microscopy and Documentation

Developing eggs were observed with an inverted microscope (Nikon, Eclipse TS100, Nikon GmbH, Düsseldorf, Germany) and photographs were taken with a Nikon Coolpix 990 digital camera mounted on the microscope. The container for the embryos had a temperature controlled compartment, so that during microscopic observations embryos were exposed to the same temperature as in their rearing tanks. After observation, embryos were placed back into their individual containers. The left side of the embryo was photographed, to exclude variation in measurements owing to asymmetrical growth.

The phylotypic period of zebrafish extends between 15 and 19 hr after fertilization in standard condition (Irmler et al., 2004). We documented the development of 839 individual embryos between 12 and 24 hr to cover the entire phylotypic period as well as time before and after. Each individual embryo was photographed up to four times, resulting in 2,392 recorded (and analyzed) images. Because different rearing conditions may cause acceleration or retardation of development, all embryos were also staged according to Kimmel et al. ('95).

Measurements

A limited set of morphological structures is accessible for repeated noninvasive measurements in early zebrafish embryo. We measured: (1) overall length of the embryo, i.e. the outline from snout to tip of tail as seen in lateral view, (2) maximum diameter of the eye (optic primordium), (3) length of the fifth somite, (4) height of the fifth somite, (5) length of the eighth somite, (6) height of the eighth somite, (7) maximum diameter of the otic vesicle, (8) distance between eye and otic vesicle, (9)

number of somites, (10) diameter of the chorda dorsalis, and (11) diameter of the yolk sac (for details, see Fig. 1 in Schmidt and Starck, 2010). Measurements were performed on digitized images using a Graphic Tablet, Summa Sketch II plus. We used SigmaScanPro (version 4.0, Jandel Scientific, SPSS Inc., Chicago, IL) for image analysis and morphometric data acquisition. Measurement error was calculated from repeated measurements of the same structures. On average, the measurement error is less than 1% of the measured value.

Breeding Design

We generated half sib crosses by mating one male (sire) to two to three females (dams; Fig. 1). From each mating, we used up to 12 embryos for morphometric measurements. For each hour of development/developmental stage, we had 5 26 full sib families (supplementary online material, appendix Table S1). The variance of the embryonic characters produced from these matings was partitioned into components of sire, dams within sire, and residual to estimate the additive genetic variances and the heritability for the embryonic characters under investigation. Estimates of variance and covariance components were obtained by restricted maximum likelihood (REML) using a linear mixed model (Conner and Hartl, 2004). Up to 11 embryonic characters have been measured and heritabilities have been estimated at several observation points between 12 and 24 hr after fertilization.
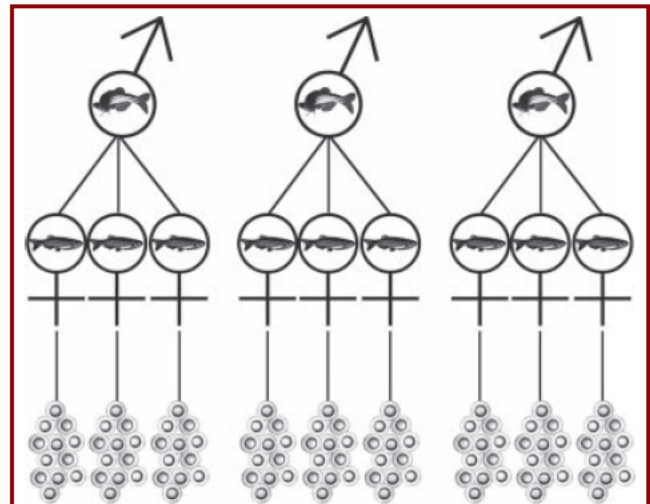

Figure 1. Schematic representation of the half-sib breeding design applied in this study. Each male was mated to two or three females. Morphometric measurements were taken from the offspring of those matings and variances of the embryonic characters were then partitioned into components of sire, dams within sire, and residual to calculate the heritability.

Heritability Analyses

To estimate the narrow sense heritability of embryonic char acters, we used a nested paternal half sib analysis as described in Conner and Hartl (2004). The narrow sense heritability is defined as the ratio of the additive genetic variance to the total phenotype variance: $h^2 = V_A/V_P$. The total phenotypic variance (V_P) of a trait can be described as $V_P = V_A + V_M + V_R$, where V_A is the additive genetic variance, V_M is the maternal effect variance, and V_R is the residual variance, consisting of environmental effects, nonadditive genetic effects, and error variance. From the nested design, we attained variance components for sire, for dam nested in sire, and the residual variance as the difference between full sibs from the individual dams. Additive genetic variances were estimated as four times the sire variance component (Falconer and Mackay, '96), which was obtained by REML using a linear mixed model. The restricted estimate maximum likelihood procedure is fairly robust toward unbalanced designs (unequal number of females mated to each male and unequal numbers of offspring per female owing to mortality), as is the case for our dataset.

We estimated heritabilities separately for each trait, time, and experimental condition. In these calculations, trait was used as the variable, and sire and dam were included as factors of analyses. To attain variance components, both were modeled as random factors where dams were nested within males. For some embryonic characters, the statistical model did not converge so that the validity of subsequent results could not be ascertained or the validity of the model fit was uncertain. Heritabilities could not be estimated from these variance partitions, and thus were omitted from our results. For 16 out of 72 heritability analyses of embryonic characters, we obtained estimates of heritability greater than 1 because of random error or owing to insufficient sample size. Heritability values greater than one have been omitted from the analyses and graphs. Standard error of heritability was calculated following the procedure described by Roff ('97). All statistical procedures were performed using SPSS version 12.0.1 (SPSS Inc., Chicago, IL).

For all measurements and stages, we also calculated the phenotypic variances. Because phenotypic variances depend on the size of the structure, we calculated the coefficient of variation for each structure and stage for all embryos reared in standard condition. The procedure was the same as in Irmler et al. (2004).

RESULTS

In standard condition, the narrow sense heritabilities of 11 embryonic characters ranged between 0.01 (Fig. 2A) and 0.94 (Table 2). Owing to the continuous development of early embryonic characters, the 12 hr embryos had only few characters that could be measured while somites, sense organs, and chorda
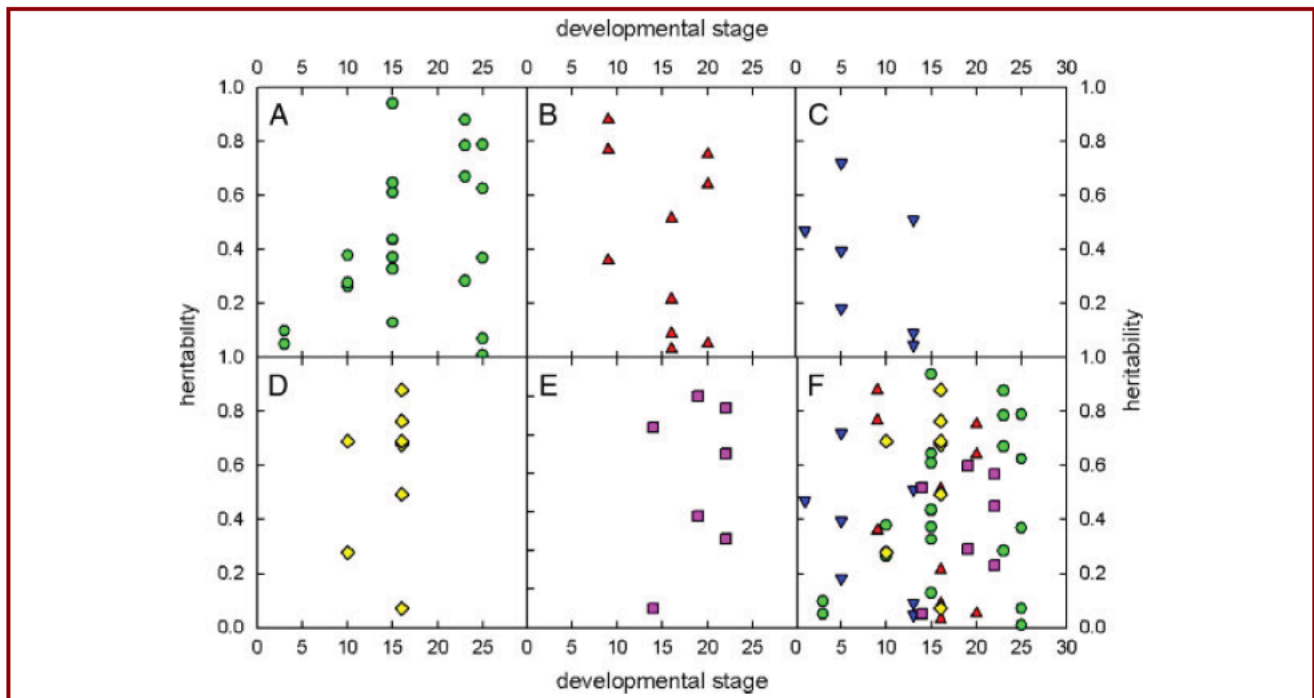

Figure 2. Heritabilities of embryonic characters when zebrafish were reared in different conditions. For direct comparison, heritabilities are plotted per developmental stage (= somite stage, following Kimmel et al.,'95). (A) standard condition, (B) 30°C, (C) 24°C, (D) high salinity, i.e. 200 mosmol L^{-1}, (E) low oxygen content (10 % of normal), and (F) all conditions together.

Table 2. Narrow sense heritability ($\pm$SE) of embryonic characters.

Time[1] (hr)	Trait	Condition	Heritability$\pm$SE
12	Yolk sac	Standard	0.10 ± 0.07
15	Embryo length	Standard	0.26 ± 0.13
18	Embryo length	Standard	0.94 ± 0.35
18	Eye	Standard	0.37 ± 0.16
23	Eye	Standard	0.37 ± 0.26
15	Fifth somite height	Standard	0.38 ± 0.18
18	Fifth somite height	Standard	0.43 ± 0.19
21	Fifth somite height	Standard	0.79 ± 0.48
23	Fifth somite height	Standard	0.79 ± 0.49
18	Fifth somite length	Standard	0.13 ± 0.06
23	Fifth somite length	Standard	0.01 ± 0.01
15	Eighth somite height	Standard	0.28 ± 0.14
18	Eighth somite height	Standard	0.44 ± 0.19
23	Eighth somite height	Standard	0.62 ± 0.40
18	Eighth somite length	Standard	0.61 ± 0.25
23	Eighth somite length	Standard	0.07 ± 0.05
18	Otic vesicle	Standard	0.33 ± 0.15
21	Otic vesicle	Standard	0.28 ± 0.20
12	Somite number	Standard	0.05 ± 0.04
21	Somite number	Standard	0.67 ± 0.42
18	Chorda	Standard	0.65 ± 0.27
21	Chorda	Standard	0.88 ± 0.52
15	Yolk sac	24°C	0.15 ± 0.08
18	Embryo length	24°C	0.72 ± 0.45
24	Embryo length	24°C	0.05 ± 0.06
24	Eye	24°C	0.51 ± 0.51
18	Fifth somite height	24°C	0.40 ± 0.41
18	Somite number	24°C	0.18 ± 0.13
24	Chorda	24°C	0.09 ± 0.10
18	Eye	30°C	0.75 ± 0.41
12	Fifth somite height	30°C	0.77 ± 0.42
12	Fifth somite length	30°C	0.88 ± 0.47
18	Fifth somite length	30°C	0.05 ± 0.03
12	Eighth somite height	30°C	0.36 ± 0.22
15	Otic vesicle	30°C	0.51 ± 0.34
15	Distance eye/otv[2]	30°C	0.03 ± 0.02
18	Distance eye/otv	30°C	0.64 ± 0.41
15	Somite number	30°C	0.21 ± 0.15
12	Chorda	30°C	0.09 ± 0.07
15	Embryo length	200 mosmol	0.28 ± 0.20
18	Embryo length	200 mosmol	0.68 ± 0.44
18	Fifth somite height	200 mosmol	0.88 ± 0.53
18	Fifth somite length	200 mosmol	0.07 ± 0.05
18	Eighth somite height	200 mosmol	0.76 ± 0.48
15	Eighth somite length	200 mosmol	0.69 ± 0.44
18	Eighth somite length	200 mosmol	0.69 ± 0.44
18	Somite number	200 mosmol	0.49 ± 0.33
18	Embryo length	10% oxygen	0.97 ± 0.85

Time[1] (hr)	Trait	Condition	Heritability $\pm$ SE
21	Embryo length	10% oxygen	0.52 $\pm$ 0.52
18	Eye	10% oxygen	0.05 $\pm$ 0.06
21	Eye	10% oxygen	0.29 $\pm$ 0.31
23	Fifth somite length	10% oxygen	0.45 $\pm$ 0.46
18	Eighth somite height	10% oxygen	0.52 $\pm$ 0.52
23	Eighth somite height	10% oxygen	0.57 $\pm$ 0.56
21	Distance eye/otv	10% oxygen	0.60 $\pm$ 0.59
23	Distance eye/otv	10% oxygen	0.23 $\pm$ 0.25

Table 2. Continued

[1]time   hours after fertilization.[2]otv, otic vesicle.

were still missing. The calculated values of the heritabilities seem to be low. Heritabilities of the later developing structures (e.g. somites length and height, measurements of sense organs) range around 0.5. Because we can calculate one heritability value only per structure and stage from many embryos, a statistical test for developmental changes in heritability of an individual structure is impossible. However, because heritabilities are dimensionless and independent of the size of the structure or the type of measurement, we can compare heritabilities of many structures across development. To test for possible differences between heritabilities at different developmental age, we calculated a univariate ANOVA with age as fixed factor and heritability as dependent variable. The model was not significant, showing that there were no changes in the heritabilities over standard development.

The same principal pattern was observed when embryos were reared in different conditions (Fig. 2B E; Table 2). The distribution of heritability values, however, is a bit patchier when embryos were reared in different conditions. This is mainly owing to heterochronic effects and rearing conditions accelerating (30°C) or retarding development (24°C), so that not all developing structures could be captured within the time frame of 12 24 hr. The important point here is that we observed a scatter of heritability values that is within the range of what was described for standard rearing condition.

The key question in this study is: does heritability (as a measure of additive genetic variance) change during development? In particular, does it show a depression during the phylotypic stage as predicted by the stabilizing selection hypothesis. Therefore, from the measured traits, we calculated mean heritabilities for each hour from 12 to 24 hr after fertilization for each of the five rearing conditions. Thus, the phylotypic period of zebrafish, ranging from 15 to 19 hr after fertilization, lies well within the developmental period covered here. Because temperature, salinity, and oxygen content affect the rate of development (acceleration, retardation), we calculated a standardized time, i.e. related time to the number of somites, to
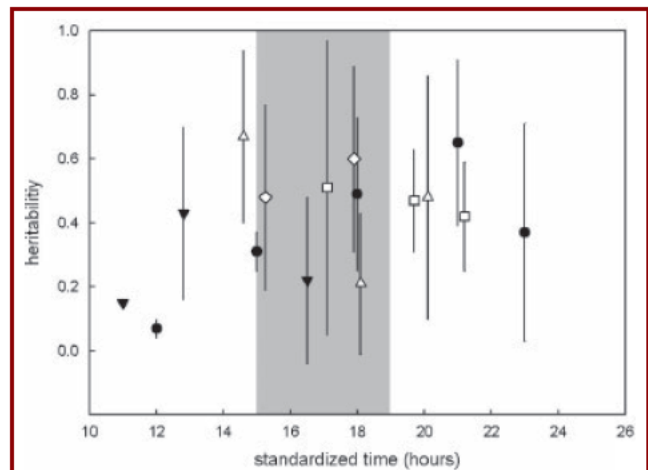

Figure 3. Heritabilities of embryonic characters between 11 and 23 hr of development. Heritabilities of zebrafish reared in different conditions are labeled with different symbols (standard condition, filled circle; 30°C, white triangle; 24°C, black triangle; high osmolarity, white diamond; low oxygen, white square). Because different rearing conditions cause acceleration or retardation in development, we standardized time to somite numbers according to a linear regression: standardized time = (21.2203*somite number)/2.0472. The gray shading indicated the phylotypic period between 15 and 19 hr after fertilization. Owing to the conversion of developmental time to standardized time, the values on the x-axis reach from 11 to 23 hr.

make development under different conditions comparable (for details, see caption of Fig. 3). For simplicity, we grouped data into heritabilities before, during, and after the phylotypic stage. To test if there were a significant effect of development on the average heritability, we used a univariate ANOVA with standardized time (grouped before, during, after the phylotypic stage) as fixed factor and heritability as dependent variable. The model was marginally significant (df = 2, F = 6.74, P = 0.048),

indicating that there are differences in the heritabilities. There fore, we calculated a Scheffe's post hoc and LSD post hoc test for comparisons of multiple means. Both tests failed to recognize significantly different subsets at $\alpha = 0.05$. The test, however, indicated that the average heritabilities for the period before, during, and after the phylotypic period increased from 0.11 to 0.43 and 0.48, respectively, though the differences between these were not significant. This result is in clear contrast to the prediction from the stabilizing selection hypothesis, which entails a depression of heritability during the phylotypic period as compared with the time before and after. Testing the same data with a linear regression analysis with standardized time as independent variable and heritability as dependent variable showed that the slope of a regression line is not significantly different from zero. Therefore, we feel quite confident that the marginally significant result reported above represents an error type I.

The coefficients of variation of phenotypic variances are given in Figure 4. During the phylotypic stage (15 19 hr), the coefficient of variation declines from an average of 13.2 ± 7.0 to 9.3 ± 1.9. These values are close to those measured in an earlier study (Irmler et al., 2004). A linear regression with the coefficient of variation as dependent variable and time during the phylotypic stage as independent variable shows a negative slope ($B = -0.5$; $R^2 = 0.97$). The negative slope is significantly different from zero (ANOVA df $= 1,3$, $F = 39.6$, $P = 0.024$). As a size indepen dent estimate of the phenotypic variance, the declining coefficient of variation is in accordance with the prediction from the stabilizing selection hypothesis and the epigenetic effects hypothesis.
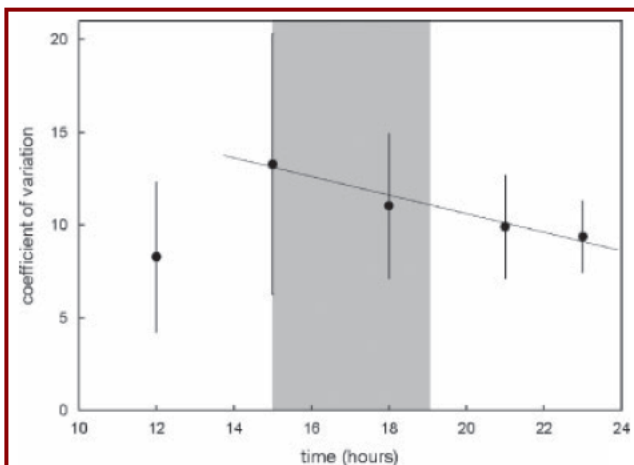

Figure 4. Coefficient of variation of phenotypic variation during the early embryonic period. Values are mean CVs of embryonic characters from embryos reared in standard condition. The gray shading indicated the phylotypic period between 15 and 19 hr after fertilization.

DISCUSSION

This study is the third in a row of studies testing evolutionary predictions about the phylotypic period. As in previous studies, we chose the zebrafish as a model system, assuming that we can test in a single species the principal reason why there is so little phenotypic variation during the phylotypic period. This study was laid out to test, in particular, the predictions from the stabilizing selection hypothesis. However, the results on pheno typic and additive genetic variances (measured as coefficient of phenotypic variation and heritability, respectively) during the phylotypic period are suitable to test all three major hypotheses, i.e. random, epigenetic effects, and stabilizing selection (Table 1). Although in this study we only have a relatively small sample size for the estimation of the heritabilities, it is large enough for the aim of the study, i.e. test predictions about the additive genetic variance of embryonic characters and about the change of heritability during the period of early development.

The heritabilities of 11 embryonic characters were within the range of heritabilities previously reported for other morphologi cal characters, mainly in adults (e.g. see: Roff, '97; Lynch and Walsh, '98; Conner and Hartl, 2004). More importantly, we could not detect any significant change of heritabilities between 12 and 24 hr after fertilization. We tested five different rearing condi tions (standard, high temperature, low temperature, high salinity, low oxygen content), but we always found that heritabilities remained stable or rather increased during development. We could not statistically test for the possible changes in heritabilities of individual characters, because this would have required multiple independent breeding experiments under identical conditions (with hundreds of breeding pairs and thousands of embryos), which would have been far beyond the scope of the study. However, the average heritabilities during the phylotypic stage, presented here, are in contrast to the predictions from the stabilizing selection hypothesis. Instead of the predicted depres sion of heritabilities, we observed stable heritabilities or even a slight, though statistically not significant, increase in heritabil ities with developmental time. The measures of phenotypic variances are in accordance with the predictions of that hypothesis. However, because both predictions must be met, we can safely reject the stabilizing selection hypothesis (Table 3). In reaching a more complete interpretation, it should be noted that we do not fully exclude that some minor effects of stabilizing selection might act on the phylotypic stage; but, then its depressive effects on additive genetic variances must be compensated for by a constant rate of mutations (Arnold, '92; Lynch and Walsh, '98) or the increasing effects of genetic interactions resulting in an overall stability of heritability values.

We can now place the results in relationship to the "random hypothesis" and the "epigenetic effects" hypothesis (Table 3). The random hypothesis predicts that there are no changes or there is increasing additive genetic variance and increasing phenotypic variances during the phylotypic stage as compared with stages

Table 3. Predictions from Table 1 and results on phenotypic variances and additive genetic variance.

Hypothesis	Phenotypic variance		Additive genetic variance	
	Predicted	Result	Predicted	Result
Random	No change	Decreasing	No change	No change
Epigenetic effects	Decreasing	Decreasing	No change	No change
Stabilizing selection	Decreasing	Decreasing	Decreasing	No change

Only the predictions from the hypothesis on epigenetic effects are met by the results of this and earlier studies. The results reject the random hypothesis and stabilizing selection hypothesis.

before and after. Both predictions are not met because additive genetic variances are constant and phenotypic variances decrease. Consequently, we reject the random hypothesis on the basis of our results. Decreasing phenotypic variances have also been reported in earlier studies of zebrafish development (Irmler et al., 2004; Schmidt and Starck, 2010), using a different data set and a tighter timing.

Predictions from the epigenetic effects hypothesis are met by the results of our study. The hypothesis predicted that genotypic variances would be stable or increase while phenotypic variances would decrease (Tables 1, 3). The results of this study support the hypothesis that the phylotypic period results from epigenetic effects that constrain the evolution of a broad variety of embryonic morphologies. The results on phenotypic variances, obtained here, confirm results from earlier studies using a similar measurement design but a tighter measurement schedule (Irmler et al., 2004; Schmidt and Starck, 2010) and considering phenotypic variances exclusively. Although this earlier study could not exclude effects of stabilizing selection, we can now suggest with some confidence that stabilizing selection does not play a major role in determining the phylotypic stage in zebrafish. As outlined in the introduction, others have also presented theoretical (Raff '94, '96; Galis and Metz, 2001; Galis and Sinervo, 2002; Horder, 2006, 2008) and empirical (Galis and Metz, 2001; Galis and Sinervo, 2002; Irmler et al., 2004; Schmidt and Starck, 2010) support for the epigenetic effects hypothesis.

It is difficult to deduce the specific nature of these epigenetic effects. This study does not provide information about this. Our earlier studies in zebrafish used morphological and morphometric methods, and we recognized multiple correlations among embryonic characters, constraints (Irmler et al., 2004), and modules (Schmidt and Starck, 2010) that canalize the develop ment of early fish embryo. Galis et al. suggested multiple inductive interactions of developing organ systems and canaliza tion through pleiotropic effects from correlations between modules as the key factors constraining early embryonic development in vertebrates (Galis, '99; Galis and Metz, 2001; Galis et al., 2002). Others have shown that the number of genetic interactions increases during the phylotypic period and have suggested that this high number of genetic interactions acts as an epigenetic effect (Duboule, '94; Sander and Schmidt Ott, 2004; Irie and Sehara Fujisawa, 2007; Compte et al., 2010; Elinson and Kezmoh, 2010), ultimately constraining the evolutionary diver sification of developmental pathways. All these suggestions are not mutually exclusive; they simply refer to different levels of development, molecular and morphological, thus possibly co occur as mechanisms constraining development.

The results presented here provide some explanation for the phylotypic period in zebrafish, but they may also have deeper implications for our understanding and perception of the phylotypic period in vertebrates in general. Indeed, the phylotypic period differs in timing, duration, and characters among different vertebrates (Richardson, '95; Richardson et al., '97; Richardson and Keuck, 2002; Bininda Emonds et al., 2003). This difference of phylotypic periods among various vertebrate taxa has been a major source of controversy about the validity and even the existence of the phylotypic period. The results of our study can offer a simple and straightforward explanation: the measured additive genetic variance during the phylotypic period suggests that the phylotypic period is accessible for evolution. Thus, differences in time, duration, and morphologies of the phylotypic period among vertebrates result from evolutionary diversification. The overall similarity, though not identity, of early vertebrate embryos results from epigenetic effects canaliz ing early embryonic development. In that view, the phylotypic period presents itself as a product of normal evolution, i.e. diversification because of additive genetic variance and similarity because of internal constraints. No phylogenetic laws or special rules are necessary to explain the similarity of early embryos. Selection acting on phenotypic variance and constraint resulting in canalization are sufficient to explain the overall similarity of vertebrate embryos. Among vertebrates, the differences in timing, duration, and stages that characterize the phylotypic period of different taxa may result from different proximate mechanisms that need to be elucidated in each taxon.

LITERATURE CITED

Arnold SJ. 1992. Constraints on phenotypic evolution. Am Nat 140: S85–S107.

Arthur W. 1988. A theory of the evolution of development. New York: Wiley.

Atchley WR. 1984. Ontogeny, timing of development, and genetic variance-covariance structure. Am Nat 123:519–540.

Bininda-Emonds ORP, Jeffery JE, Richardson MK. 2003. Inverting the hourglass: quantitative evidence against the phylotypic stage in vertebrate development. Proc R Soc B Biol Sci 270: 341–346.

Cohen J. 1977. Reproduction. London: Butterworths.

Compte A, Roux J, Robinson-Rechavi M. 2010. Molecular signaling in zebrafish development and the vertebrate phylotypic period. Evol Dev 12:144–156.

Conner JK, Hartl DL. 2004. A primer of ecological genetics. Sunderland, MA: Sinauer.

de Beer GR. 1940. Embryos and ancestors. Oxford: Claredon Press.

Duboule D. 1994. Temporal colinearity and the phylotypic progression: a basis for the stability of a vertebrate Bauplan and the evolution of morphologies through heterochrony. Development 120:135–142.

Elinson RP, Kezmoh L. 2010. Molecular Haeckel. Dev Dyn 239: 1905–1918.

Falconer DS, Mackay TFC. 1996. Introduction to quantitative genetics. London, UK: Longman.

Galis F. 1999. Why do almost all mammals have seven cervical vertebrae? Developmental constraints, Hox genes, and cancer. J Exp Zool (Mol Dev Evol) 285:19–26.

Galis F, Metz JAJ. 2001. Testing the vulnerability of the phylotypic stage: on modularity and evolutionary conservation. J Exp Zool (Mol Dev Evol) 291:195–204.

Galis F, Sinervo B. 2002. Divergence and convergence in early embryonic stages of metazoans. Contrib Zool 71:101–113.

Galis F, Van Dooren TJM, Metz JAJ. 2002. Conservation of the segmented germband stage: robustness or pleiotropy? Trends Genet 18:504–509.

Gould SJ. 1977. Ontogeny and phylogeny. Cambridge: Harvard University Press.

Haeckel E. 1866. Generelle morphologie. Berlin: G. Reimer.

Hall BK. 1997. Phylotypic stage or phantom: is there a highly conserved embryonic stage in vertebrates? Trends Ecol Evol 12: 461–463.

Hall BK. 2003. Evo-devo: evolutionary developmental mechanisms. Int J Dev Biol 47:491–495.

Horder TJ. 2006. Gavin Rylands de Beer: how embryology foreshadowed the dilemmas of the genome. Nat Rev Genet 7: 892–898.

Horder T. 2008. A history of evo-devo in Britain. Theoretical ideals confront with biological complexity. Ann Hist Phil Biol 13:101–174.

Huxley JS. 1942. Evolution. The modern synthesis. London: George Allen and Unwin.

Irie N, Sehara-Fujisawa A. 2007. The vertebrate phylotypic stage and an early bilaterian-related stage in mouse embryogenesis defined by genomic information. BMC Biology 5:1. DOI: 10.1186/1741-7007-5-1.

Irmler I, Schmidt K, Starck JM. 2004. Developmental variability during early embryonic development of zebra fish, *Danio rerio*. J Exp Zool B Mol Dev Evol 302:446–457.

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. 1995. Stages of embryonic development of the zebrafish. Dev Dyn 203: 253–310.

Kirschner M, Gerhart J. 1998. Evolvability. Proc Natl Acad Sci USA 95: 8420–8427.

Lynch M, Walsh B. 1998. Genetics and analysis of quantitative traits. Sunderland: Sinauer Associates.

Meckel JF. 1811. Entwurf einer Darstellung der zwischen den Embryonalzuständen der höheren Thiere und den permanenten niederen stattfindenden Parallele. Beiträge Vergleichende Anatomie 2:1–60.

Medawar PB. 1954. The significance of inductive relationships in the development of vertebrates. J Embryol Exp Morphol 2:172–174.

Raff RA. 1994. Developmental mechanism in the evolution of animal form: origins and evolvability of body plans. In: Bengston S, editor. Early life on earth. New York: Columbia University Press. p 489–500.

Raff RA. 1996. The shape of life: genes, development, and the evolution of animal form. Chicago: University of Chicago Press.

Raff RA, Sly BJ. 2000. Modularity and dissociation in the evolution of gene expression territories in development. Evol Dev 2:102–113.

Richardson MK. 1995. Heterochrony and the phylotypic period. Dev Biol 172:412–421.

Richardson MK. 1999. Vertebrate evolution: the developmental origins of adult variation. BioEssays 21:604–613.

Richardson MK, Keuck G. 2002. Haeckel's ABC of evolution and development. Biol Rev Camb Philos Soc 77:495–528.

Richardson MK, Hanken J, Gooneratne ML, Pieau C, Raynaud A, Selwood L, Wright GM. 1997. There is no highly conserved embryonic stage in the vertebrates: implications for current theories of evolution and development. Anat Embryol 196:91–106.

Richardson MK, Minelli A, Coates M, Hanken J. 1998. Phylotypic stage theory. Trends Ecol Evol 13:158.

Richardson MK, Jeffery JE, Coates MI, Bininda-Emonds ORP. 2001. Comparative methods in developmental biology. Zoology 104:278–283.

Roff DA. 1997. Evolutionary quantitative genetics. New York, NY: Chapman & Hall.

Sander K. 1983. The evolution of patterning mechanisms: gleanings from insect embryogenesis and spermatogenesis. In: Goodwin BC,

Holder N, Wylie CC, editors. Development and evolution. Cambridge: Cambridge University Press. p 137–159.

Sander K, Schmidt-Ott U. 2004. Evo-devo aspects of classical and molecular data in a historical perspective. J Exp Zool (Mol Dev Evol) 302:69–91.

Schmidt K, Starck JM. 2010. Developmental plasticity, modularity, and heterochrony during the phylotypic stage of the zebra fish, *Danio rerio*. J Exp Zool (Mol Dev Evol) 314B:166–178.

Seidel F. 1960. Körpergrundgestalt und Keimstruktur. Eine Erörterung über die Grundlagen der vergleichenden und experimentellen Embryologie und deren Gültigkeit bei phylogenetischen Untersuchungen. Zoologischer Anzeiger 164:245–305.

Slack JMW, Holland PWH, Graham CF. 1993. The zootype and the phylotypic stage. Nature 361:490–492.

von Baer KE. 1828. Über die Entwicklungsgeschichte der Thiere. Königsberg: Beobachtung und Reflexion.

Waddington CH. 1940. Organizers and genes. Cambridge biological studies. Cambridge, MA: Cambrige University Press.

Waddington CH. 1956. Principles of embryology. London, UK: George Allen and Unwin Ltd.

Waddington CH. 1957. The strategy of genes. A discussion of some aspects of theoretical biology. London: George Allen and Unwin.

Williams GC. 1992. Natural selection. Domains, levels, and challanges. Oxford: Oxford University Press.

3 General Discussion

One of the main critiques of the phylotypic stage is that the phylotypic stage contains still a certain amount of variation. This variation is generated by sequence hetero-chrony. Thus, opponents did argument, that it cannot be a conserved stage. The def-inition of the phylotypic stage, speaks of the stage where all (vertebrate) embryos look most similar to each other e.g. resemble each other more than at an early or later stage. None of the proponents speaks anymore of "identical" embryos and thus variation is certainly allowed at the phylotypic stage. So the major critique that there are sequence heterochronies at the phylotypic stage cannot collapse the concept. In the end it all boils down to the question put up by Sander and Schmidt-Ott (2004): how much heterochrony can be allowed without ruining the concept of the phylotypic stage.

In the zebra fish, the typical landmarks, characteristic for the phylotypic stage, occur during a 16h hour period of embryonic development.

I have analysed patterns of variation and co-variation. Therefore, I calculated the phenotypic variance and the number of significant correlations among embryonic traits. This data enabled me to define a timeframe for the phylotypic stage for the zebrafish.

The phenotypic trait variation during the early embryogenesis is restrained by functional/developmental interactions among traits. Thus, addressing my first ques-tion, one can see a discrete pattern for the observed variance and observed covaria-tion for the period between 15h and 19h post fertilization. The observed pattern ful-fils the 2nd prediction: internal constraints lead to reduced phenotypic variance and increased covariation among traits.

This period must be relatively resistant to selection because changing any trait will affect all others that are functionally linked. I can describe the phylotypic stage in zebrafish as a period, characterized by a high number of internal correlations.

Nevertheless, in my next study, I also found plasticity, heterochrony and dis-crete modules within the period of the phylotypic stage. Here, my intention was to find out if changes in external raising conditions may induce phenotypic variation during the phylotypic stage. Coming to my 2nd question, I could demonstrate that changes in raising conditions affect the development of the zebrafish on three levels

(1) developmental rate (2) size and shape, and (3) developmental dissociation. Thus, plasticity can be found during the phylotypic stage.

Different treatments affect different traits of the developing embryo and in some cases the same traits in different directions. For example, raising the zebrafish embryo at a low temperature or at low oxygen concentration both resulted in a reduced developmental speed; however, at a low temperature the size of certain traits increased, while it decreased when embryos were raised under low oxygen concentration. Thus growth and development were affected. Similar, but less strong effects were found when embryos were raised in elevated salinity.

Furthermore, under hypoxic conditions, there was also a profound effect on the timing of development leading to developmental dissociation. Thus, growth and development were affected.

My results displayed a distinct heterochronic displacement of the onset of the development of the eye and the otic vesicle. The observed shift in relative timing resulted in a changed sequence of organ formation. The delayed onset of eye and otic vesicle development meets all criteria of heterochrony (Raff 1996). An important difference to Raff's definition of heterochrony is that, in my thesis, I do not compare ancestral and descendant ontogenies, but ontogenies of the same species developing in different environmental conditions. Therefore, in this study, heterochrony is induced by an environmental change and considered a special case of developmental plasticity.

Addressing my third and fourth questions, I suggest that the dissociation of eye development and otic vesicle development from the remaining embryo, under the condition of low oxygen concentration, identifies eye and ear as module(s), which can be dissociated from the other forming organs and structures/modules during development. This dissociation is a heterochronic event during the phylotypic stage. However, under normal developmental conditions, I could not detect modules either because they are not effective or because my method could not elucidate modules. But if developmental conditions were pushed to the margins, modules could be detected and became effective. This observation reminds of a postulation of Waddington (1940), (1956) who said that developmental trajectories are certainly flexible within certain margins. Modularity at the margins of development may act as

a stabilizing mechanism that ensures an undisturbed development of the embryo by buffering it against disruptive external influences.

Coming back to my table of prediction, I found that plasticity is indeed present during the phylotypic stage. My results support the predictions of number 2, acceleration of growth/development, number 3, changes in size and shape, and number 4, developmental dissociation. Constraints as suggested by several researchers, acting during the phylotypic stage, cannot avoid a plastic response of the early embryo. I suggest that eye and otic vesicle are clearly defined modules during the phylotypic stage of the zebrafish. Consequently, I suggest these modules make changes during the phylotypic stage possible. However, I have only been able to describe dissociation of modules for the eye and otic vesicle during the phylotypic stage. In accordance with Raff's hypothesis (1996), I agree that without modules, there is little plasticity, possibly constrained by the high connectivity of all the other, not modularized structures.

These findings are in contrast to a theory of Domazet-Loso and Tautz (2010). They argue that one reason for the conservation of the phylotypic stage is that environmental cues favouring evolution cannot act during the phylotypic stage. They suggest that embryos at this period are not in contact with the environment. They draw the conclusion that constraints might not be so essential for the phylotypic stage, although they still consider constraints as an alternative option for the conservation of the phylotypic stage. I showed, in my study, that environmental cues have an effect under laboratory conditions on the development of the phylotypic stage and thus embryos are not isolated during development from the environment. The environmental cues I used in the laboratory (temperature, salinity and oxygen) are also found in nature.

My final study, addressing my last questions, also stresses that constraints play an important role for the conservation of the phylotypic stage. The aim of the final study was to analyse the change in additive genetic variance of embryonic characters by measuring the change of heritability during the period of early development.

The heritabilities of 11 embryonic characters were within the range of heritabilities previously reported for other morphological characters, mainly in adults (Roff

1997, Lynch and Walsh 1998, Conner and Hartl 2004). More importantly, I could not detect any significant change of heritabilities between 12h and 24h after fertilization. I tested five different rearing conditions (standard, high temperature, low temperature, high salinity, low oxygen content), but I always found that heritabilities remained stable or rather increased during development. I observed stable heritabilities or even a slight, though statistically not significant, increase of heritabilities with developmental time. The measures of phenotypic variances are in accordance with the measure in my first study of zebrafish development, in which I was using a different dataset and a tighter timing.

While this earlier study could not exclude effects of stabilizing selection, I can now combine the results of all my studies. Though I am able to test three different hypotheses, which I have drafted around the evolutionary origin of the phylotypic stage: (1) stabilizing selection, (2) random and (3) epigenetic effects (in the sense of Waddington) acting as internal constraints. One sees that the average heritabilities as a measure for additive genetic variances presented here are excluding the stabilizing selection hypothesis as the prediction is violated. The measures of phenotypic variances are excluding the random hypothesis as the prediction is violated. Finally, the results of the correlation analysis also exclude the stabilizing selection and random hypothesis.

As all predictions must be met, I can safely reject the random and stabilizing selection hypotheses. To be careful and conservative with my interpretations, I do not fully exclude that some minor effects of stabilizing selection might act on the phylotypic stage, but looking at my results, I can now suggest that stabilizing selection does not play a major role in determining the phylotypic stage in zebrafish.

Thus, only the predictions from the epigenetic effects hypothesis are met by the results of my study. The hypothesis predicted that genotypic variances would be stable or increase, while phenotypic variances would decrease with a peak of correlations at the phylotypic stage.

Interestingly, I could show that stabilizing selection plays only a minor role in conserving the phylotypic stage of zebrafish, whereas in the past, stabilizing selection has been a prime candidate for the conservation of the phylotypic stage in the hypothesis of several authors (Hall 1997, Kirschner and Gerhart 1998, Galis and Metz

2001, Galis and Sinervo 2002, Galis, Van Dooren et al. 2002, Kalinka, Varga et al. 2010).

4 Conclusion

I have been able to show that zebrafish embryos pass through a phylotypic stage during early development and to identify the time period in which the phylotypic stage occurs in this species.

Plasticity could be detected throughout embryonic development and thus also during the phylotypic stage. I recognized multiple correlations among embryonic characters, constraints, and modules (eye and otic vesicle) that canalize the development of early fish embryos.

Combining the results of all my studies, I was able to test the following three evolutionary hypotheses, which aim at explaining the phylotypic stage: (1) stabilizing selection, (2) random, and (3) epigenetic effects in the sense of Waddington (Table 1).

The results of my study can offer a simple and straightforward explanation: The overall similarity, though not identity, of early vertebrate embryos results from epigenetic effects canalizing early embryonic development. In that view, the phylotypic stage presents itself as a product of evolution. In other words, there is diversification because of additive genetic variance and similarity because of internal constraints.

Selection acting on phenotypic variance and constraints resulting in canalization are sufficient to explain the overall similarity of vertebrate embryos. Among vertebrates, the differences in timing, duration and stages that characterize the phylotypic stage of different taxa, may result from different proximate mechanisms, which need to be elucidated in each taxon.

This study defined the term of the phylotypic stage in theory and practice evaluating this part of developmental stage in zebrafish. I do not only deliver a quantitative analysis of the phylotypic stage in zebrafish, but also present a new idea for an evolutionary concept explaining the phylotypic stage in vertebrates.

However, it is still possible that Richardson's (2012) theory, that the phylotypic stage not only represents an evolutionary lockdown but is a key target for evolution and a powerhouse of speciation, is valid.

The presence of additive genetic variance allows that selection alters the phylotypic stage and constraints that canalize development, which might be overcome when developmental condition are pushed to its margin.

The results presented here provide some explanation for the phylotypic stage in zebrafish, but they may also have deeper implications for our understanding and perception of the phylotypic stage in other vertebrates.

References

Armant, O., M. März, R. Schmidt, M. Ferg, N. Diotel, R. Ertzer, J. C. Bryne, L. Yang, I. Baader, M. Reischl, J. Legradi, R. Mikut, D. Stemple, W. V. Ijcken, A. van der Sloot, B. Lenhard, U. Strähle and S. Rastegar (2013). "Genome-wide, whole mount in situ analysis of transcriptional regulators in zebrafish embryos." Developmental Biology **380**(2): 351-362.

Ballard, W. W. (1981). "Morphogenetic movements and fate maps of vertebrates." Am. Zool. **21**: 391-399.

Bininda-Emonds, O. R. P., J. E. Jeffery, M. I. Coates and M. K. Richardson (2002). "From Haeckel to event-pairing: The evolution of developmental sequences." Theory in Biosciences **121**(3): 297-320.

Bininda-Emonds, O. R. P., J. E. Jeffery and M. K. Richardson (2003). "Inverting the hourglass: Quantitative evidence against the phylotypic stage in vertebrate development." Proceedings of the Royal Society B: Biological Sciences **270**(1513): 341-346.

Bininda-Emonds, O. R. P., J. E. Jeffrey and M. K. Richardson (2003). "Is sequence heterochrony an important evolutionary mechanism in mammals?" Journal of Mammalian Evolution **10**(4): 335-361.

Bogdanović, O., A. Fernandez-Miñán, J. J. Tena, E. De La Calle-Mustienes, C. Hidalgo, I. Van Kruysbergen, S. J. Van Heeringen, G. J. C. Veenstra and J. L. Gómez-Skarmeta (2012). "Dynamics of enhancer chromatin signatures mark the transition from pluripotency to cell specification during embryogenesis." Genome Research **22**(10): 2043-2053.

Bolker, J. A. (2000). "Modularity in development and why it matters to evo-devo." American Zoologist **40**(5): 770-776.

Brigandt, I. (2006). "Homology and heterochrony: the evolutionary embryologist Gavin Rylands de beer (1899–1972)." Journal of Experimental Zoology Part B: Molecular and Developmental Evolution **306B**(4): 317-328.

Bromley, J. S. (1971). The new Cambridge modern history: The rise of Great Britain and Russia, 1688-1715/25, Cambridge University Press.

Chipman, A. D., A. Haas, E. Tchernov and O. Khaner (2000). "Variation in anuran embryogenesis: Differences in sequence and timing of early developmental events." Journal of Experimental Zoology **288**(4): 352-365.

Cohen, J. (1963). Living embryos, 1st edn. Oxford, Pergamon.

Cohen, J. (1977). Reproduction. London, Butterworth.

Collazo, A. (2000). "Developmental variation, homology, and the pharyngula stage." Systematic Biology **49**(1): 3-18.

Collins, P. (1995). Embryology and development. Gray's anatomy, 38th edn. C. P. London, Churchill Livingstone.

Compte, A., J. Roux and M. Robinson-Rechavi (2010). "Molecular signaling in zebrafish development and the vertebrate phylotypic period." Evolution and Development **12**(2): 144-156.

Conner, J. K. and D. L. Hartl (2004). A primer of ecological genetics. Sunderland, MA, Sinauer.

de Beer, G. (1940). Embryos and Ancesters. Oxford, Clarendon Press.

Domazet-Loso, T. and D. Tautz (2010). "A phylogenetically based transcriptome age index mirrors ontogenetic divergence patterns." Nature **468**(7325): 815-819.

Duboule, D. (1994). "Temporal colinearity and the phylotypic progression: A basis for the stability of a vertebrate Bauplan and the evolution of morphologies through heterochrony." Development **120**(SUPPL.): 135-142.

Elinson, R. P. and L. Kezmoh (2010). "Molecular Haeckel." Developmental Dynamics **239**: 1905-1918.

Fordyce, J. A. (2006). "The evolutionary consequences of ecological interactions mediated through phenotypic plasticity." Journal of Experimental Biology **209**(12): 2377-2383.

Galis, F. and J. A. J. Metz (2001). "Testing the vulnerability of the phylotypic stage: On modularity and evolutionary conservation." Journal of Experimental Zoology **291**(2): 195-204.

Galis, F. and B. Sinervo (2002). "Divergence and convergence in early embryonic stages of metazoans." Contributions to Zoology **71**(1-3): 101-113.

Galis, F., T. J. M. Van Dooren and J. A. J. Metz (2002). "Conservation of the segmented germband stage: Robustness or pleiotropy?" Trends in Genetics **18**(10): 504-509.

Galis, F., G. P. Wagner and E. L. Jockusch (2003). "Why is limb regeneration possible in amphibians but not in reptiles, birds, and mammals?" Evolution and Development **5**(2): 208-220.

Garstang, W. (1922). "The theory of recapitulation: A critical restatement of the biogenetic law." J Linn Soc Zool **35**: 81-101.

Gilbert, S. F. (2013). Ernst Haeckel and the Biogenetic Law (An informed Opinion). Developmental Biology. Sunderland, MA, Sinauer Associates.

Goldschmidt, R. B. (1940). The material basis of evolution. New Haven, Yale Univerisity Press.

Gould, S. J. (1977). Ontogeny and Phylogeny. Cambridge, Harvard University Press.

Gould, S. J. (1980). The return of the hopeful monsters. The panda's thumb. New York, W. W. Norton & Co**:** 186-193.

Groeters, F. R. and D. D. Shaw (1992). "Association between Latitudinal Variation for Embryonic Development Time and Chromosome Structure in the Grasshopper Caledia captiva (Orthoptera: Acrididae)." Evolution **46**(1): 245-257.

Haeckel, E. (1866). Generelle Morphologie. Berlin, G. Reimer.

Haeckel, E. (1874). Anthropogenie, oder Entwickelungsgeschichte des Menschen. Leipzig, Engelmann.

Hall, B. K. (1996). "Bauplane, phylotypic stages, and constraint - Why there are so few types of animals." Evolutionary Biology **29**: 215-261.

Hall, B. K. (1997). "Phylotypic stage or phantom: Is there a highly conserved embryonic stage in vertebrates?" Trends in Ecology and Evolution **12**: 461-463.

Hall, B. K. (1999). Evolutionary Developmental Biology. Dordrecht, Kluwer Academic Press.

Hall, B. K. (2000). "Guest Editorial: Evo-devo or devo-evo — does it matter?" Evolution & Development **2**(4): 177-178.

Hall, B. K. (2003). "Evo-Devo: Evolutionary developmental mechanisms." International Journal of Developmental Biology **47**(7-8): 491-495.

Hazkani-Covo, E., D. Wool and D. Graur (2005). "In search of the vertebrate phylotypic stage: A molecular examination of the developmental hourglass model and von Baer's third law." Journal of Experimental Zoology Part B: Molecular and Developmental Evolution **304**(2): 150-158.

Irie, N. and S. Kuratani (2011). "Comparative transcriptome analysis reveals vertebrate phylotypic period during organogenesis." Nature Communications **2**(1).

Irie, N. and A. Sehara-Fujisawa (2007). "The vertebrate phylotypic stage and an early bilaterian-related stage in mouse embryogenesis defined by genomic information." BMC Biology **5**.

Jeffery, J. E., O. R. P. Bininda-Emonds, M. I. Coates and M. K. Richardson (2002). "Analyzing evolutionary patterns in amniote embryonic development." Evolution and Development **4**(4): 292-302.

Jeffery, J. E., O. R. P. Bininda-Emonds, M. I. Coates and M. K. Richardson (2005). "A new technique for identifying sequence heterochrony." Systematic Biology **54**(2): 230-240.

Kalinka, A. T., K. M. Varga, D. T. Gerrard, S. Preibisch, D. L. Corcoran, J. Jarrells, U. Ohler, C. M. Bergman and P. Tomancak (2010). "Gene expression divergence recapitulates the developmental hourglass model." Nature **468**(7325): 811-816.

Kirschner, M. and J. Gerhart (1998). "Evolvability." <u>Proc. Natl. Acad. Sci USA (PNAS)</u>: 8420-8427.

Levin, M., T. Hashimshony, F. Wagner and I. Yanai (2012). "Developmental Milestones Punctuate Gene Expression in the Caenorhabditis Embryo." <u>Developmental Cell</u> **22**(5): 1101-1108.

Lillie, F. R. (1919). <u>The development of the chick</u>. New York, Holt.

Love, A. C. and R. A. Raff (2003). "Knowing your ancestors: themes in the history of evo-devo." <u>Evolution & Development</u> **5**(4): 327-330.

Lynch, M. and B. Walsh (1998). <u>Genetics and Analysis of Quantitative Traits</u>. Sunderland, Sinauer Associates.

Maynard Smith, J., R. Burian, S. Kauffman, P. Alberch, J. Campbell, B. Goodwin, R. Lande, D. Raup and L. Wolpert (1985). "Developmental Constraints and Evolution: A Perspective from the Mountain Lake Conference on Development and Evolution." <u>The Quarterly Review of Biology</u> **60**(3): 265-287.

Mayr, E. (1981). <u>The growth of biological thought: Diversity, Evolution, and Inheritance</u>, Harvard University Press.

McNamara, K. J. (1997). <u>Shapes of Time: The Evolution of Growth and Development</u>. Baltimore, MD, The John Hopkins University Press.

Meckel, J. F. (1811). "Entwurf einer Darstellung der zwischen den Embryonalzuständen der höheren Thiere und den permanenten niederen stattfindenden Parallele." <u>Beiträge vergleichende Anatomie</u> **2**: 1-60.

Meckel, J. F. (1821). <u>System der vergleichenden Anatomie, 1. Teil. Allgemeine Anatomie.</u>. Halle, Renger.

Medawar, P. B. (1954). "The significance of inductive relationships in the development of vertebrates." <u>J Embryol Exp Morphol</u> **2**: 172-174.

Mueller, T., P. Vernier and M. F. Wullimann (2006). "A phylotypic stage in vertebrate brain development: GABA cell patterns in zebrafish compared with mouse." <u>Journal of Comparative Neurology</u> **494**(4): 620-634.

Müller, W. A., Hassel, M, (2005). <u>Entwicklungsbiologie und Reproduktionsbiologie von Mensch und Tieren</u>. Berlin, Springer.

Myers, P. Z. (2003). "Wells and Haeckel's Embryos. A review of Chapter 5 of Icons of evolution." Retrieved 05.05.2014, 2014, from http://www.talkorigins.org/faqs/wells/haeckel.html.

Needham, J. (1950). <u>Biochemistry and morphogenesis</u>. Cambridge, At the University Press.

Nelson, A. C. and F. C. Wardle (2013). "Conserved non-coding elements and cis regulation: Actions speak louder than words." <u>Development (Cambridge)</u> **140**(7): 1385-1395.

Neukamm, M. (2002). "Die Ähnlichkeiten zwischen den Arten. Homologie als Evolutionsbeleg." <u>Praxis der Naturwissenschaften - Biologie</u> **51**(7): 39-42.

Neukamm, M. (2007). "Ernst Haecksels Biogenetisches Grundgesetz und das Konzept der ontogenetischen Rekapitulation. AG Evolutionsbiologie. S.1-48.
URL: .
." Retrieved 15.06.2014, from ag-evolutionsbiologie.net/pdf/2007/ernst-haeckels-biogenetisches-grundgesetz.pdf.

Neukamm, M. (2007). Wissenschaft und ontologischer Naturalismus. Eine Kritik antievolutionistischer Argumentation. <u>Kreationismus in Deutschland. Fakten und Analysen</u>. U. Kutschera. Münster, Lit-Verlag**:** 163-231.

Newman, S. A. (2011). "Animal egg as evolutionary innovation: A solution to the "embryonic hourglass" puzzle." <u>Journal of Experimental Zoology Part B: Molecular and Developmental Evolution</u>.

Osche, G. (1982). "Rekapitulationsentwicklung und ihre Bedeutung fu?r die Phylogenetik - Wann gilt die "biogenetische Grundregel"?" <u>Verh. Naturwiss. Ver. Hamburg (N.F.)</u> **25**: 5-31.

Pigliucci, M. (2001). <u>Phenotypic Plasticity - Beyond Nature And Nurture</u>. Baltimore, Maryland, The John Hopkins University Press.

Pigliucci, M., C. J. Murren and C. D. Schlichting (2006). "Phenotypic plasticity and evolution by genetic assimilation." <u>Journal of Experimental Biology</u> **209**(12): 2362-2367.

Poe, S. (2006). "Test of Von Baer's law of the conservation of early development." <u>Evolution</u> **60**(11): 2239-2245.

Poe, S. and M. H. Wake (2004). "Quantitative tests of general models for the evolution of development." <u>American Naturalist</u> **164**(3): 415-422.

Quint, M., H. G. Drost, A. Gabel, K. K. Ullrich, M. Bönn and I. Grosse (2012). "A transcriptomic hourglass in plant embryogenesis." <u>Nature</u>.

Raff, R. A. (1996). <u>The Shape of Life: Genes, Development, and the Evolution of Animal Form.</u> Chicago, University of Chicago Press
.

Richardson, M. K. (1995). "Heterochrony and the phylotypic period." <u>Developmental Biology</u> **172**(2): 412-421.

Richardson, M. K. (1999). "Vertebrate evolution: The developmental origins of adult variation." <u>BioEssays</u> **21**(7): 604-613.

Richardson, M. K. (2012). "A Phylotypic Stage for All Animals?" <u>Developmental Cell</u> **22**(5): 903-904.

Richardson, M. K., J. Hanken, M. L. Gooneratne, C. Pieau, A. Raynaud, L. Selwood and G. M. Wright (1997). "There is no highly conserved embryonic stage in the vertebrates: Implications for current theories of evolution and development." <u>Anatomy and Embryology</u> **196**(2): 91-106.

Richardson, M. K., J. E. Jeffery, M. I. Coates and O. R. P. Bininda-Emonds (2001). "Comparative methods in developmental biology." <u>Zoology</u> **104**(3-4): 278-283.

Richardson, M. K. and G. Keuck (2002). "Haeckel's ABC of evolution and development." <u>Biological Reviews of the Cambridge Philosophical Society</u> **77**(4): 495-528.

Richardson, M. K., A. Minelli, M. Coates and J. Hanken (1998). "Phylotypic stage theory." <u>Trends Ecol. Evol.</u> **13**: 158-158.

Roff, D. A. (1997). <u>Evolutionary quantitative genetics</u>. New York, NY, Chapman & Hall.

Roux, J. and M. Robinson-Rechavi (2008). "Developmental constraints on vertebrate genome evolution." <u>PLoS Genetics</u> **4**(12).

Sander, K. (1983). The evolution of patterning mechanisms: gleanings from insect embryogenesis and spermatogenesis. <u>Development and Evolution</u>. B. C. Goodwin, Holder N., Wylie CC,, Cambridge University Press**:** 137-159.

Sander, K. (1994). "Spuren der Evolution in den Mechanismen der Ontogenese - Neue Facetten eines zeitlosen Themas." <u>Leopoldina (R.3)</u> **39**: 297-319.

Sander, K. (2002). "Ernst Haeckel's ontogenetic recapitulation: Irritation and incentive from 1866 to our time." <u>Annals of Anatomy</u> **184**(6): 523-533.

Sander, K. (2004). "Ernst Haeckels ontogenetische Rkeapitulation - Leitbild und Ärgernis bis heute? IN: Höxtermann E et al. Von der „Entwicklungsmechanik" zur Entwicklungsbiologie." <u>Verhandlungen zur Geschichte und Theorie der Biologie</u> **10**: 163-176.

Sander, K. and U. Schmidt-Ott (2004). "Evo-Devo aspects of classical and molecular data in a historical perspective." <u>Journal of Experimental Zoology Part B: Molecular and Developmental Evolution</u> **302**(1): 69-91.

Sauermost, R. (1994). <u>Herder - Lexikon der Biologie</u>. Heidelberg Berlin Oxford, Spektrum Akademischer Verlag GmbH.

Scheiner, S. M. (2002). "Selection experiments and the study of phenotypic plasticity." <u>Journal of Evolutionary Biology</u> **15**(6): 889-898.

Schep, A. N. and B. Adryan (2013). "A Comparative Analysis of Transcription Factor Expression during Metazoan Embryonic Development." PLoS ONE **8**(6).

Schlosser, G. and G. P. Wagner (2004). Modularity in Development and Evolution. Chicago, Univ. of Chicago Press.

Sedgwick, A. (1894). "On the law of development commonly known as von Baer's law; and on the significance of ancestral rudiments in embryonic development." Quarterly Journal of Microscopical Science **36**: 35-52.

Seidel, F. (1960). "Körpergrundgestalt und Keimstruktur. Eine Erörterung über die Grundlagen der vergleichenden und experimentallen Embryologie und deren Gültigkeit bei phylogenetischen Untersuchungen." Zoologischer Anzeiger **164**: 245-305.

Slack, J. M. W., P. W. H. Holland and C. F. Graham (1993). "The zootype and the phylotypic stage." Nature **361**(6412): 490-492.

Smith, K. K. (1996). "Integration of craniofacial structures during development in mammals." American Zoologist **36**(1): 70-79.

Smith, K. K. (1997). "Comparative patterns of craniofacial development in eutherian and metatherian mammals." Evolution **51**(5): 1663-1678.

Smith, K. K. (2001). "Heterochrony revisited: The evolution of developmental sequences." Biological Journal of the Linnean Society **73**(2): 169-186.

Stearns, S. C. and R. F. Hoekstra (2001). Evolution. Oxford, Oxford University Press Inc.

von Baer, K. E. (1828). Über die Entwicklungsgeschichte der Thiere. Beobachtung und Reflexion. Königsberg.

Von Dassow, G., E. Meir, E. M. Munro and G. M. Odell (2000). "The segment polarity network is a robust developmental module." Nature **406**(6792): 188-192.

Von Dassow, G. and E. Munro (1999). "Modularity in animal development and evolution: Elements of a conceptual framework for EvoDevo." Journal of Experimental Zoology **285**(4): 307-325.

Waddington, C. H. (1940). Organizers and Genes. Cambridge Biological Studies. Cambridge, MA, Cambrige University Press.

Waddington, C. H. (1942). "Canalization of development and the inheritance of acquired characters." Nature **150**(3811): 563-565.

Waddington, C. H. (1953). "Genetic assimilation of an acquired character." Evolution **7**: 118-126.

Waddington, C. H. (1956). Principles of embryology. London, Allen & Unwin.

Wagner, G. P., M. Pavlicev and J. M. Cheverud (2007). "The road to modularity." Nature Reviews Genetics **8**(12): 921-931.

Wang, Z., J. Pascual-Anaya, A. Zadissa, W. Li, Y. Niimura, Z. Huang, C. Li, S. White, Z. Xiong, D. Fang, B. Wang, Y. Ming, Y. Chen, Y. Zheng, S. Kuraku, M. Pignatelli, J. Herrero, K. Beal, M. Nozawa, Q. Li, J. Wang, H. Zhang, L. Yu, S. Shigenobu, J. Liu, P. Flicek, S. Searle, J. Weng, S. Kuratani, Y. Yin, B. Aken, G. Zhang and N. Irie (2013). "The draft genomes of soft-shell turtle and green sea turtle yield insights into the development and evolution of the turtle-specific body plan." Nature Genetics **45**(6): 701-706.

West-Eberhard, M. J. (2003). Developmental Plasticity and Evolution, Oxford University Press. New York.

West-Eberhard, M. J. (2005). "Developmental plasticity and the origin of species differences." Proceedings of the National Academy of Sciences **102**(suppl 1): 6543-6549.

Williams, G. C. (1992). Natural Selection. Domains, Levels, and Challanges. Oxford, Oxford University Press.

Wolpert, L. (1969). "Positional information and the spatial pattern of cellular differentiation." Journal of Theoretical Biology **25**(1): 1-47.

Wolpert, L. (1991). The triumph of the embryo. Oxford, Oxford University Press.

Yassin, A., E. K. Lienau, A. Narechania and R. DeSalle (2010). "Catching the phylogenic history through the ontogenic hourglass: A phylogenomic analysis of Drosophila body segmentation genes." Evolution and Development **12**(3): 288-295.

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Schmidt K, Starck JM. Developmental plasticity vs. phylotypic constraints during early embryogenesis of zebrafish, *Danio rerio*. Society for Integrative and Comparative Biology 2006 Annual Meeting, Orlanda, Florida, USA.

Schmidt K, Starck JM. Developmental plasticity and heterochronic shifts induced by hypoxic conditions during early embryogenesis of zebrafish, *Danio rerio.* Society for Integrative and Comparative Biology 2007 Annual Meeting, Phoenix, Arizona, USA.

Schmidt K, Starck JM. Dissociated Modules and Developmental Plasticity during early Embryonic Development of *Danio rerio*. 8th International Congress of Vertebrate Morphology, Paris, France, 16-21 July 2007.

Schmidt K, Starck JM. Examining the Influence of Plasticity, Heterochrony, Modularity and Heritability on the Phylotypic Stage in *Danio rerio*, defined by Patterns of Variation. . Society for Integrative and Comparative Biology 2011 Annual Meeting, Salt Lake City, Utah, USA.

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