



Phenotyping in Evo-Devo

Nico Posnien

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Abstract

The description and comparison of morphological features has been an integral part of evolutionary developmental biology (evo-devo) from the early comparative embryology in the late nineteenth century to the revival of the discipline in the late twentieth century. The success of modern evo-devo research was fueled by an exciting accumulation of experimental data revealing central mechanisms underlying developmental processes in a few well-established model organisms. Recent advances in imaging and sequencing technologies allow for an in-depth genome-wide and highly quantitative comparison of developmental processes in a variety of organisms. The combination of this quantitative data with the establishment of theoretical and mathematical frameworks to integrate and analyze such data provides an excellent starting point to reveal the evolutionary, genetic, developmental, and ecological forces underlying the morphological diversification in Nature. In this chapter, I summarize key features of qualitative and quantitative phenotyping methods, highlighting advantages and potential limitations. Additionally, I argue that gene expression represents an intermediate

N. Posnien (✉)

Abteilung für Entwicklungsbiologie, Johann-Friedrich-Blumenbach-Institut für Zoologie und Anthropologie, Universität Göttingen, Georg August University, Göttingen, Germany
e-mail: nposnie@gwdg.de

phenotype that has the potential to link genotypic and epigenetic changes with the evolution of developmental processes and thus adult morphology.

Keywords

Evo-devo · Phenotyping · Morphology · Gene expression · Geometric morphometrics · Mathematical modelling

Introduction

It is one of the greatest challenges of biological research to reveal general concepts underlying the evolution of the breathtaking morphological variation in animals and plants. In the last decades, it has been established that the functional morphology of organisms or organs that interact with the environment and thus provide the basis for evolutionary adaptations are the result of changes during embryonic and post-embryonic development. Evolutionary developmental biology (evo-devo), therefore, provides an excellent conceptual framework to study the evolution of the size and shape of living organisms and their individual organs.

In most animals, key morphological structures upon which selection can act are restricted to specific stages of the life cycle. For instance, external copulation organs are important for species-specific reproduction and are present in sexually mature adults. Similarly, sexually selected exaggerated morphologies, such as stag beetle horns, the colorful feathers of peacocks, or the antlers of male deer, are only fully formed at adult stages. At different stages of the life cycle, different sensory organs may be present to perceive environmental information. The visual system in mammals, for instance, is only necessary and functional at the adult stage. In contrast, holometabolous insects have different requirements for the perception of visual cues; the eyes are usually small during larval stages, while the adults of flying insects can develop highly sophisticated and large compound eyes. Similarly, functional properties of the olfactory or chemosensory system vary clearly with the life style of insects at different developmental stages. Some structures that show a large morphological variety, such as insect extraembryonic membranes play only transiently important roles during embryonic development. In contrast to animals, plants usually grow throughout their life and they are exposed to environmental changes throughout that period.

Depending on the stage and morphological trait to study, different methods and tools may be necessary to capture and digitize them. For very small structures, high magnifications are necessary, while larger structures are easier to image and compare using conventional microscopy. For internal structures, one may need noninvasive methods such as computed tomography (CT) imaging. And for morphological traits that are only transiently present, it would be advantageous to have access to noninvasive life imaging methods in combination with fluorescent live dyes or transgenic organisms. Depending on the desired resolution of the trait under investigation, it may be necessary to reach tissue or single-cell resolution based on histological sections or transmission electron microscopy (TEM). Once the trait of

interest is captured, the most important decision is whether a qualitative assessment is sufficient for a proper comparison or whether quantitative differences should be evaluated. For the latter, plenty of mathematical and statistical methods, such as geometric morphometrics, to compare shape differences across species or populations have been established and improved in the recent years (see the chapter on “► [Morphometrics and Evo-Devo](#)”).

Another important readout of the genotype of a given organism is the spatial and temporal expression of developmental genes. Those gene products act in gene regulatory networks to direct developmental processes such as cell growth and proliferation, tissue patterning, and morphogenesis, and their expression needs to be tightly controlled at any time point. Key conceptual achievements of evo-devo research are based on the comparison of gene expression and gene sequences. The availability of molecular and genetic methods established in a few model organisms such as the fruit fly *Drosophila melanogaster*, the nematode *Caenorhabditis elegans*, or the mouse *Mus musculus* provided a substantial basis for the revival of evo-devo research in the late twentieth century.

Note that the development of an organism also influences, for instance, its behavior, life history, and physiology – three important phenotypes that also vary because of natural variation in developmental processes. Although the integration of these disciplines will inevitably contribute to exciting advances in the field, I will not discuss these aspects in this chapter.

In this chapter, I will first summarize major achievements obtained using thorough qualitative phenotyping. Afterwards, I will discuss advantages and applications of quantitative phenotyping methods. Gene expression as intermediate readout of the genotype-phenotype map will be briefly discussed, and the advantages of mathematical modelling in evo-devo will be highlighted. Eventually, I will provide some practical considerations aiming to foster the integration of a thorough experimental design.

Qualitative Phenotyping Is Prevalent in Macro-Evo-Devo Studies

Classical research questions in evo-devo dwell around the understanding of how different or similar body plans evolved. To understand the mechanisms underlying body plan changes, it is very often sufficient to study the entire organism by qualitative means. For instance, the groundbreaking identification of the highly conserved HOX cluster was possible by qualitatively comparing the impact of alterations in HOX gene expression on the body plan (see the chapter on “► [Hox Genes](#)”). The observation that some arthropod groups lack posterior body regions has been associated with the loss of posterior HOX genes in mites and tardigrades (Smith et al. 2016 and references therein). The homeotic nature of this gene family could, for instance, be observed by the qualitative comparison of different types of appendages that grow in different arthropod segments (Hughes and Kaufman 2002). In vertebrates, the regionalization of the vertebral column is also defined by the action of HOX genes. Recently, the region-specific vertebral morphology has been

quantitatively described and related to the activity of region-specific HOX protein activity in extant species. The identification of these region-specific unique features in extinct species from the fossil record allowed for the reconstruction of the evolution of regional patterning in fossils (Böhmer et al. 2015). Gene products with homeotic functions have also been shown to be involved in basic plant development. The MADS box genes are expressed in a combinatorial manner to define flower morphology. Loss of MADS box gene function has been implicated in different naturally occurring flower morphologies, which are easily identified by qualitative assessments (see the chapter on “► [Evolution of Floral Organ Identity](#)”).

Other traits that are easily accessible by qualitative measurements are gain and loss of structures. Prominent examples are pelvic skeleton structures in fresh and salt water stickleback fish that have been shown to be key adaptations to environmental changes, for example, the presence or absence of predators. The reduction or complete loss of eyes and body pigmentation in cave animals such as fish, crustaceans (Protas and Jeffery 2012) represents another qualitative trait variation. The loss of eyes in different fish lineages has been extensively studied and the underlying developmental mechanisms seem to be variable in different fish groups (Menuet et al. 2007; Stemmer et al. 2015). Body pigmentation is an adaptive trait that varies significantly in different animal groups. For instance, the evolution of the colorful wings of butterflies fascinated researchers for a long time. Research on the developmental basis of wing spot development and evolution has contributed to the formulation of central concepts in evo-devo (see the chapters on “► [Butterfly Wing Eyespots](#)” and “► [Evo-Devo of Butterfly Wing Patterns](#)”). Among them is the observation that redeployment of certain developmental genes and subnetworks is a reoccurring method to increase functional complexity, while the number of developmental gene products encoded for most organisms is rather limited (i.e., toolkit). This has been shown for the involvement of the homeobox genes *Distal-less* (*Dll*) and *optix/six3* in wing spot formation. A similar observation was obtained for wing pigmentation spots in different *Drosophila* species that involve the redeployment of the *Dll* gene during pupal wing development to initiate the pigmentation cascade.

Besides adult traits, numerous morphological structures that are only transiently present have been studied applying qualitative methods. Prominent examples are extraembryonic membranes (i.e., the amnion and serosa) in insects. These membranes have been implicated in immune response (Jacobs et al. 2014), and they are relevant to support coordinated movement of embryonic tissue. Intriguingly, the size and morphology of these two membranes is highly variable among different insects ranging from clearly distinguishable amnion and serosa in beetles such as *Tribolium castaneum* to highly reduced amnioserosa in *Drosophila* and other higher Diptera (Panfilio 2008).

In summary, the qualitative analysis of morphological traits provides a powerful methodological framework to study the evolution of developmental programs in plants and animals and thus obtain valuable insights into phenotypic evolution. It is obvious, though, that qualitative measures are restricted to traits that are easily accessible and differ clearly between studied species or taxa. Therefore, these approaches are mainly applied if macroevolutionary events are studied.

Quantitative Phenotyping Is Prevalent in Micro-Evo-Devo Studies

While morphological variation on a macroevolutionary scale is in many cases accessible by qualitative means, small-scale changes in morphology between closely related species (micro-evo-devo, *sensu lato*) or even across populations of the same species (micro-evo-devo, *sensu stricto*; Nunes et al. 2013) can only be identified unequivocally by applying quantitative methods.

The identification of morphological differences among genetically closely related (i.e., less diverged) organisms very often provides the opportunity to establish genotype-phenotype associations using quantitative genetics approaches (see the chapters on “► [Genotype-Phenotype Map](#)” and “► [Quantitative Genetics and Evo-Devo](#)”). This combination already resulted in valuable insights into the developmental basis of variation in traits such as trichome number across various *Drosophila* species and populations (Stern and Frankel 2013). The accumulation of studies aiming at identifying causative genetic differences responsible for trichome number variation already allowed to draw general conclusions about the architecture of the developmental gene regulatory networks underlying trait formation. Most importantly, all identified loci converge in or are connected to the transcription factor Shaven baby (Svb) that seems to be a central hub in the underlying gene regulatory network. This finding was used to infer some level of predictability for genotype-phenotype associations (Stern and Orgogozo 2009). Recently, this view has been challenged by new data showing that trichome formation and evolution strongly depends on the developmental context and the architecture of the underlying gene regulatory network (Kittelman et al. 2017), further highlighting the importance of a combination of developmental and quantitative genetics studies.

Similar general observations were obtained for pigmentation variation expressed as wing spots or body coloration. Intriguingly, quantitative mapping approaches repeatedly identified similar loci responsible for the observed variation in wing or body pigmentation in different *Drosophila* (Massey and Wittkopp 2016) and vertebrate (Hoekstra 2006) lineages. A more detailed analysis of the enzymatic pathway leading to the production of black pigment in *Drosophila* showed that the cascade is linear and rather simple and thus highly constrained. In the light of this knowledge, it is not surprising that only a limited number of evolutionary targets have been identified over the years and some level of predictability may be indeed possible. However, it remains to be established whether similar trends will be observed for different life stages or in different insect lineages.

Although the genetic architecture of traits like trichome number or pigmentation intensities may be highly complex with various loci interacting to shape the phenotypic outcome during development, the quantification of the morphological feature remains rather simple. But what about complex morphological traits such as leaf and flower shape in plants (Vuolo et al. 2016), eye size and shape (Posnien et al. 2012), or exaggerated weapons in insects (Lavine et al. 2015), or skull shape in mice (Pallares et al. 2015)? To quantify morphological differences, much more sophisticated mathematical and statistical approaches are necessary. Especially the analysis of size and shape of complex organs has attracted much attention in the last 20 years

since the advent of geometric morphometrics (see the chapter on “► [Morphometrics and Evo-Devo](#)”). More and more mathematical approaches are being established to quantitatively compare complex morphological features and most importantly to provide a solid statistical framework for these comparisons. Advanced noninvasive imaging technologies such as computed tomography (CT), even for small individuals, starts to allow for semi-high throughput imaging of complex morphological traits in three-dimensional space (see the chapter on “► [Image Bioinformatics and Digital Embryo](#)”). Such imaging methods also provide new opportunities to capture high-resolution 3D information for fossils (Böhmer et al. 2015). Since the mathematical framework has been adopted to quantify and compare 3D image information, geometric morphometrics in combination with new imaging technology provides a fantastic opportunity to study complex trait evolution on a hitherto impossible detailed level.

Additionally, geometric morphometrics does not only provide the background to quantify differences in morphology, but it also allows visualizing variations in shape in even complex structures among groups. Another major advantage of geometric morphometric methodology is the fact that size and shape of a given organism or organ can be studied independently. This may allow to disentangle the developmental basis of shape differences related to overall growth regulation and those related to differential patterning processes or compartment-specific growth (see the chapter on “► [Morphometrics and Evo-Devo](#)”).

The combination of shape quantification with genetic information has a great potential to reveal the genetic architecture of divergence in complex traits. For instance, a thorough combination of genetic information and shape analyses allowed to establish a link between genetic variation, developmental plasticity, and sexual shape dimorphisms in wings (Testa and Dworkin 2016) in *Drosophila*. Similarly, the integration of quantitative mapping with three-dimensional shape analyses revealed candidate loci responsible for craniofacial shape variation in outbred mice (Pallares et al. 2015).

Once candidate loci are identified, a thorough functional characterization of the respective gene products allows to decipher the developmental basis underlying the evolution of shape differences. This latter aspect has been restricted to a few well-established model organisms such as mouse, *Drosophila*, or *Caenorhabditis elegans* until reverse genetics tools such as RNA interference (RNAi) and very recently the broadly functional genome editing method based on CRISPR/Cas9 started to change this picture. These days, functional manipulations to test and validate candidate genes are open to a much wider array of systems. The availability of these tools even allows, in the long run, to test the functional relevance of a given allelic variant in natural or seminatural conditions to include environmental and ecological information to assess the fitness-related aspects.

Gene Expression as Intermediate Phenotype

While purely morphological comparisons dominated the highly fruitful early years of comparative developmental biology, the resurrection of the discipline as evo-devo was triggered by advances in molecular biology. Active genomic loci are tissue- and time-specifically transcribed and translated into the functional proteins that control developmental processes. Therefore, the detection of gene expression throughout embryonic and postembryonic development provides an excellent readout of the genotype. For instance, transcription factors are fundamental DNA-binding proteins necessary to control the activity of many other genes, while structural proteins are integral parts of the cells of a given tissue. Hence, identifying the presence of a given gene product at a given time point in a given cell provides a powerful way to link the genotype and the resulting phenotype.

In the early days, molecular and genetics tools were primarily limited to a few prime model systems, such as *Drosophila melanogaster*, *Caenorhabditis elegans* and mouse. However, the establishment of cross-species whole mount in situ hybridization protocols in 1989 (Tautz and Pfeifle 1989) fueled the transfer of this method to plenty of emerging model systems. Since that time, gene expression has been extensively studied qualitatively by comparing expression patterns based on whole mount in situ hybridizations and in more rare cases on the protein level using immunohistological methods. Famous examples of the latter method were the use of cross-reacting antibodies against Engrailed (En) (Patel et al. 1989) and Distal-less (Dll) (Panganiban et al. 1997) in a variety of animals that resulted in the unexpected finding that the spatial distribution of the proteins was highly conserved in all studied species. This and many other studies led to the identification of a central finding in evo-devo research, namely the observation that the development of all living organisms is regulated by a limited set of genes, the so called developmental toolkit (Carroll 2008). Research in evo-devo dwells around this central dogma and tries to either use this toolkit to draw conclusions about organ, tissue, or even cell homologies or to understand, how the astonishing morphological diversity present on earth evolved based on a limited set of toolkit genes. Hence, the qualitative assessment of spatial and temporal gene expression has been and still is a central instrument of evo-devo to develop new hypotheses. For instance, a comprehensive expression study in different stages of the life cycle in the sea anemone *Nematostella vectensis* allowed developing a new hypothesis about the evolution of the three germ layers present in Bilateria. Until recently, it was accepted textbook knowledge that the Mesendoderm and the Ectoderm of Cnidaria gave rise to the three bilaterian germ layers, ectoderm, mesoderm, and endoderm. The authors, however, found astonishing evidence for the presence of all three germ layers already in Cnidaria (Steinmetz et al. 2017).

The advent of next generation sequencing technologies and dropping sequencing costs in the last decade helped lifting gene expression studies to the genome wide level. These days, genomic resources can easily be generated, massively increasing the accessibility of more and more non-model systems for comparative molecular studies. For instance, various large-scale consortia aim at providing genomic data for

a broad taxon spectrum to support comparative genomics analyses and the reconstruction of phylogenetic relationships. A solid phylogenetic framework is crucial for evo-devo research to allow a proper reconstruction of developmental processes on a macroevolutionary scale (Telford et al. 2015). Additionally, the availability of genomic information will become highly valuable for various evo-devo questions; for instance, aiming at understanding the epigenetic underpinnings of developmental plasticity. Genome-wide expression comparisons are already broadly used to either answer specific questions or to facilitate the establishment of new hypotheses. For instance, the classical hourglass model based on comparative embryology observations has recently been revived and supported by gene expression studies in animals and plants (see the chapter on “► [The Hourglass Model](#)”). Additionally, RNA sequencing helps reconstructing developmental gene regulatory networks on a more system wide level (Thompson et al. 2015). The generation of genome-wide expression profiles on the single-cell level will inevitably foster more in-depth analyses of the molecular fingerprint of various cell types (see the chapter on “► [Evo-Devo of Cell Types](#)”) and a highly detailed understanding of developmental processes (Karaïskos et al. 2017).

Besides advances in nucleotide sequencing, better protocols for fluorescent in situ hybridizations, even to the single-molecule level, and protein detection methods in combination with major innovations in microscopy technologies (see the chapter on “► [Image Bioinformatics and Digital Embryo](#)”) significantly improved the imaging resolution to quantitatively study temporal and spatial gene expression. This expression data can be used to feed mathematical models (Sharpe 2017) (see chapters on “► [Modelling Evolution of Developmental Gene Regulatory Networks](#)” and “► [Computational Approaches in Evo-Devo](#)”) to infer predictions that can be tested in the lab. This way, longstanding questions in Evo-Devo can be addressed with new methodology. It is, for instance, long known that segments are added sequentially in short germ insects and in other arthropods such as spiders and myriapods. This process is based on a gene expression oscillator mechanism reminiscent of that known in vertebrate somatogenesis (see the chapter on “► [The Evolution and Development of Segmented Body Plans](#)”). Only a combination of thorough gene expression quantification with reverse modelling recently revealed that oscillatory segmentation gene expression does also exist in the long germ model system *Drosophila*, lending further support for the conservation of segmentation mechanisms in animals (Verd et al. 2018) (see chapter on “► [Modelling Evolution of Developmental Gene Regulatory Networks](#)”).

While we can learn a lot from purely studying gene expression and function, it should be the ultimate goal to understand in detail how gene expression influences developmental processes. Mathematical modelling approaches integrating observed or predicted gene expression dynamics and cell and tissue behaviors have a great potential to sufficiently link the genomic architecture with developmental processes. Such models can subsequently also be used to implement an evolutionary scale to reconstruct the history of developmental changes on a mechanistic level. First results are already highly encouraging, for instance, the understanding of the evolutionary forces that shape tooth morphology in seals (Salazar-Ciudad and Jernvall 2010).

Practical Considerations

While technological innovations seem to proceed with high speed, and new methods to assess organismal phenotypes pop up nearly every week, one should not forget about some central limitations in their application.

Since phenotypes are studied in living organisms, it is of major importance to obtain basic knowledge about the ecology and life cycle of the animal or plant in question. For instance, for some applications, it may be relevant to rear the organisms in laboratory conditions to have access to different life stages throughout the year, or organisms that have extremely long development times may be less well suited for in-depth phenotyping during development. While new genome editing technologies lower the obstacles of functional perturbations, one should not underestimate the time needed to transfer existing protocols from other species to a new one. Ironically, although the time needed to establish a new phenotyping method for a new model system is often a limiting factor, this information is rarely accounted for in the final publications summarizing the results.

Another focus of attention should be the amount of phenotypic data generated. Recent imaging and sequencing technologies result in massive amounts of data that needs to be stored, transferred, and handled. For most of these new applications, no easy-to-use analysis software is available since the analysis of such data requires special algorithms and analytical frameworks that are still under active development. One should thus be prepared to have access to high-performance computer clusters, and programming knowledge will become more and more relevant for biologists handling and especially integrating phenotyping data. Eventually, to be able to follow the rules of good scientific practice, it gets more and more relevant to share the large datasets in public databases and repositories to ensure on one side the reproducibility of the performed analyses and on the other side to allow the analysis of the data in another context or the integration in future meta-analyses.

As part of the experimental design and project planning, one should take special care about the actual phenotype to be studied. This applies specifically to the analysis of complex morphological traits. For instance, if one wants to understand the molecular and developmental basis underlying compound eye size variation in insects, it may be informative to measure and compare the area of the entire eye. However, since compound eyes are composed of functional subunits, called ommatidia, one should rather ask, whether different insects show differences in the number or the size of the individual ommatidia. This data may be much more informative for the establishment of a hypothesis about the underlying developmental differences. And what about the shape of the eye? Are different insect compound eyes always oval, or do different eyes show different proportions along different body axes? Answers to these questions may be helpful in revealing differences in patterning processes during development. Hence, depending on the exact research question, the right phenotyping method should be applied.

In summary, we are living in exciting times with various new methodologies on the horizon to study the evolution of developmental processes in great detail. However, especially with the increase in the amount of data that can be generated,

it is getting increasingly important to invest time in proper experimental design and extensive sharing of the data.

Cross-References

- ▶ [Butterfly Wing Eyespots](#)
- ▶ [Computational Approaches in Evo-Devo](#)
- ▶ [Evo-Devo of Butterfly Wing Patterns](#)
- ▶ [Evo-Devo of Cell Types](#)
- ▶ [Evolution of Floral Organ Identity](#)
- ▶ [Genotype-Phenotype Map](#)
- ▶ [Hox Genes](#)
- ▶ [Image Bioinformatics and Digital Embryo](#)
- ▶ [Modelling Evolution of Developmental Gene Regulatory Networks](#)
- ▶ [Morphometrics and Evo-Devo](#)
- ▶ [Quantitative Genetics and Evo-Devo](#)
- ▶ [The Evolution and Development of Segmented Body Plans](#)
- ▶ [The Hourglass Model](#)

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