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Doctoral Thesis

Environmental influence on the phenotype:

morphological variation in human dentition

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"Unanimity of opinion may be fitting for a rigid church, for the frightened or greedy victims of some (ancient, or modern) myth, or for the weak and willing followers of some tyrant. Variety of opinion is necessary for objective knowledge."

Feyerabend (1975, pp. 31-32)

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Abstract

Doctor of Philosophy

Environmental influence on the phenotype: morphological variation in human dentition

by Alessandro Riga

This work is about the role that environment plays, through development, in the production of evolutionary significant variations. It is structured in 5 chapters, and it starts with an historical introduction about two topics: the concept of variation and the role of environment in its production. From a multiplicity of conceptions in the 19^{th} century, there is a dramatic bottleneck between 1930s and 1970s, and then a return to a wider view. In Chapter 2, I show how a lack of attention to the concept of variation may lead, in anthropological research, to serious mistakes in the interpretation of the data. A statistical re-analysis of published data on the effects of malnutrition on dental eruption, shows that what has been interpreted as an increase in the mean value, actually is linked to an increase of variability in an asymmetric distribution with a left wall. In Chapter 3 I present the topic of development as a link between variability and environmental influence. Moreover, I give a review of the possible mechanisms, by which development (and, by the mean of development, environment) may influence evolutionary dynamics. Chapter 4 is the core chapter of the thesis; studying the crania from the Frassetto collection, I tried to understand the role of environment in the development of dental morphology. I used dental hypoplasia as a marker of stress and dental development disruption. An accurate study of its presence in the studied population, allowed to characterize two groups with different stress levels. Then, I studied dental morphology in the upper molars, using the ASU-DAS (Arizona State University - Dental Anthropology System) standard plaques. Comparing the two groups, three major results came out: (i) there is a significant effect of environmental stressors like malnutrition and/or systemic diseases on the overall morphology of upper molars; (ii) the stressor generates a developmental response which increases the morphological variability of the stressed population; (iii) the increase of variability is directional, since stressed individuals have increased cusps dimensions and number. In the discussion section, I also hypothesized the molecular mechanisms

that could be responsible of the observed effects. Finally, in Chapter 5, I present future perspectives for developing this research. In particular, I noted that the direction of dental development response to stress is in the same direction of the trend in mammalian dental evolution. Since malnutrition triggers the developmental response, and this particular kind of stressor must have been very common in our class evolutionary history, I propose the possibility that environmental stress actively influenced mammals evolution, and I recall some of the historical concepts presented in Chapter 1. Moreover, I discuss the possibility of reconsidering the role of natural selection in the evolution of dental morphology.

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Chapter 1

Variation and Environment before the Modern Synthesis

"No single theory ever agrees with all the known facts in its domain [...]
Facts are constituted by older ideologies, and a clash between facts and theories may be proof of progress"
Feyerabend (1975, p. 39)

1.1 The progress of science: an example

To stand against the clash between theory and facts, scientists often conceal the existing troubles "by *ad hoc* hypothesis, *ad hoc* approximations and other procedures" (Feyerabend 1975, p. 49). In my opinion, the greatest example in biological science comes from Darwin's theory of natural selection (Darwin 1859). His theory predicted continuity among fossil forms, while all the evidences coming from palaeontology strongly suggested a pattern of stasis and abrupt change. Darwin was aware of this danger: "Why [...] is not every geological formation and every stratum full of such intermediate links? Geology assuredly does not reveal any such finely graduated organic chain; and this, perhaps, is the most obvious and gravest objection which can be urged against my theory" (Darwin 1859). He supported his theory thanks to the introduction of an *ad hoc* hypothesis: "The explanation lies, as I believe, in the extreme imperfection of the geological record". In other words, Darwin maintained a refuted theory by the mean of a hypothesis not supported by facts.
One of the effect of a procedure like the one described above is that the theory can be maintained and can have time to collect supporting data and change the mind-set in which researchers work: indeed Darwin's theory of natural selection

survived [1] and proliferated with a lot of positive (from an evolutionary biologist point of view) results, although a Popper of the 19th century would have rejected it. Another effect of this procedure is that the hypothesis introduced, making available new data, also hides others. After a long time its "*ad hocness*" can be forgotten and the unsupported hypothesis become part of the "natural interpretations" of the new conceptual framework.

"Natural interpretations" are "ideas so closely connected with observations that it needs a special effort to realize their existence and to determine their content" (Feyerabend 1975, p.54). In the case described above, the natural interpretations of the palaeontologists of the 20th century, working in the framework of phyletic gradualism, was to interpret morphological stasis as a gap in the fossil record. When Eldredge and Gould (1972) proposed punctuated equilibria they made the "special effort" needed to discover the natural interpretation and, through a new theory, they changed the expectations of palaeontologists. Now morphological stasis represents available data.

In this section we have seen two examples of how science can make progress. In the first one, Darwin used "counterinduction" to sustain his theory against the known facts; in the second one, Eldredge and Gould discovered a "natural interpretation" changing the perception of the facts. Both these methods (and many others) are equally valid and one is not better than the other. A proliferation of theories is beneficial to science, no matter if those theories, or the methodology by which they are supported, might seem to be irrational. More than irrational methods and non-orthodox theories, a scientist should be frightened by the uniformity of opinion. Indeed, "*unanimity of opinion may be fitting for a rigid church, for the frightened or greedy victims of some (ancient, or modern) myth, or for the weak and willing followers of some tyrant. Variety of opinion is necessary for objective knowledge*" (Feyerabend 1975, pp. 31-32).

1.2 Pensée unique

Single thought, or *pensée unique* in its original French, is a term used to describe the hegemony in today's economic world of neoliberistic ideology. The single thought is the "translation in ideological terms, claiming to be universal, of the

[1] I use the word "survive" because, between the 19th and 20th centuries, very few researchers accepted natural selection as the cause of evolution. Among them Alfred Russel Wallace, the co-author of Natural Selection Theory (Darwin and Wallace 1858) and August Weissman, who carried to the extreme the logic of natural selection as the only evolutionary force responsible for evolution (Weissman 1893). The greatest part of evolutionists, however rejected Darwin's hypothesis and preferred explanations based on different mechanisms (cfr Chap 1).

interests of a set of economic forces, and specifically those of international capital" (Ramonet 1995).

Comparing to the variety of ideas and evolutionary mechanisms admitted before the '30s, the neo-Darwinian orthodoxy called Modern Synthesis, seems to deserve the label of *pensée unique*. Also, many authors evidenced and criticized ideological aspects of the Modern Synthesis, which claimed to be universal, for example: genetic determinism (Gould 1981; Lewontin 1991); gene-centric view and evolution as change in allele frequency (Eldredge 1985; Gould 2002, Jablonka and Lamb 2005); development as a genetic program (Kauffman 1993; West-Eberhard 2003); natural selection as the only evolutionary force (Gould and Lewontin 1979; Kimura 1983).

With the birth of the Modern Synthesis alternatives to the neo-Darwinian view of variability and environment have been strongly rejected. In particular, "in the three major branches of science that were the foundation of the modern synthesis - genetics, systematics, and palaeontology - [the sensitiveness of the phenotype to environment] has been an intractable nuisance, rather than a subject of productive interest" (West-Eberhard 2003, p. 4).

Ernst Mayr, wrote: "In the short run, it was perhaps the refutation of a number of misconceptions that had the greatest impact on evolutionary biology. This include soft inheritance [Lamarckism], saltationism, evolutionary essentialism [with reference to the first Mendelians], and autogenetic theories [orthogenism]" (Mayr 1982, p. 570). With these words, one of the fathers of the Modern Synthesis shows how the Synthesis was born by drastically reducing the number of possible evolutionary explanations. Can this be described as a progress for biological science?

Science is a human product, which have a history, and its development often follows the main rule of the history: contingency. The separation between the history of a science, its philosophy and the science itself it's deleterious to the "progress" of science (Feyerabend 1975). All the ideas and the weird theories coming from the history of a subject should be "utilized in the attempt to improve its most recent and most 'advanced' stage"(p.33).

The two themes of my thesis, variation and environment, both had recent developments, which have been possible also thanks to the re-integration of old theories. Therefore, in this chapter I want to give an historical overview of these two themes.

In a neo-Darwinian perspective variability is an immanent characteristic of species. Variation between individuals allows evolution; without differences between individuals there would be no evolution. The role of the environment is to apply selective pressures to a population to make natural selection working. But it wasn't always like this. Indeed, before neo-Darwinism spread, in the evolutionary theory

there were a huge variety of interpretations both about the concept of variation and about the role of environment in shaping the organic world.

1.3 Different concepts of variation

Mayr (1959) emphasized the epistemological fracture on the concept of species realized by Darwin; naturalistic philosophies before Darwin had an essentialist concept of species, coming straight from Plato, through Aristotelian logic (Mayr 1982). Darwin overturned this view and developed what Mayr calls a populational concept of species; in this new mind-set, normal variation in a population becomes central to the new natural philosophy.
However, some authors do not agree with this view and suggest a revision of the topic because Mayr's words "have carried such authority that his claims have been rarely questioned" (Winsor 2006, p.3). The opposition of essentialism and populationism tends to oversimplify the complexity of all the different views about variation in natural history. For this reason, instead of putting altogether the non-Darwinian schools in contrast with Darwin's ideas, I briefly present separately the different concepts of variation of the 19^{th} and 20^{th} century.

1.3.1 Variation in Darwinian logic

Variability has been the central topic in Darwin's one long argument. In the 6_{th} edition of the "Origin" (Darwin 1872) three chapters are dedicated to the understanding of variation: *Variation Under Domestication* (chap. 1), *Variation Under Nature* (chap. 2), *Laws of Variation* (chap. 5). This theme is then extended in "The Variation of Animal and Plants Under Domestication" (Darwin, 1868) where Darwin also presented his original theory of pangenesis in the attempt of explaining his observations on variation and inheritance.
Darwin's attention on this topic becomes clear when we consider that, in his theory, Darwin introduced a view of variation, which was in open contradiction with all the other views prevailing between the 19^{th} and the 20^{th} century.
In the *Origin* (1859), Darwin proposed two different theories:

- the common origin of all the living beings
- natural selection as the most important evolutionary force.

While the first point had a great success and the most of the authors after 1859 embraced the idea of evolution, the second point didn't achieve the same destiny. Indeed very few authors accepted natural selection as a creative force in evolution,

whereas most of them preferred other explanations.
To make the natural selection effective as a creative force, heritable variation must possess certain characteristics (Gould 2002). It must be *abundant*, otherwise the raw material for natural selection lacks; it must be of *minor entity*: variation in the order of magnitude of a macro-mutation deprives natural selection of its creative power and it will become a negative force which just remove the non-adapted new forms. Finally, heritable variation must be *isotropic*, occurring with the same probability in all directions, where else, the direction of evolution wouldn't be determined from natural selection, but from some internal factor.
In Darwinian logic the population is the place where evolution occurs. Normal heritable variation in a population is the raw material on which natural selection acts.

1.3.2 Variation in pre-Darwinian formalism

In Germany, the first half of 19$^{\text{th}}$ century was dominated by the *Naturphilosophie*, which was an idealistic and romantic conception of nature. Nature was a manifestation of the Absolute, a vital and divine principle, which can be known through intuition (Cioffi et al. 1998); "the most important result of a rational inquiry into nature is, therefore, to establish the unity and harmony of this stupendous mass of force and matter, to determine with impartial justice what is due to the discoveries of the past and those of the present, and analyze the individual parts of natural phenomena without succumbing beneath the weight of the whole" (Humboldt 1858).
With this aim, Goethe (1749-1832) tried to lead back all the variation in the organic world to a few archetypal forms; these archetypal forms were the symbols of the unity of nature. In his botanical studies, Goethe proposed the leaf as the archetypical form of all the parts of a plant. Each part of a plant is a deviation from the metaphysical archetype represented at best by the leaves. The archetype is not the same than Plato's Ideas, in that the latter represents the perfect form underneath an imperfect representation, while the former is a totipotential principle which may give birth to all the variation observed (the leaf is the less specialized part of a plant, from which petals, sepals, and the other part of the plant can be derived). In the same way, in its zoological works, Goethe proposed the archetypal vertebra as the basic unit, from which the entire vertebrate skeleton can be derived (Fig.1.1).

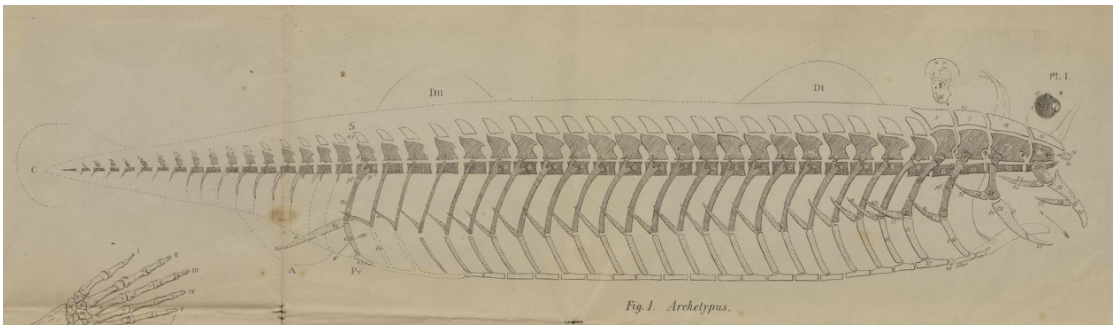

FIGURE 1.1: The archetypal vertebrate for Owen (1849). The skeleton of all the vertebrates is derived by modification of each segment.

1.3.3 Variation for Natural Theologians

In the same time, in England a few authors agreed to the formalist view[2]; the most of them agreed to a functionalist philosophy, like the Natural theologians. For them, nature reflects the plan of God in all the perfect adaptation of each species to its habitat. This philosophy was strongly influenced by Plato: species represents the plan of God as long as they resemble to God's Idea of that species. Species, in their distinctiveness, were representative of the platonic ideal Form and each individual was an imperfect copy, a misrepresentation of the platonic Idea of its species. This essentialist mind-set is resumed best in the First Principle of McCosh and Dickie (1857): these two natural theologians assess that one of the principles which run through the structure of the cosmos is "the Principle of Order, or a General Plan, Pattern or Type, to which every given object is made to conform with more or less precision"(p. 1).
Hence, variability is random variation around a mean, which represents the essence of the species because it is what more resembles the ideal Form. Variation was nothing more than a background noise hiding the true essence of a species.

1.3.4 Variation in orthogenesis

One of the most popular alternatives to Darwinism was orthogenesis, which developed in the second half of the 19th century; orthogenesis is the principle for which variation cannot move freely in every direction (the third requirement of Darwinian variation - *isotropy*), but it is restrained to follow the direction imposed by development. Therefore the causes of evolution are internal to the individuals and natural selection has no role in evolutionary dynamics. This scientific trend was born with Haeckel's theory of recapitulation and has its philosophical ancestors in

[2]One of those authors was Richard Owen (1804-1892) who developed Goethe's theory on the vertebra as the archetype for the vertebrate skeleton.

the German *Naturphilosophist*.

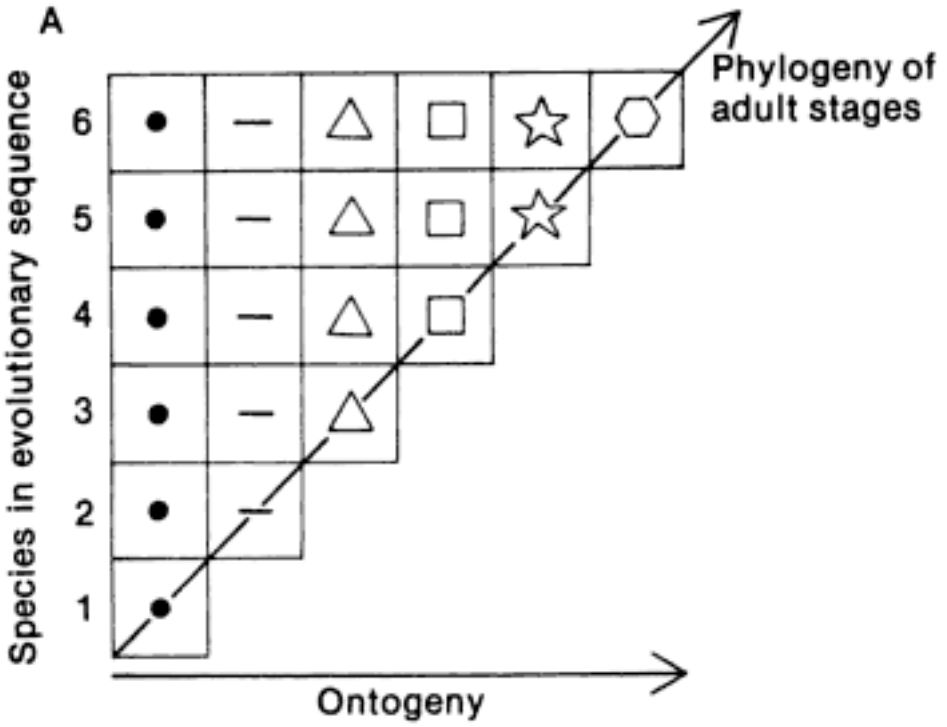

FIGURE 1.2: In this picture Gould represents Haeckel's principle of the addition of a trait to the end of the ontogenetic process. More evolved species (upper rows) during their ontogeny pass through the adult stage of their ancestors (from Gould 1977, p. 75).

Haeckel (1834-1919) believed that new species arise by addition of new characters at the end of individual ontogenesis (Fig.1.2). Evolution is not random variation and selection of the best trait in a contingent environment, but it is a great chain of beings where the most evolved species recapitulate their phylogeny in their ontogeny. A human being during his ontogeny pass through the adult stage of a fish, than of a reptile, of a mammal and, only at the end of his ontogeny, he gains the specific characteristics of humans. For this reason, evolution is a finalistic process, which tends towards the most evolved form, the man. However, not all the orthogenetic theories had a finalistic interpretation of evolution.

Orthogenesis survived also in some theories of the 20th century such as the fetalization theory proposed by Bolk (1926) in his studies on human evolution. He thought that natural selection couldn't explain several human traits such as the long intrauterine permanence, the shape of the cranium and the scarce body hair,

which could be explained by a delay in development with respect to the other apes. For a complete panoramic of the very interesting history of orthogenesis, see Gould (1977, 2002).

1.3.5 Variation for saltationists

Saltationism is characterized by the belief that species evolve abruptly and in a discontinuous fashion. Continuous variation cannot produce a progressive evolution, because it is destined to regress towards the mean: in his studies on seeds size (and then on human height), Galton (1886) showed how "the offspring did *not* tend to resemble their parent [...] in size, but to be always more mediocre than they - to be smaller than the parents, if the parents were large; to be larger than the parensts if the parents were very small" (p. 246). Only discontinuous variation can change a species.

Saltationist ideas gained strength when De Vries rediscovered Mendel's experiments in the early 20th century. Indeed, the most of the geneticists interpreted Mendel's result as evidence of the discontinuous nature of heritable variation. If heritable variation is discontinuous, it lacks Darwin's second requirement of variation (*of minor entity*); therefore one species can origin from another only by abrupt change, consequent to a macromutation (Fig.1.3). In this framework, continuous variation is accidental and linked to environmental disturbance, without any important role in evolutionary mechanisms.

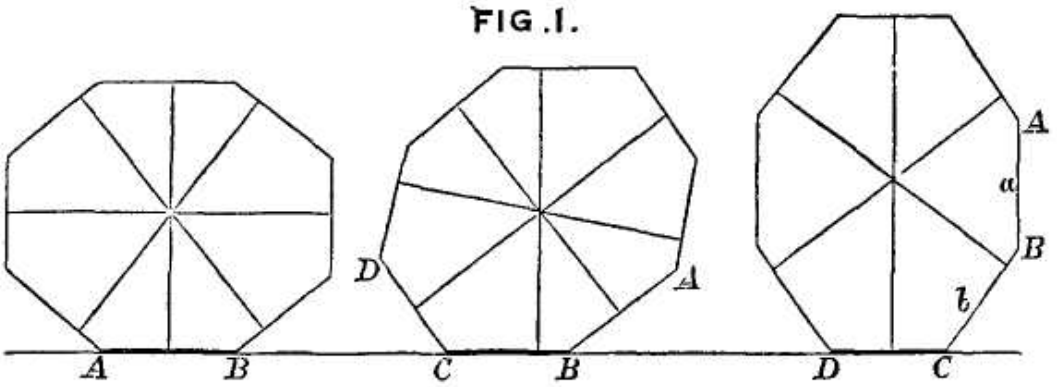

FIGURE 1.3: Galton's Polyhedron, represents the union of the theme of saltation with the internal drive for evolutionary variation. Species change is equal to face flipping in a polyhedron. Accidental variation is not important and cannot change the face where the polyhedron lies, producing an oscillation which promptly regress toward the stable condition. A macromutation, on the contrary, can flip the face. The new species arises abruptly (saltation) and depends on the form of the polyhedron itself (internal drive). from Gould (2002).

1.4 Different roles for environment

1.4.1 What is environment

Environment is a general term, which change the meaning on the basis of what it is referred to. Trying to state a general definition, environment is everything that is external to a certain object. If we refer to a population environment is the climate, the chemical component of the soil where the population lives, the composition of its diet, the species which the population interacts with. If we consider a single individual, environment is everything listed above and also its social position and its role in the society. If we manage cells or genes, then we can speak of cellular environment, with regards to the molecules in the cytoplasm, and genomic environment, referring to the gene network where a single gene operates. In the history of natural science, authors attributed very different roles to the environment in shaping living forms (Fig.1.4). Some of them opted for a total absence of environment from evolutionary dynamics, whereas others saw the environment as the main force shaping living beings; Darwin stood somewhat in the middle of these two extreme positions.

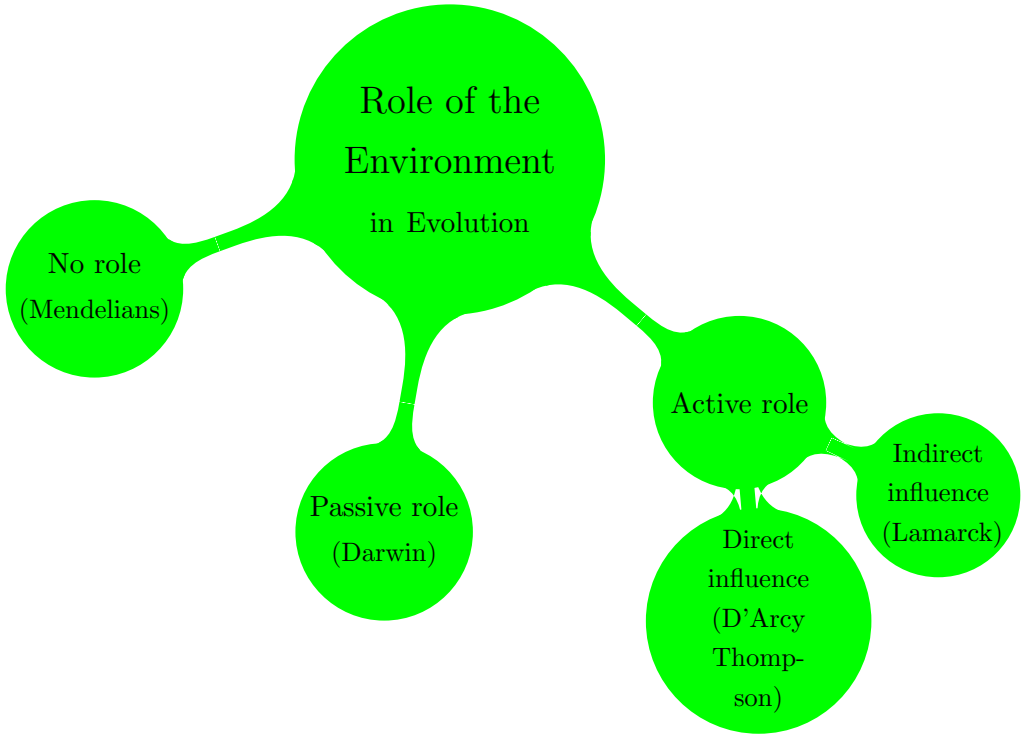

FIGURE 1.4: Outline of the different roles attributed to environment in shaping living forms in the 18^{th} and early 19^{th} centuries

1.4.2 No role for environment

Some authors attributed no role to the environment in shaping living forms. As in the example of Galton's polyhedron (Fig1.3), evolution depends on factors that are internal to the individual. For Mendelians, for example, the form that a new species would have depends on the results of a macromutation; a macromutation can occur only if it is compatible with life. Environmental variation is accidental variation and does not contribute to evolutionary dynamics; neither, environment has any role as it has for example in Darwinism.
In this framework the form predates the function, in that internal factors give birth to new forms, independently on the function they will have or on environmental conditions. Natural selection will act *a posteriori* eliminating the forms that aren't well adapted, but it has no power in shaping those forms.

1.4.3 Environment in Darwinism: a passive role

Darwin accepted a certain grade of environmental influence in evolutionary dynamics. In Chapter 5 of the Origin, Darwin dedicates a paragraph to the effects of the use and disuse combined with natural selection, where he accepts also the inheritance of acquired characteristics. However, in Darwin's thought the role of these processes is of minor importance: "Such considerations as these incline me to lay less weight on the direct action of the surrounding conditions, than on a tendency to vary, due to causes of which we are quite ignorant" (Darwin 1872, p.107).
This "tendency to vary", that is variation, is an immanent characteristic of a population and it occurs independently of the environmental conditions. In Darwinism, natural selection is the evolutionary force shaping the living world. It works every generation sorting the best variations of a population, in a contingent environment.
Environment has no role in shaping directly or indirectly variability; however it maintains an importance in that it drives the selective pressures in a population so that natural selection can work. Its role in evolutionary dynamics is passive and it doesn't shape variation nor directly or indirectly.

1.4.4 Active role for environment

The most of the scientists, until the early 20^{th} century, recognized an active role to environment in evolution (Mayr 1982). Scientists of the 19^{th} and early 20^{th} century working in this framework are often improperly called "neo-Lamarckians", even

if in certain cases the only thing they had in common with the French naturalist was the belief that acquired characters could be inherited. Neo-Lamarckians were a heterogeneous group of scientist with very different views and whereas some of them attributed an indirect role to environmental influence on variation, others believed that environment could shape directly living forms.

1.4.4.1 Indirect environmental influence

Jean-Baptiste Pierre Antoine de Monet, Chevalier de Lamarck (1744-1829) was a French naturalist, between the firsts to propose a consistent and materialistic theory of evolution (Gould 2002). His idea of evolution is characterised by two distinct processes:

- an increase of perfection and complexity along the taxonomic order and over time;
- the adaptation of each species to its environment.

The first process, often interpreted as a proof that Lamarck had a vitalistic view of evolution, is actually derived from the way Lamarck interpreted physics, in contrast with Lavoisier theories (Gould 2002). An analysis of the first factor is not pertinent to this thesis, so I won't dwell on it. As for the second factor, Lamarck thought that adaptation to the particular conditions of each species, originated through two principles:

- *use and disuse*;
- *inheritance of acquired characteristics.*

These two principles were largely accepted until the early 20th century (Mayr 1982) and also Darwin thought that use and disuse, and inheritance of acquired characteristics, could help in explaining evolutionary phenomena as the vestigial organs (Darwin 1859).
Through the combination of these two principles, evolution could produce new shapes and new species. Evolution could occur through a few steps (the example is hypothetic):

1. Environmental conditions change;
   Example: the flow of a stream pushes some larvae of a species of salamanders into an underground cave, with an underground lake with no exit.
2. The species adapt to the new conditions changing behaviour;
   Example: salamanders tend to stay in the water independently of the season because of the abundance of food and the absence of predators.

3. New behaviours push the individuals to use some organs more (and some others less) than before;
   Example: in the total absence of light eyes become useless; the same is for the limbs, while muscles involved in swimming become more important. Also, gills are needed in the whole life cycle and not only in the larval phase.

4. The organs change after the new use (what today is called plasticity);
   Example: eyes and limbs become atrophic, while the muscles of the tail become bigger and more functional. Gills are retained also in the adult phase.

5. The changed organs can be inherited by the new generation;
   Example: the offspring of this population inherit the traits their parent acquired through the use and the disuse: atrophic eyes and limbs, strong tail, permanent gills.

As showed above, environment plays an indirect role in shaping living forms. It acts on the species changing its living conditions and inducing a change in the behaviour; then the species itself adapts the form to the new function it has to fulfil.
The main difference with Darwin is that, in this case, variation is induced by a behaviour and it is not isotropic, but the change occurs in the direction of a better functionality of the trait.
Between 19th and 20th century many scientists agreed with the idea that use and disuse combined with inheritance of acquired characteristics could drive evolution. Many orthogenists, for example, used this two principles to explain how new traits could arise at the end of an ontogenetic trajectory (Gould 1977).

1.4.4.2 Direct environmental influence

Other neo-Lamarckians agreed to a different view, in which environment acted on the living beings shaping directly their forms.
The theories of D'Arcy Thompson (Thompson, 1917) represent one of the latest examples of the interest on the direct influence of environment on the forms. The main thesis of the Scottish morphologist was that physical forces shape directly the adaptive forms (Gould 2003). For example, the trabecular bone in the proximal epiphysis of the femur distributes according to the physical forces, which act on the femur; the bone is strengthened exactly on the lines where the compressive load is applied. When the physical forces change, for example subsequently to a fracture of the femur, the trabecules rearrange to obtain the most physically stable form. In D'Arcy Thompson's view, environment impose directly phyletic changes

on the organisms through physical forces; differences between related species could be explained by different physical forces acting on the different species (Fig.1.5).

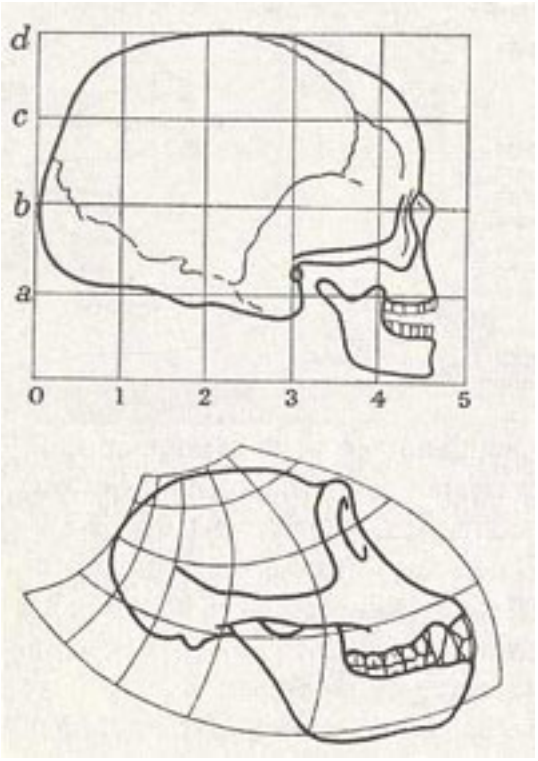

FIGURE 1.5: Deformation grids representing how physical forces differentiate human and chimpanzee skulls. Today, this kind of representation are used in geometric morphometrics to show morphological differences. From Thompson (1945).

Chapter 2

The meaning of the mean

Statistics is a useful tool when we try to face complex problems, which are difficult to deal with. Statistics helps to simplify certain features of complex entities, so that we can represent general concepts otherwise indescribable.
However, statistics can be misleading when used in a wrong or superficial way; one of the most common errors is reification: attributing a real entity to an abstract concept. Gould (1981) showed how this happened (and continues to happen) in studies on intelligence, where the correlation between multiple mental tests has been represented by a principal component, a new variable expressly created to represent as much variation as possible in one variable; this new variable doesn't exist, it is an abstraction and the identification of the principal component with a physical entity (without any other clue of causality) is a huge mistake.

> Spearman calculated such a component indirectly in 1904 and then made the cardinal invalid inference that has plagued factor analysis ever since. He reified it as an "entity" and tried to give it an unambiguous causal interpretation. He called it g, or general intelligence, and imagined that he had identified a unitary quality underlying all cognitive mental activity - a quality that could be expressed as a single number and used to rank people on a unilinear scale of intellectual worth.
> (Gould 1981, p. 281)

The problem of reification is also evident for the simplest statistics like the arithmetical mean. In this chapter I will face how a wrong use of the mean can mislead the interpretation of data in human biology.

2.1 The mean in Platonic and Darwinian worlds

If we lived in a Platonic world, the mean would be a good method to eliminate the noise of variation to reach the essence. As long as the mean of multiple measurements is useful to get nearer to the "true" measure and to erase measurement errors, so the mean between individuals would be a valid tool to erase the deviations from the essence that produce variation and to get nearer to the "true" individual, the Idea of the species. But we live in a Darwinian world and, as I showed in Chapter 1, variation has a central role in Darwinian thought. When we deal with a population it is important accounting for all the variability and a study of variability cannot be reduced to the study of the mean. The arithmetical mean is no more the approximation to the essence, but a mere abstraction; the interest of evolutionary studies from the Darwinian perspective must be focused on the variation around the mean. Focusing on the mean without considering variation around it can mislead the interpretation of the data, as we'll see in the next paragraphs.

2.2 Socio-economic conditions and dental development

Between the studies on the effects of environment on development, many focus on the effects of socio-economic condition on different aspects of human development. These studies usually compare two groups with different socio-economic levels and they are always unanimous in stating that socio-economic environment affects (positively or negatively) the mean of one group (Bjerregaard et al. 2010; Boas 1927; Enwonwu 1973; Folayan et al 2007; Garn et al. 1971; Gaur and Kumar 2012; Hatton 1955; Kapoor et al. 2010; Malina et al. 2009; Mukhopadhyay 2010; Pathak and Parashar 2010; Reddy and Papa Rao 2010; Saleemi et al. 1994; Sheppard et al. 2009; Sinha and Kapoor 2010; Stinson 2009; Tyagi and Kapoor 2010; Woodroffe 2010). In particular, studies on dental eruption show a delay of teeth eruption in malnourished groups or, more in general, in groups with low socio-economic conditions (Boas 1927; Enwonwu 1973; Folayan et al 2007; Garn et al. 1971; Gaur and Kumar 2012; Hatton 1955; Saleemi et al. 1994; Woodroffe 2010).
In all these studies the mean is considered a representative measure of the group, without any other clue suggesting that it is really so. The distribution of the variation around the mean is systematically ignored. If we take the same data as the studies cited above and consider not only the mean, but also all the variability, it is possible that we reach very different conclusions.

2.3 A new interpretation of an old study

2.3.1 Materials

The study by Enwonwu (1973) is the only one that presents data in a way that allows reorganizing them to make a new statistical analysis. In this study two groups of Nigerian children were compared for the timing of deciduous teeth eruption.

Table 2.1 shows the data relative to the timing of eruption respectively for an optimal group and a malnourished group. Individuals are subgrouped for age class (classes of 3 months from 4 to 30 months) For each class of age these variables are available: mean age, number of individuals examined, percentage of erupted teeth (and standard error) for each of the 5 deciduous teeth of the mandible and of the maxilla (both labeled with letters from A to E).

2.3.2 Methods and results

It is possible to transform the data as presented in Table 2.1 so that we can arrange them to understand better the variability of the population examined without focusing only on the average values. For a better understanding here we present only the analysis done for the mandibular deciduous tooth D. Data and results for all the other teeth are similar and are presented in Appendix A.

TABLE 2.1: Data for the optimal and malnourished groups of Nigerian children. Percent values are calculated to the nearest whole number (Enwonwu 1973).

Optimal group			Percentage of children with erupted									
			Mandibular					Maxillary				
Age (months)	Mean age (months)	Number examined	A	B	C	D	E	A	B	C	D	E
4-64	5.1	20	84	-	-	-	-	13	-	-	-	-
7-9	8.2	38	100	11	-	-	-	80	26	-	-	-
10-12	10.9	18	100	73	-	6	-	100	86	-	8	-
13-15	13.8	17	100	100	30	63	-	100	100	30	57	-
16-18	17.1	11	100	100	68	100	-	100	100	82	100	-
19-21	20.0	10	100	100	89	100	-	100	100	89	100	22
22-24	22.9	16	100	100	100	100	66	100	100	100	100	71
25-27	26.0	11	100	100	100	100	84	100	100	100	100	88
28-30	29.1	14	100	100	100	100	97	100	100	100	100	87
Malnourished group			Percentage of children with erupted									
			Mandibular					Maxillary				
Age (months)	Mean age (months)	Number examined	A	B	C	D	E	A	B	C	D	E
4-64	5.3	88	15	-	-	-	-	-	-	-	-	-
7-9	8.0	118	63	2	-	-	-	19	4	-	-	-
10-12	11.0	98	94	26	-	1	-	80	35	-	-	-
13-15	13.8	80	99	61	2	19	-	96	72	1	19	-
16-18	17.0	54	100	88	27	60	2	98	92	36	69	-
19-21	20.2	50	100	100	55	98	17	100	100	66	96	15
22-24	23.0	34	100	100	74	95	18	100	99	80	97	16
25-27	26.0	45	100	100	95	100	65	100	100	100	100	64
28-30	28.7	41	100	100	98	100	92	100	100	100	100	84

2.3.2.1 Transformation of the data

The first step is to transform the percentage values of erupted deciduous teeth in absolute values of non-erupted deciduous teeth. Let's take as example the case of the mandibular tooth D. In the optimal group, age class 13-15 has 17 children examined, 63% of which has the mandibular deciduous tooth A erupted; that is to say 6 children have non-erupted mandibular D (Table 2.2).

Age (months)	Mean age (months)	Number examined	non-erupted mandibular D
13-15	13.8	17	6

TABLE 2.2: Percentage values of erupted teeth are transformed in absolute values of non-erupted teeth.

The second step is to create a new database where the absolute frequencies of non-erupted teeth are transformed in single cases; age class and health status will be the new variables. Continuing the example above we will have 6 healthy children between 13 and 15 months old, with mean age equal to 13.8 (Table 2.3).

Id	Health status	Class age (months)	Mean age (months)
1	Healthy	13-15	13.8
2	Healthy	13-15	13.8
3	Healthy	13-15	13.8
4	Healthy	13-15	13.8
5	Healthy	13-15	13.8
6	Healthy	13-15	13.8

TABLE 2.3: Part of the new database, referring to the healthy children with non-erupted mandibular deciduous D tooth.

At the end of this process we will have a database for each deciduous tooth. These new databases show the number of children for each age class who doesn't have a certain tooth erupted.

2.3.2.2 Comparison between healthy and malnourished children

We can plot these new datasets and observe the distributions and the differences between healthy and malnourished children. Figure 2.1 show the distribution for the mandibular deciduous tooth D. Indeed, as Enwonwu (1973) stated, teeth of the malnourished group erupt later, on average, than those of the healthy group. However, if we take a look at the distributions we can understand why it is a bad idea using the mean to describe the effect of malnourishment on dental eruption.

First, we can observe that the distributions are not normal, but asymmetrical.

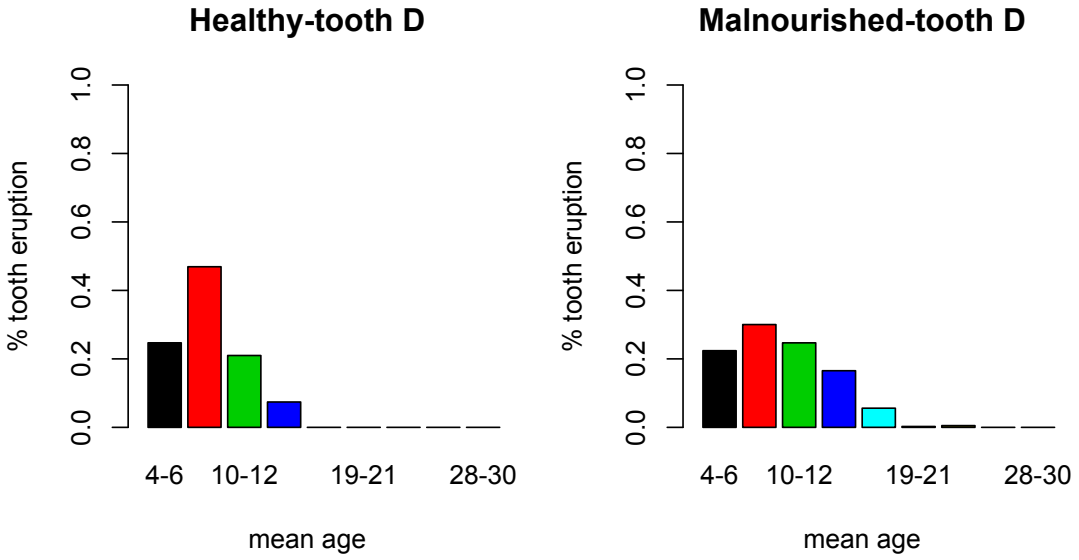

FIGURE 2.1: Comparison between healthy and malnourished children for the mandibular deciduous tooth D

They have a left wall, due to the fact that, for developmental reasons, a tooth cannot erupt before a certain age. Therefore, variation cannot occur in every direction, but only in the direction of a later eruption.

Also, the mode of the distribution is next to the left wall, and, in the case of mandibular deciduous tooth D, it is 7-9 months. A non-directional increase of variation in distributions like these causes an increase of the mean; if one looks only at the mean can be misled and could think to a directional change, whereas there is only a non-directional increase of variability. The mode is a more stable statistics and should be used for distributions like those described above. Indeed, if we look to the new dataset we obtained the mode does *never* change between in malnourished children compared to the healthy ones.

In all the plots the mode is constant and what changes is the number of age classes

represented. Malnourished children distributions have a longer right tail compared to the healthy group, that is to say they are more variable in their eruption age. The increase of the mean seems to be a side effect of an increase of variation in asymmetrical distributons.

2.3.3 Statistical tests

After the first observations on the plots we tried to understand if the distributions of eruption age in malnourished and healthy children are statistically significant. To avoid the possibility that the different mean ages in the two groups influenced the analysis we first scaled the values of each distribution, by subtracting them by their mean and dividing the value obtained by their standard deviation. In this way both malnourish and healthy sample have mean eruption age equal to 0 and standard deviation equal to 1. All the differences revealed by statistical test would be due to the shape of the distribution and would confirm or reject the observation we took on the plots. We used the Kolmogorov-Smirnov test to compare the couples of distributions. Significance threshold was set to $p = 0.05$ and then corrected using Bonferroni's method for repeated tests ($p = 0.005$). Except for mandibular deciduous tooth A, which has too few cases to make sense using a statistical test, all the K-S tests gave significant $p - values$ (Table 2.4). This results show that differences between malnourished and healthy children are related to differences in the shape of the distribution. In particular, while the mode of the distribution doesn't change and it is always near to the left wall, the right tail is considerably longer in malnourished group. This indicate a non directional increase of variability in the malnourished group which, as a side effect, generate an increase in the mean.

	$p - values$	
Tooth	Mandibula	Maxilla
A	na	0.00068
B	0.00002	0.00000
C	0.00032	0.00021
D	0.00007	0.00003
E	0.00066	0.00015

TABLE 2.4: Differences between healthy and malnourished children are significant. Tested with Kolmogorov-Smirnov test; significance threshold set at $p = 0.05$ ($p = 0.005$ after applying Bonferroni's correction for multiple tests)

2.3.4 Conclusions

The reanalysis of the data published by Enwonwu (1973), considering all the variation instead that only the mean values, lead us to a very different interpretation of the effects of malnutrition on dental eruption. Whereas all the studies point out that the effect of malnutrition is a delay in dental eruption, our analysis shows that the delay is a side effect of an increased variability in the stressed group.
This result is also consistent with the current knowledge on development. Indeed, development is a complex process, where genetic inputs, environmental inputs and the developing phenotype itself interact in a non-linear way (West-Eberhard 2003). Simple correlations between a genetic or environmental parameter and the phenotype are highly improbable and the same input can have very different effects in different conditions (Salazar-Ciudad 2007). For this reason it is unlikely that malnutrition affects dental eruption always in the same way in all the individuals; more reasonably, malnutrition, affecting different individuals in different times and with different intensity, produces an increase of variability in the timing of dental eruption.

Chapter 3

The role of development in evolution

As we stressed in Chapter 1, in the second half of the 19^{th} century there was a great interest in development. In the years of the Modern Synthesis, between the 1930s and the 1970s, this topic was almost erased from the research agenda since, in the neo-Darwinian paradigm, dominated by population genetics, development became only a necessary step to obtain the phenotype programmed by genes.
Also today "two popular ideas dominate the current views of developmental and evolutionary change. In evolutionary biology, the textbook Neodarwinian paradigm has instigated a narrow view of evolution as the sum of changes in allelic frequencies through time. In developmental biology, half a century of progress in the study of mechanisms of gene expression has resulted in the popularity of a model of development interpreted as the actualization of a *program* written in the genes" (Minelli 2009, p. 141).
However, starting from the 1970s, with the publication of *Ontogeny and Phylogeny* (Gould 1977), a new interest in development arose. In this book, after an historical overview of recapitulation and other orthogenetic theories, the author proposed its theory of how development could be the way by which great evolutionary changes can occur. Changing the regulation of development, fastening or slowing the development of certain traits with respect to the others (heterochrony), living organism can evolve a variety of new forms, also in a few generations. In the following years there was an increase of studies on this topic and the relationship between development and evolution became again a central theme in evolutionary biology (Wake 1996). At the same time, in developmental biology became evident how development is a complex interaction in which environment participates the production of phenotypic variations. The debate about phenotypic plasticity and its role in evolution has recently received new attention in evolutionary biology (de

Jong 2005; Pigliucci et al. 2006; West-Eberhard 2003).
In this chapter I will review some of the mechanism by which development can affect evolution. There is a lot of literature on development and evolution; many authors use different terms to describe the same process and sometimes they give different importance to different mechanisms; I do not claim giving a complete review of all the mechanisms called in cause in the literature, for example I will let out of the discussion the very important topic of modularity, which have great interest in development and evolution. My review is incomplete and partisan: I will discuss only the processes I'm interested most in and that are relevant to the central part of my theses (Chapter 4).

3.1 Regulatory genes

One of the main achievements of the evolutionary-developmental biology (evo-devo) is the identification of the genes that regulate development. The most impressive feature of these genes is that they are shared, with very few alterations, between very different organisms. For example the gene *Pax-6* is one of the regulatory genes that allow the development of eyes in arthropods and very similar homologous genes have been identified in mammals and cephalopods (Gehring 1996; Tomarev et al. 1997).
These deep homologies shared between very different taxa give us an idea of the power of development in driving evolution. In particular, the similar form of cephalopods and mammals eyes, which has always been interpreted as an evidence of the power of natural selection in shaping living forms (Salvini-Plawen and Mayr, 1977), must be reinterpreted as cases of parallel evolution. The same developmental pattern allowed the evolution of similar structures in different taxa. The origin of functional structure didn't follow the laws of natural selection in an isotropic variation, but followed the path marked by development. In the same way, the repeated evolution of similar traits in different taxa, such as the hypocone in mammalian evolution (Hunter and Jernvall 1995), is likely due to shared developmental mechanisms for dental development that lead evolution in a certain direction and doesn't necessarily have a functional meaning.
The other lesson of evo-devo research is that with a few changes in regulatory genes it is possible to achieve great changes in morphology. Indeed, acting on these genes it is possible to alter, in the early phases, the developmental cascade that produces phenotypes, amplifying the phenotypic effects on the later phases of development. In this way a single mutation is able to lead drastic changes in morphology (Gould 2002).

3.1.1 Allometry and heterocrhony

Richard Goldschmidt (1878-1958) developed the themes of orthogenism and saltationism in a complete alternative to neo-Darwinism: in his view evolution occurs abruptly by the occasional birth of "hopeful monsters": jumps in the morphology of a species produced by systemic rearrangements of the genome or mutations in the developmental cascades (Goldschmidt 1940); his work caused a strong reaction by the neo-Darwinian scientists; Mayr (1942), for example, wrote his *Systematics and the Origin of Species* primarily as a refutation of Goldschmidt's ideas (Mayr 1980). Between 1940s and 1970s Goldschmidt became the "embodiment of all that pure Darwinism must oppose" (Gould 2002, p. 451).
A new interest in the themes developed by Goldschmidt arose in the late 1970s when Ontogeny and Phylogeny was published (Gould 1977). The main argument to make development the preferred channel for evolution was about allometry and heterochrony. Allometry is the change in the form of different parts of an organism correlated to dimensions, for example during ontogeny; any evolutionary change that implies an alteration of allometry is heterochrony (Gould 1977). Heterochrony is an easy way for evolution to produce a great variety of new functional forms, working only on the regulation of development. The development of each trait can be fastened or slowed, depending on the functional necessities. Recapitulation and paedomorphosis are two aspects of heterochronic change and their pervasive presence in nature, for example neoteny in salamanders and progenesis in insects, is a mark of the importance of development as a cause of evolution.

3.2 Phenotypic plasticity

Until now I reviewed some mechanism that, although they are not part of the neo-Darwinian paradigm, they are still coherent with a view of development as a genetic program and the evolutionary variation needed is only genetic. In the next paragraphs I'll try to go beyond this view and I'll review a mechanism that includes environmental variation in evolutionary dynamiscs.
Some authors describe phenotypic plasticity as a "property of individual genotypes to produce different phenotypes when exposed to different environmental conditions" (Pigliucci et al. 2006). I strongly reject this view since it recalls a view of development as genetic program, while development is "phenotypic change in a responsive (plastic) phenotype due to inputs from the environment and the genome. [...] Genomic and environmental factors have equivalent and potentially

interchangeable developmental effects, effects that depend as much on the structure of the responding phenotype as they do on the specific inputs themselves." (West-Eberhard 2003, p. 89) Therefore I prefer defining phenotypic plasticity as a property of organisms: it is "the ability of an organism to react to an environmental input with a change in form, state, movement, or rate of activity" (West-Eberhard 2003, p. 34). This definition includes also behavioural changes, but in these paragraphs I will focus on reactions to environmental inputs involving a change in form. This kind of plasticity operates through changes on the developmental cascade that build up a phenotype; therefore it is also called developmental plasticity.

3.2.1 Developmental plasticity as a source of variation

Developmental plasticity allows to an organism to change its form in response to environmental inputs. Therefore, in this perspective, the variation which natural selection and the other evolutionary forces act on is not genetically determined, but it is the result of complex interaction in which environment is as important as the genome.

Also, this kind of variation is different from the classic Darwinian variation in that it doesn't necessarily respect two of the three requirements described in Chapter 1: to be of *minor entity* and *isotropic*.

Indeed, development allows to obtain large phenotypic changes acting on a few starting parameters (that can be both genetic or environmental). Waddington's experiment on the *bithorax-like* phenotype (Waddington 1956) is a good example of a great entity variation produced by environment through development. Many studies demonstrated that small changes in the regulation of developmental pathways might promote large phenotypic variations. These studies focused on the genetic basis of developmental regulation and mostly ignored that "genomic and environmental factors have equivalent and potentially interchangeable developmental effects" (West-Eberhard 2003, p. 89).

Also, developmental variation rarely is *isotropic*. New variations depend on the developmental network and must be congruent with it. Many works show how development may decide the direction of evolution (Gould 1977; Gould 2002; West-Eberhard 2003). Another important aspect regarding directionality of variation is that, many times, developmental plasticity is adaptive, like in the case of the reorganization of bone structure during physical stress (Burr et al. 2002; Roux 1881; Ruff et al. 2006; Wolff 1892). In this framework the role of natural selection in shaping living forms is secondary to the role of development.

3.2.2 Developmental plasticity in evolution

Phenotypic and developmental plasticity are hot topics of the modern biology and there is a heated debate about their role in evolution (for example: de Jong 2005; Pigliucci et al. 2006). There is an increasing amount of evidences suggesting the importance of plasticity in evolutionary changes (Aubret and Shine 2009; Chapman et al. 2000; Emlen et al. 2007; Gibson and Hogness, 1996; Price 2003; Price 2006; Rutherford and Lindquist 1998; Sollars et al. 2003).
One of the mechanisms, through which phenotypic plasticity affects evolution, is known as genetic assimilation and it was proposed by the Scottish geneticist Waddington (1942). Waddington's argument starts from the observation that, in some cases, a phenotype that in the adult originates by environmental stimulus, is already formed in the embryo. Let's take an example of Waddington: in ostriches, a crouched position causes the formation of a callus in the ventral part of the animal. This trait is already present in the embryo of the ostrich. The classical neo-Darwinian explanation states that the action of natural selection has acted on random variations of the trait in question. The author considers this explanation insufficient because it doesn't take into consideration that the given phenotype typically develops as the result of an environmental stimulus. The alternative explanation suggested by Waddington is that in the ancestors of todays' ostriches the "callus" trait was only present as a developmental response induced by an environmental stimulus, and in the course of evolution, the environmental induction of the phenotype has been replaced by genetic factors.
The process described by Waddington, named genetic assimilation, is divided into 4 main steps:

1. variation of a trait is canalized, i.e. there is a buffering mechanism that allows the absorption of small genetic or environmental variations below a certain threshold in such a way as to keep the "optimal" development of the phenotype;

2. an environmental stimulus above a certain threshold causes the loss of the buffering mechanism and a modification of development, which leads to a new phenotype;

3. if one of the new phenotypes is better than the others, selection will favour the canalization of that particular phenotype;

4. once the phenotype is canalized, mechanisms such as genetic factor take over to stabilize the trait (genetic assimilation of the phenotype).

In the following years, Waddington performed several experiments to test the hypothesis of genetic assimilation of acquired characteristics (Waddington 1953, 1956 and 1959).

3.2.2.1 Assimilation of the *crossveinless* phenotype

In the first of these experiments Waddington (1953) treated a stock of the fruit fly *Drosophila melanogaster* by a thermal shock during the pupal stage. This treatment, in some individuals led to abnormal development of certain veins of the wings in a phenotype called *crossveinless* (*cvl*, Fig. 3.1). There is no doubt that this was a phenotype induced by environmental perturbation due to the application of thermal shock during development; if a shock was not applied, pupae developed normally. Waddington in this way produced two lines: a line of positive selection of the *cvl* phenotype (referred to by the term *upward*) in which the only individuals allowed to reproduce were those *cvl*, and a line of negative selection (*downward*) in which only individuals that, after the thermal shock, did not develop the emphcvl phenotype were allowed to reproduce. Selection proceeded in the expected direction and the *cvl* phenotype increased its frequency after the heat shock in the upward line and decreased in the downward line.

Every generation, in the upward line, the offspring of some *cvl* individuals were

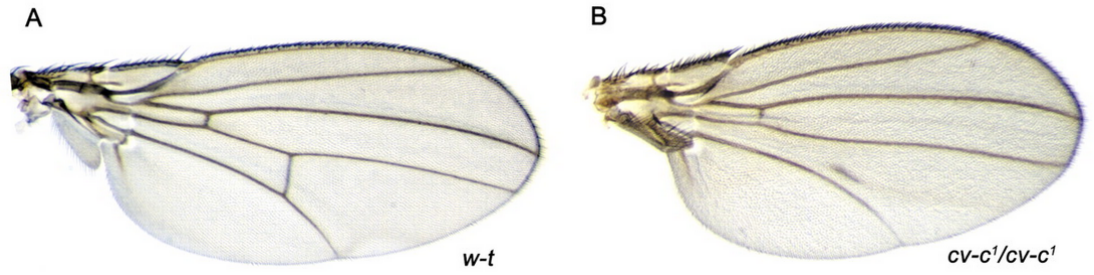

FIGURE 3.1: Difference between the *D. melanogaster* wild type (left) and the *crossveinless* phenotype (right). From Denholm et al (2005)

allowed to develop without any heat shock. Starting from the 14^{th} generation *cvl* individuals begin to appear among untreated individuals. At the 16^{th} generation *cvl* individuals in the untreated part of the upward line were between 1% and 2%. Breeding together those individuals, the cvl trait was inherited by the offspring even in the absence of the environmental stimulus. In this way Waddington demonstrated for the first time the possibility that a phenotype, whose development is induced by the environment, becomes a stable phenotype even in the absence of environmental stimuli.

3.2.2.2 Assimilation of the *bithorax-like* phenotype

In a later experiment (Waddington 1956; repeated 40 years later by Gibson and Hogness 1996) genetic assimilation was tested on another trait obtained by treatment of the embryo of *D. melanogaster* with ether: in treated individuals, the meta-thoracic disc becomes similar to a mesothorax and also halters are enlarged forming true wings, in a phenotype called *bithorax-like* (Fig. 3.2), which can have different degrees of development.

FIGURE 3.2: An adult of *D. melanogaster* with the *bithorax-like* phenotype. Note that the specimen has two pairs of true wings.

The aim of the experiment was to test the possibility that genetic assimilation could work on substantial changes of the phenotype: "if such a change occurred during phylogenesis it would certainly be accounted a macro-evolutionary phenomenon. It was felt that, if such a fundamental modification as this can be genetically assimilated, then one would have some grounds for confidence that the process was powerful enough to be invoked to explain quite far-reaching evolutionary changes." (Waddington 1956, p.1).
This time, two experiment were carried on, starting from two different stocks, and obtaining 4 different selection lines: one upward and one downward for each stock. Even if the two stock had initial different frequency of developmental response, selection worked regularly, leading to an increase of the *bithorax-like* phenotype in the upward, and a decrease in the downward lines. Again, after a certain number of generations (8 in experiment II , 29 in experiment I, in which the treatment with ether was reduced) the phenotype *bithorax-like* began to appear in upward individuals who developed without treatment. The trait, initially acquired as a result of environmental stimulus, became hereditary.

3.2.3 Criticisms to the concept of canalization

Waddington's experiment, replicated and confirmed 40 years later by Gibson and Hogness (1996), show that an acquired characteristic might be genetically assimilated. Furthermore phenotypes induced are always phenocopies or, in other words, can be produced either by environmental induction or gene mutation. This fact helps us to understand how a phenotypic change, acting on the developmental pathways, may precede and help the genetic change in a certain direction. Development, one of the forgotten themes of the Modern Synthesis, is central to Waddington's view; the genotype can take into account environmental inputs through development, which become the preferred channel along which evolution acts. However, Waddington's view falls completely inside the limits of development as a genetic program, thanks to the concept of canalization.
Canalization is the property of a genotype to resist minor environmental and genetic variation to produce the optimal phenotype (Wadington 1942, 1957); optimal phenotypes are canalized thanks to the action of natural selection. Canalization involves lack of phenotypic variation with respect to a genetic variability hidden by a buffering mechanism that is genetic too (Salazar-Ciudad 2007); in Waddington's genetic assimilation, the role of environment is to remove the buffer making available a previously hidden genetic variability. Natural selection will act on this new variability and will tend to canalize the optimal phenotype in the new environmental conditions. Environmental stress over a certain threshold acts as a switch mechanism between two or more genetically determined phenotypes.
With the concept of canalization development is deprived of any autonomy from the genes. On the contrary, if we put development at the centre of the argument, it becomes clear that it is the morphogenetic process, in its complexity, that produces variability (Salazar-Ciudad et al. 2003). The fact that not every environmental input implies a phenotypic change is not a consequence of canalization, but of the complex nature of developmental networks and the non-linear interactions that produce the phenotype.

3.2.4 Genetic accommodation

Following Braendle and Flatt (2006) genetic assimilation is a special case of a more general mechanism that produces adaptive evolution thanks to the introduction of phenotypic novelties originated by development. This mechanism, known as genetic accommodation, is described by West-Eberhard (2003) and involves 4 main step:

1. *origin of the trait*: a mutation or an environmental change triggers the expression of a new variation in developmental pathways, which brings a phenotypic change or re-organization;

2. *phenotypic accommodation*: the immediate adjustment of the phenotype to the change, due to the adaptive flexibility of the phenotype. In the example of the two-legged goat (West-Eberhard 2003), a goat born with only two legs re-adapted the whole musculoskeletal system to walk upright;

3. *initial spread*: because the recurrence of the cause of origin of the trait (for example new environmental conditions) the new phenotypic variation, initially rare, spread in a subpopulation of individuals where the trait expresses;

4. *genetic accommodation*: change in allele frequency due to selection on the variability in regulation, form or side effects of the new trait.

Waddington's genetic assimilation is a special case of genetic accommodation that occurs when:

- the trigger of the expression of the new phenotypic variation is environmental;

- selection bring to the genetic fixation of the phenotypic variation produces new variability.

Chapter 4

Environmental influence on dental morphology

4.1 Introduction

In developmental studies, teeth represent an excellent model, since once their development is completed, they undergo no further changes in size and shape (except from wear, traumas or cultural practices), thus recording the developmental process in their phenotype. Our knowledge on the mechanisms of dental development has increased exponentially over the past 20 years. In particular, it has become evident how dental development, like the development of other organs, is regulated by inductive interactions between epithelial and mesenchymal cells (Ruch, 1995; Thesleff and Hurmerinta, 1981; Thesleff et al., 1995). The bulk of the research agenda has focused on the genetic and molecular bases (reviewed in Bei 2009; Jernvall and Jung 2000): detailed knowledge is available today on over 300 genes expressed in mouse dental tissues and their patterns of expression from the beginning of the tooth bud to the end of its development (see for example the Gene expression in tooth - WWW database, 1996). The focus on the genetics of dental development has made it possible to make progress in our understanding of morphogenesis and variation in dental morphologies; at the same time, it has overlooked other important aspects, such as the environmental influence on dental development and its contribution to the final phenotype. It is probably because of this bias toward the genetic component of dental development research that is common to find in the literature such statements as "the development of teeth is under strict genetic control" (Galluccio et

al., 2012; Thesleff and Nieminen, 1996) which, from a developmental biology perspective, greatly underestimate the role of the environmental component. Indeed, "development is phenotypic change in a responsive (plastic) phenotype due to inputs from the environment and the genome. [...] Genomic and environmental factors have equivalent and potentially interchangeable developmental effects, effects that depend as much on the structure of the responding phenotype as they do on the specific inputs themselves" (West-Eberhard, 2003, p. 89).

4.1.1 Known environmental effects on dental development

The significant role of environmental factors in determining dental variation was reviewed by Butler (1983), who acknowledged that "dental phenotypic differences *between* populations do reflect genetic differences" (p. 288, Italics ours), but at the same time emphasized that the genes "that affect the dentition do so only through the mediation of a complex ontogeny in which the environment takes part" (p. 288). Different aspects of the environmental influence on tooth development in human populations have been studied. One is the relationship between malnutrition and tooth eruption, where malnourished children reveal a delay in the eruption of their teeth (Boas, 1927; Enwonwu, 1973; Garn et al., 1965; Gaur and Kumar, 2012; see also Chapter 2 of this thesis). Another way to study the environmental influence on dental development is to correlate tooth size and environmental stress. McKee and Lunz (1990) studied the correlation between enamel hypoplasia occurrence, a marker of developmental disruption, and dental size reduction: they found an association between dental reduction and the presence of enamel hypoplasia in the individual. Garn and Russel (1971) showed how maternal health status can influence the tooth size of the deciduous and permanent dentition of the offspring. Other authors demonstrated that tooth size depends also on environmental variables, both in family studies (Kolakowski and Bailit, 1981; Townsend and Brown, 1979) and in studies on populations with different economies (Perzigian 1984). Luke et al. (1979) found that malnourished pigs have both delayed tooth eruption and reduced size in the third molar.

Similar results come from the study of fluctuating asymmetry, which is considered a measure of stress and developmental instability also influenced by environmental variables (Carter and Houle, 2011; Parsons, 1990). Kieser

(1992), studying fluctuating odontometric asymmetry, used alcohol consumption by the mother during the gestation period as an environmental variable, and found a high level of asymmetry in children of alcoholic mothers compared to children of non-alcoholic mothers. In another paper, (Kieser et al., 1997) fluctuating asymmetry in children was considered in relation to maternal obesity and smoking; again asymmetry was higher in children of obese and smoking mothers. Corruccini et al. (2005) studied the correlation between the two markers of developmental disruption mentioned above: enamel hypoplasia and dental asymmetry. They didn't find a good correspondence at the individual level, however, they suggested that "a comparison across stressed and unstressed samples (rather than individuals) could reveal shared differences in asymmetry and hypoplasic calcification" (p.180). Indeed they found that the hypoplasic sample showed more variation in dental asymmetry.

The study of monozygotic twin pairs is another research approach to address how differences in the presence, size, and shape of teeth are linked to environmental influence. "[...] Supernumerary tooth formation is influenced not only by genetic factors but also by environmental and epigenetic influences" (Townsend et al. 2012, p. 6). For example, Townsend et al. (2005) found monozygotic twins to be discordant in the number and position of supernumerary teeth. Other studies on monozygotic twins focused on the heritability of discrete traits; in the first half of the last century, the prevailing view was that morphological traits on tooth crowns were under strong hereditary control (Scott and Turner 1997, p.161). For example, in the case of Carabelli's trait, the most studied trait on upper molar crowns, starting from the 1950s this view began to shift from a simple single-locus model to a more complex one, invoking multi-loci genetic control or environmental effects. As studies on twins increased in number, it became evident how trait expression often differs between identical twins (Scott and Turner 1997, p.162) and recently a study demonstrated, in a developmental biology perspective, how variation in the expression of Carabelli's trait depends also on environmental factors (Hunter et al. 2010). Lastly, the effects of pathological conditions on dental development (Atar and Körperich, 2010) have been described. Several pathological conditions (e.g. cystic fibrosis, AIDS, leukemia) cause a number of enamel and dentine defects, acting during tooth development, in the pre-natal and post-natal periods.

4.1.2 Problems in evaluating environmental influence

All these studies discussed above (except Hunter et al. 2010) have some limitations when considered from an evolutionary developmental biology perspective. First of all, the majority of them concentrates on metrical traits; when discrete traits are considered, the focus is on characters of the whole dentition, such as congenital absence and supernumerary teeth (Townsend et al., 2005), or on pathological defects (Atar and Körperich, 2010). In studies on environmental influence, normal individual variation which describes the morphology of a single tooth is usually not taken into account. Secondly, in evolutionary biology, the relevant variation is that occurring at the population level, since morphological differences among taxa often stem from evolutionary processes taking place at the population level (Mayr, 1982; Jernvall, 2000). Few studies have taken into account environmental influences on teeth at the population level: among them, some were on populations who migrated into new environments (Lasker, 1945; Scott and Alexandersen, 1992), but they failed to demonstrate plasticity in dental traits, probably because of the kind of stressor tested (Scott and Turner, 1997). Thirdly, even when some degree of plasticity is recognized (e.g. differences in trait expression between MZ twins), these studies lack a developmental explanation of the mechanism through which this plasticity is produced. This difficulty is particularly evident in heritability studies, where the issue is trying to distinguish between genetic and environmental variance, as if one genotype were bound to always produce the same phenotype and, as such environmental variance is a mere noise altering the correct correspondence between genotype and phenotype. In this framework, any attempt to explain the developmental process becomes sterile. On the other hand, if we accept that variability on which evolutionary factors act is the product of development (Salazar-Ciudad et al., 2003) and that development is the result of the interactions between genetic and environmental inputs and the developing phenotype (West-Eberhard, 2003), the mechanism involved in the shaping of variability becomes central to our investigation.

4.1.3 Dental morphogenesis

In recent years, thanks to remarkable advances in the field of molecular biology, it has been possible to begin to understand the molecular basis of this mechanism and how the developmental process leads to the final

phenotype. Studies on experimental animals have provided information on genes expressed in different tissues during tooth development; this research, together with a broad scale analysis of morphological variability in mammalian dentition, has led to the proposal of a specific model of development for multicuspidate teeth. The developmental unit for cusp development is the primary enamel knot (Jernvall and Jung, 2000), "a cluster of cells in the central part of dental epithelium, facing the dental mesenchyme" (Butler, 1956; Vaahtokari et al., 1996). The primary enamel knot acts like an embryonic signaling center and expresses the same genes as involved in the organogenesis of other ectodermal organs such as feathers, nails, and glands (Bei, 2009; Jernvall and Thesleff, 2000; Jernvall and Jung, 2000); the majority of these genes belong to one of the signaling pathways of four gene families: BMP, FGF, SHH, and WNT. Furthermore, the dimensions of the primary enamel knot determine the position and dimensions of the secondary enamel knots (Jernvall and Jung, 2000; Jernvall and Thesleff, 2000), from which the cusps originate. Lastly, secondary enamel knots express the same genes as expressed in the primary enamel knot, i.e., in the gene network there are no specific signals for each cusp (Jernvall and Jung, 2000). Starting from these clues, Jernvall (1995) developed a model for secondary enamel knots initiation: a new enamel knot initiates when there are enough epithelial cells available to form them; the number of epithelial cells is regulated by the expression of FGFs in extant enamel knots. The formation of cusp patterns is therefore a cascade of events beginning with the formation of the primary enamel knot: "the relative sizes, numbers, and heights of the cusps [in seal molars] are symmetrically correlated, anterior and posterior, to a major central cusp, as if the development of these additional cusps depends on rate of growth relative to a threshold in time, beyond which further development does not occur" (Weiss et al. 1998, p. 390; Jernvall 2000). The dimension of the primary enamel knot regulates the formation of subsequent secondary enamel knots and the dimensions of those regulate the initiation of other enamel knots (and cusps) later in the developmental cascade. Tooth morphogenesis is therefore a self-organizing system or a Bateson-Turing Process (Weiss et al., 1998), where "once a set of differentiation factors is established [...] further exogenous signaling is no longer needed" (Weiss et al. 1998, p. 376). When the first enamel knot appears, the tooth primordium has all the information it needs to complete its development. This model for tooth development is usually referred to as the patterning cascade mode for cusp

development, or the morphodynamic model. Today fairly accurate computational models for tooth development are available, taking into account both the genetic network and the cellular interactions (Osborn, 1993, 2008; Salazar-Ciudad and Jernvall, 2002, 2010). Starting from the cascade model, these attempt to reproduce extant variability in tooth diversity. For example, Salazar-Ciudad and Jernvall (2010) developed a model accounting for the morphological diversity in seal molars. Following the suggestions of the cascade model, this diversity is obtained by acting on very few initial inputs representing genetic information: one inhibitor and one activator for the differentiation of enamel knots, and one parameter representing growth factors, upregulated in enamel knots, regulating cell proliferation or differentiation.

4.1.4 Aim of the work and expectations

With this background, the aim of this paper is to test whether environmental inputs can affect dental morphology acting on the developmental cascade. Scott and Turner (1997) suggested that stressors such as undernutrition and infectious diseases, already known to disrupt dental development leaving marks on teeth, could affect dental morphology as well. The occurrence of dental enamel hypoplasia is a good marker for these kind of stressors, since it is known to be linked to physiological stress, such as malnutrition or systemic diseases, during tooth development (Guatelli-Steinberg and Lukacs, 1999; Ten-Cate, 1998; Skinner and Goodman, 1992). The response of the developing tooth to such stress is a slowing down of its development and the visible result on the tooth surface is an enamel hypoplastic defect. Jernvall (2000) suggested that studies on variability at the population level can be useful in testing hypotheses in development biology: our hypothesis is that, in a homogeneous population, individuals with severe hypoplasia, that is to say, individuals whose development was probably disrupted by an environmental stress, are more variable in their molar morphology than individuals with no hypoplasia. The expectation we want to test is that a developmental disruption, due to an environmental stressor, occurring in a population increases variability in molar morphology. In a single organism, a disturbance during the morphogenetic cascade should cause subtle “errors” in the morphological output. These changes will be more evident as the “errors” accumulate in the developmental cascade, that is to say, in later developing cusps. If we look at the whole population, where disruption has occurred at different times and with different intensity in each individual, the logical

expectation is an increase in morphological variability, particularly evident in later developing cusps.

4.2 Materials and methods

The specimens analyzed derive from the "Fabio Frassetto Collection" housed in the Museum of Anthropology at the University of Bologna. The largest part of this collection is made up of over 600 skeletons recovered from the cemeteries of the town of Sassari (Sardinia, Italy) in the early 20th century. For the vast majority of the skeletons, individual information is available (name, sex, date of birth, date of death, job, and cause of death). This sample can thus be considered homogenous in terms of genetic structure of the population and physical environment. Selection criteria required the presence of the maxillary dentition, with at least two molar teeth from the same side. Specimens where attrition precluded analysis of cusp morphology were excluded.

Scott and Turner (1997) suggested that stressors such as undernutrition and infectious diseases, already known to disrupt dental development and leaving marks on teeth, could affect dental morphology as well. The occurrence of dental enamel hypoplasia is a good marker for these kind of stressors, since it is known to be linked to physiological stress, such as malnutrition or systemic diseases, during tooth development (Guatelli-Steinberg and Lukacs, 1999; Ten-Cate, 1998; Skinner and Goodman, 1992). The response of the developing tooth to such stress is decrease in the amount of tissue formed and the visible result on the tooth surface is an enamel hypoplastic defect. The occurrence of enamel hypoplasia was employed here as marker of environmental stress (*sensu lato*). The presence of linear enamel hypoplasias was recorded following Buikstra and Ubelaker (1994), taking into account the apparent prominence of the hypoplastic bands with a two-grade scale (Fig. 4.1). Two groups were then identified, with different stress levels: the first group included individuals with no signs of enamel hypoplasia in any of the present teeth; the second group included individuals with at least one tooth with one prominent or three faint hypoplastic events. Individuals with only one or two faint hypoplastic bands were excluded from the sample in order to reduce as much as possible the error of attribution to the group with hypoplasia without compromising the sample size: only individuals with a clear pattern of presence/absence of hypoplasia have been included. The

final sample included 75 individuals (34 females, 41 males). Group 1 (no hypoplasia) included 30 individuals (13 females, 17 males); group 2 (prominent or diffuse hypoplasia) included 45 individuals (21 females, 24 males). For the sake of convenience, from now on group 1 and group 2 will be referred to as "non-stressed" group (NSG) and "stressed" group (SG), respectively. For

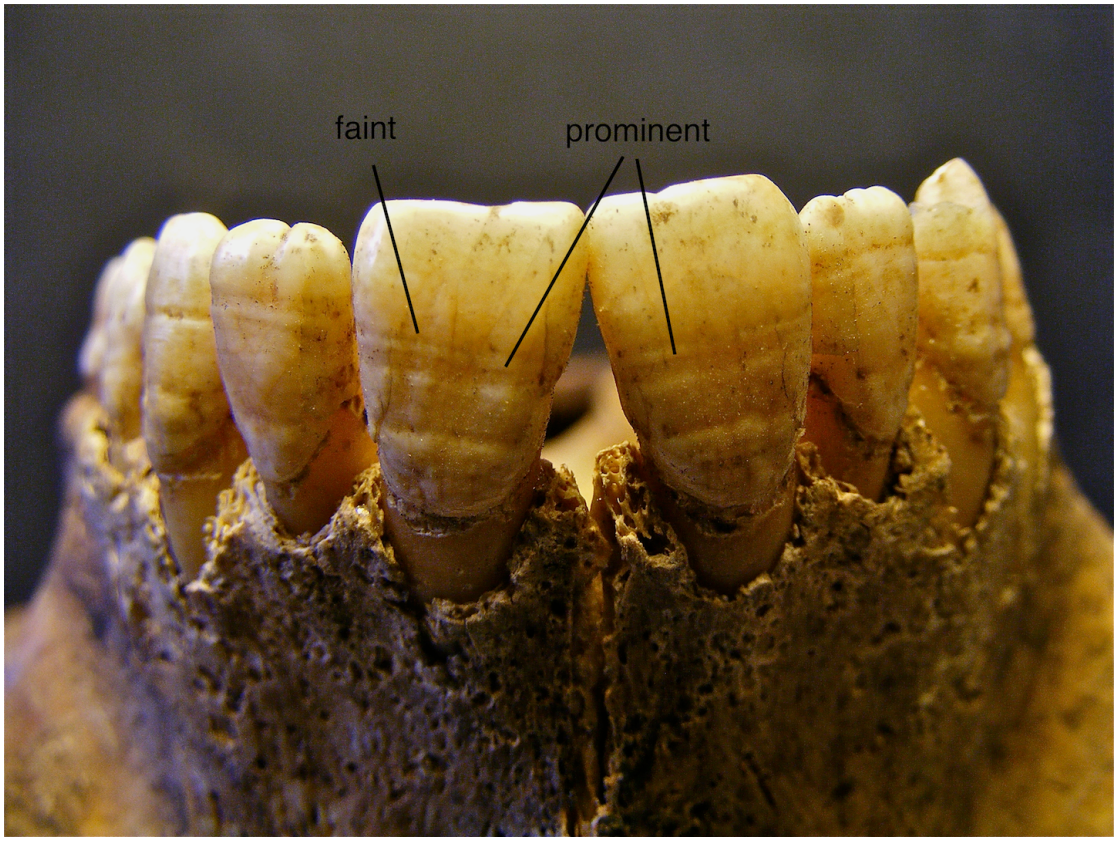

FIGURE 4.1: Linear enamel hypoplasia on the maxillary dentition: on the basis of the breadth and depth of the bands, prominent and faint grades are distinguished (following Buikstra and Ubelaker, 1994).

each individual of each group, the variability in the expression of maxillary molar cusps was observed. Expression of the relative development of the cusps was recorded in a semi-quantitative way using the ASU-DAS standard (Turner et al., 1991), based on ranked-scale plaster cast and descriptions for each discrete trait. Discrete traits considered were: metacone, hypocone, cusp 5, Carabelli's trait, and parastyle. Each trait was scored and ranked on a scale from 0 to 5 (metacone, hypocone and cusp 5), 0 to 6 (parastyle), or 0 to 7 (Carabelli's trait), where 0 is the absence of the trait and the maximum value represents a well formed cusp, separated from adjacent ones (following Turner et al., 1991). The two sets of data (hypoplasia occurrence and morphological traits expression) were all collected by the first author.

Morphological data have been collected two weeks later than hypoplasia and teeth were blinded to the observer relatively to the stress level. In a random subsample of 30 teeth we calculated the error of assessing the presence of the trait as percentage of mismatching between the two observations. We then run a Kendall tau rank correlation to test the agreement between the two sessions of data collection. A two-sample Wilcoxon test was used to compare each discrete variable between the two groups. T^2 Hotelling test and two-sample MANOVA (Pillai test) were used for multivariate analysis. The significance threshold for $p - values$ was set at 5% and, where necessary, corrected for repeated tests with Bonferroni's method. All the statistical analyses were carried out using the software R (R Development Core Team, 2010).

4.3 Results

Intra-observer error in assessing the presence/absence of the traits, was under 5% for each of the 5 traits considered. Kendall rank correlation gave significant correlations between the two observations with all the taus above 0.7, indicating a strong correlation. Frequencies of dental traits in our sample are comparable with those observed in other European populations. The percentage of presence/absence fall into the variability observed in Western European population for cusp 5, Carabelli's trait and hypocone in their focal tooth (Scott and Turner, 1997).
The analysis was carried out for each group (NSG and SG) with sex pooled; any further subdivision would have reduced sample sizes and affected reliability of the statistical analysis. The pooling is justified by two facts: ratios between sexes in the two groups is largely similar; and differences in dental traits distribution between sexes in our sample are not significant (Wilcoxon test and Bonferroni's correction) for each of the traits considered.
Table 4.1 shows, for each molar and for each morphological trait (cusp), the comparison between NSG and SG in the frequencies of expression of each category. A general trend appears in most cases: in the SG, the variability of expression of the different grades (the frequency of each category) is greater than in the NSG and the higher grades of expression are more frequent.

TABLE 4.1: $p - values$ for the Wilcoxon test applied to compare the distributions of cusps development between the non-stressed group (NSG) and the stressed (SG). The threshold of significance was set at 0.05; the values that maintain their significance after Bonferroni's correction ($p - value < 0.0033$) are in bold.

	M^1		M^2		M^3	
Metacone	$(p = 0.037)$***		$(p = 0.431)$ns		$(p = 0.289)$ns	
	NSG	SG	NSG	SG	NSG	SG
0	0	0	0	0	0	0
1	0	0	0	0	0	0
2	0	0	0	1	1	0
3	0	0	4	8	10	18
4	12	9	35	39	15	16
5	31	64	12	26	0	9
	M^1		M^2		M^3	
Hypocone	$(p = 0.302)$ns		**(p=0.002)*** **		$(p = 0.408)$ns	
	NSG	SG	NSG	SG	NSG	SG
0	0	0	16	5	11	16
1	0	0	8	9	4	6
2	0	0	3	11	5	3
3	4	4	10	18	6	14
4	15	22	14	17	1	4
5	23	46	0	11	0	0
	M^1		M^2		M^3	
Cusp 5	$(p = 0.362)$ns		$(p = 0.580)$ns		$(p = 0.008)$***	
	NSG	SG	NSG	SG	NSG	SG
0	40	64	46	68	26	33
1	0	5	3	4	0	5
2	1	0	0	0	0	1
3	1	1	1	0	0	2
4	0	1	0	0	0	0
5	0	0	0	0	0	2

Table 4.1: continued on the next page...

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	M^1		M^2		M^3	
Carabelli's trait	**(p=0.000)*** **		**(p=0.001)*** **		$(p = 0.031)$***	
	NSG	SG	NSG	SG	NSG	SG
0	25	9	48	50	26	31
1	6	15	2	14	1	2
2	7	8	1	4	0	5
3	3	9	0	2	0	0
4	2	12	0	1	0	1
5	0	13	0	0	0	1
6	0	1	0	0	0	0
7	0	3	0	0	0	0
	M^1		M^2		M^3	
Parastyle	na		**(p=0.416)ns**		$(p = 0.440)$ns	
	NSG	SG	NSG	SG	NSG	SG
0	43	73	51	73	27	41
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	1
4	0	0	0	1	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0

That is, in 10 of the 15 cusps considered (metacone on M^1 and M^2, hypocone and cusp 5 on M^1 and M^3, Carabelli's trait and parastyle on M^2 and M^3), the mode of the distribution is constant, while the mean increases (see figures in Appendix B). This condition is suggestive of a directional increase in cusp expression variability in the SG. These differences in the distributions are significant (two-samples Wilcoxon test,$p - value < 0.05$) for the following cusps: Carabelli's trait on each of the three molars; metacone on the first, hypocone on the second and cusp 5 on the third molar. When the $p-values$ are corrected using Bonferroni's method, the values under the threshold of significance are those of hypocone on the second molar and Carabelli's trait on the first and second molars. The variability for parastyle is too low to make sense of it using any statistical test, but it is worth noting that the only two parastyles observed in the sample belong to two individuals of the SG. As a further step in the study it was deemed suitable to perform a multivariate analysis in order to take into account at the same time the grade of development of every cusp in each tooth or in each single individual and compare it between the two groups. This was done since the scope of the work is to address the overall variability in cusp expression of the molar teeth, rather than the relative development of each single cusp. A principal component analysis considering, for each individual and each group, all three molar teeth and the grade of expression of each cusp (except for the parastyle because of its very low frequency in the sample) was carried out. Figure 4.2 shows scatterplots of the first three principal components in pairs, where individuals of the NSG and SG are indicated in different colors. The first three principal components explain the 77% of the variability of the dataset (PC1 = 39%, PC2 = 23%, PC3 = 15%). In Figure 4.2 the weight of each variable on principal components is also shown (black arrows). Variation along PC1 is linked to Carabelli's trait on M1, hypocone on M2 and M3, and cusp 5 on M3; variation on PC2 is mostly explained by Carabelli's trait on M1 and hypocone on M2 and M3; lastly, cusp 5 and Carabelli's trait on M3 explain most of the variation along PC3. The same analysis was also performed separately for each molar tooth. A similar pattern in the spatial distribution of the individuals belonging to the two groups emerges in both types of analysis. Individuals from the NSG tend to cluster together in a relatively delimited area of the plot, whereas those from the SG are more broadly distributed on the Cartesian plane, partially overlapping the NSG. PC1 values for the NSG vary between -3.19 and 0.42, while in the SG between -2.88 and 5.86; PC2 values between -1.62 and 2.10 for the NSG and between

-4.12 and 2.17 for the SG; PC3 values between -0.70 and 0.17 (NSG) and -2.18 and 4.09 (SG). This indicates a higher variability in the SG as compared to the NSG, more easily detectable on PC1, which best distinguishes the two groups, followed by PC3. Also, individuals in the NSG tend to fall into the margin of the distribution, suggesting that the enhanced variability of the SG is not isotropic but directional. Once again, the direction toward which variability increases is the direction of more developed cusps (indicated by the direction of the arrows in Figure 4.2).

In order to test whether the differences in the observed distributions were

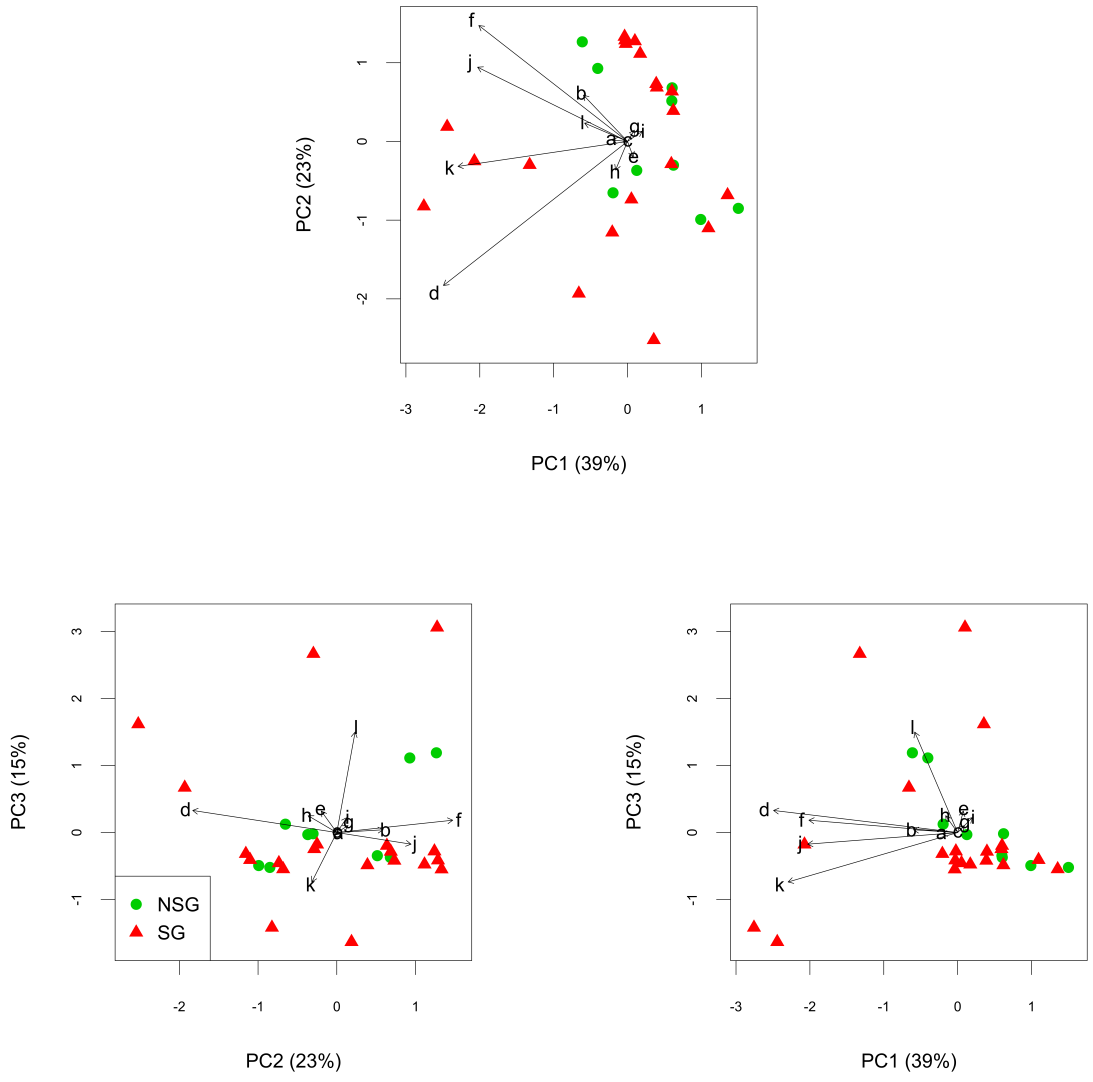

FIGURE 4.2: Plots of the first three PCs for all the traits in upper molars (parastyle was excluded because of the low frequency in the sample). The arrows represent the weight of each variable on the PCs (a=metacone on M^1; b=hypocone on M^1; c=cusp 5 on M^1; d=Carabelli's trait on M^1; e=metacone on M^2; f=hypocone on M^2; g=cusp 5 on M^2; h=Carabelli's trait on M^2; i=metacone on M^3; j=hypocone on M^3; k=cusp 5 on M^3; l=Carabelli's trait on M^3).

statistically significant, a Hotelling's T^2 test was employed; first with the three molars pooled, and then for each molar separately. In the first case, parastyle and cusp 5 had to be excluded from the analysis, because of their overall very low frequencies and the reduced number of cases due to the omission of strings with NAs data. On the other hand, in the analysis of each molar tooth (where reduced variables for each case made it possible to omit fewer strings), it was possible to include cusp 5, but not parastyle, in the variables considered. In both analyses, differences between NSG and SG are statistically significant (Tab. 4.2), even after Bonferroni's correction for repeated tests.

Tooth	$p - value$ Hotelling T^2
$M^1 + M^2 + M^3$	0.00059
M^1	0.00000
M^2	0.00004
M^3	0.00178

TABLE 4.2: Multivariate comparison (Hotelling T^2 test) between SG and NSG, for the molars pooled (M^1+M^2+M^3) and for each molar separately. Significance threshold is set to $p = 0.05$ ($p = 0.0125$ after Bonferroni's correction)..

4.4 Discussion

The different degree of expression of some dental morphological traits in the two groups of a modern human population (SG and NSG) seems to suggest the existence of a link between enamel hypoplasia occurrence and upper molar tooth morphology. We do not mean that there is a causal nexus between hypoplasia and tooth morphology, but that both are the result of the same cause: a developmental disruption, likely due to a non-genetic disturbance such as malnutrition or a systemic disease (among the most common causes of enamel hypoplasia). In other words, an environmental disturbance affecting dental development has an influence on the final phenotype. Two aspects of the results are worthy of note: first, as we hypothesized, a developmental disruption resulted in an increased variability in dental morphology; secondly, the increase in variability is directional, toward an expression of larger cusps. These two aspects will now be discussed in detail.

4.4.1 Variability increase in the stressed group

The first effect of the developmental disruption on the expression of upper molars non metric traits is an increase of variation in the SG. The increase of variability in the expression of morphological traits under a stressful condition has usually been attributed to two different factors. The first is related to the existence of a hidden genetic variability that is canalized by a buffering mechanism in normal development (Waddington, 1959); if an environmental stressor exceeds a threshold, the buffering mechanism breaks down and new genetic variability arises (Rutherford, 2000). However, there are few cases in which this buffering mechanism has been discovered (Rutherford and Lindquist, 1998; Sollars et al., 2003) and there are no clues indicating that a similar genetic buffering mechanism may be active during dental development. The other possibility discussed in the literature is that new variability arises from the developmental mechanism itself (Salazar-Ciudad, 2007). As mentioned in the introduction, the current developmental model proposed for the molars has, as a logical consequence, an increase of variability in a developmentally disrupted population. Indeed, molar tooth development is a morphodynamic process in which morphogenesis and induction mechanisms are interdependent (Jernvall, 1995; Salazar-Ciudad et al., 2003). In morphodynamic systems a small change in the inputs can be amplified during the morphodynamic process (developmental cascade) and have unexpected results on the final morphology, because the relationship between the parameters' change and morphology is not linear, but depends on the developmental network, on the phase of morphogenesis, and on all the other inputs involved in tooth development. Therefore, the effect we observed in our sample on the increase of morphological variability in the SG is compatible with this current view of tooth development. Dental development is regulated by cellular and genetic interactions (Salazar-Ciudad and Jernvall, 2010): both these factors (cellular parameters and gene expression) can be affected by environmental inputs. As for the first factor, environmental stress is known to produce a downregulation of cellular metabolism (Hand and Hardewig, 1996). The effects of this downregulation can be multiple; for example during hypometabolic states (such as starvation or lack of nutrients), downregulation can affect both energy production and energy consumption, and can inhibit macromolecule synthesis and turnover. In a similar situation it is likely that other parameters important for tooth development may also change, such as epithelial and mesenchymal growth and differentiation rates.

In this regard, some authors (Johnston and Gallant, 2002; Katso et al., 2001; Kozma and Thomas, 2002; Schmelze and Hall, 2000) have suggested that cell growth is affected by environmental and developmental conditions and that, in some cases, it can change in response to nutrient levels. Kim et al. (2002) identified Raptor, a protein sensitive to nutrient levels participating in the regulation of cell size. As for the importance of gene expression, there is a vast literature on how environmental stressors can alter gene expression, even just considering nutritional stressors. In bees, for example, queens and sterile workers differentiate from genetically identical larvae thanks to a different nutrition that probably influences the expression of some genes through DNA methylation (Kucharski et al., 2008). Also, in *Drosophila*, gene expression is regulated by starvation (Zinke et al., 2002). In mammals, diet can influence the pattern of methylation and the stable expression of some genes (Jaenisch and Bird, 2003); nutrition is important during the gestational period and can affect the phenotype of the offspring, for example inducing changes in methylation in rats (Burdge et al., 2007). Most of the literature on the effects of the environment on gene expression focuses on regulation by changes in DNA methylation, but it is not the only possibility. Transposable elements can also regulate gene expression under stressful situations (Slotkin and Martienssen, 2007). For example in mammals, apoptosis regulation is participated by a LTR retrotransposon, (Romanish et al., 2007) and retrotransposon activation is known to be stress-induced (Frucht et al., 1991; Hampar et al., 1976; Hohenadl et al., 1999; Ruprecht and Lanzavecchia, 2006; Sutkowski et al., 2004). In humans, undernutrition can alter the growth trajectory of babies; also, their morphology and physiology is affected by the nutritional state of the mother (Bateson et al., 2004). Somel et al. (2008) found that differences in diet can produce differences in gene expression. They replicated human and chimpanzee diets in laboratory mice and, after two weeks, they analyzed the differences in gene expression in the liver and in the brain. They found that 4-8% of genes expressed in the liver changed their expression; considering that only 15% of the genes expressed in the liver are differently expressed between humans and chimpanzees, these data are impressive. Thus, during dental development, from teeth primordia in intrauterine life up to calcification of the permanent teeth, the environment can act on all the factors listed above. Changes in one or more of those inputs can affect tooth development, producing a wide range of morphological variation.

4.4.2 Directionality of variability increase

The finding of directionality in the increase of the variability was unexpected. This is most apparent in the PC plots, where individuals belonging to the SG are scattered in the Cartesian plane non randomly with respect to the NSG individuals. In particular, along PC1, NSG individuals tend to fall mostly toward negative values of the first component, whereas SG individuals are uniformly distributed along the axis. Thus variation increases in the direction of more developed cusps (Fig. 4.2), a pattern which is also the most common in the univariate comparisons of each cusp.
We then considered two options to evaluate the source of the directionality observed: (i) it is a mathematical artifact due to the presence of a left wall in the distribution of ASU-DAS grades; (ii) it is a true directionality, given by the particular stressor considered, which alters development in a specific direction.

4.4.2.1 Directionality as a mathematical artifact

In general terms, when comparing two distributions with different mean values, it is reasonable to consider the distribution with a larger mean value as the effect of some directional factor. However, in some cases, it may happen that a non-directional increase in variability results in a directional change in the distribution, without any influence of a directional factor (Gould, 1988). This is the case for asymmetrical distributions with a left wall, a mode standing constantly near the wall and a right tail of variable length. The increase in the mean may be explained as an increase in variability which, because of the asymmetric distribution, is resolved in the right tail. In our sample (considering only statistically significant comparisons between NSG and SG), Carabelli's trait on M^2 and cusp 5 on M^3 seem to fall in this category. The mode of the NSG is 0 (absence of the trait) and variations from this state are absent or very rare; in the SG the mode does not change and more individuals fall into the right tail, which is also longer than in the NSG (Fig. 4.3). The increase in variability is visible only in the right tail, because the lowest value in our scale is 0 (absence of the trait), and this gives the impression of directionality. Therefore, the directionality in changes in morphology in the SG might derive from some non-directional factor. This could mean that the environmental stressor considered acts on a wide range of epigenetic parameters such as gene expression and cellular

growth, without any specificity, randomly with respect to the effects on the developmental cascade.

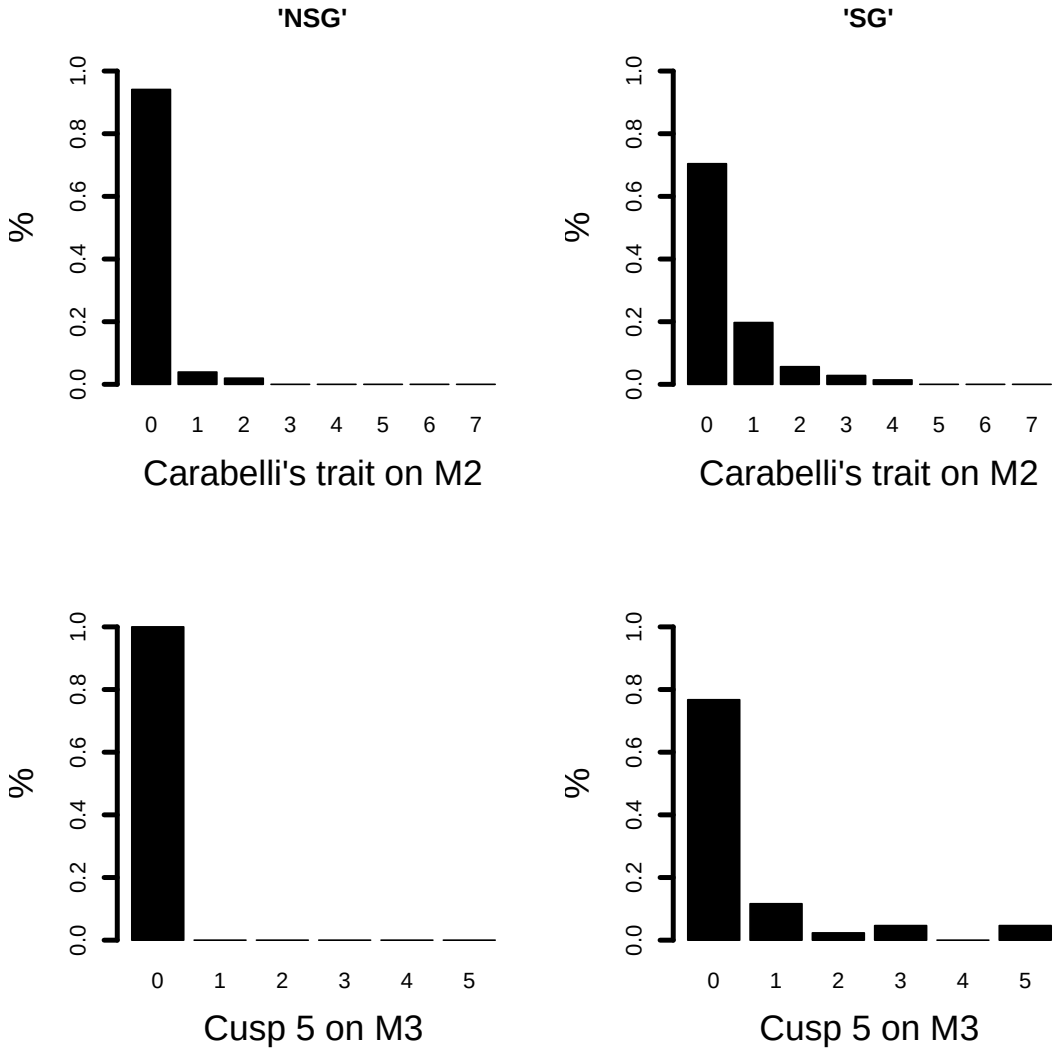

FIGURE 4.3: Differences between NSG (left column) and SG (right column) for Carabelli's trait on M^2 and cusp 5 on M^3. The directional increase in the mean of the SG could be a mathematical artifact due to a non-directional increase in variability in an asymmetric distribution with a left wall (absence of the trait) and a mode standing next to the wall.

4.4.2.2 True directionality

The likelihood of directionality as a mathematical artifact, however, does not fit with other results. Again, considering statistically significant results only, Carabelli's trait on M^1 and hypocone on M^2 show a different pattern of distribution in the comparison between NSG and SG (Fig. 4.4).

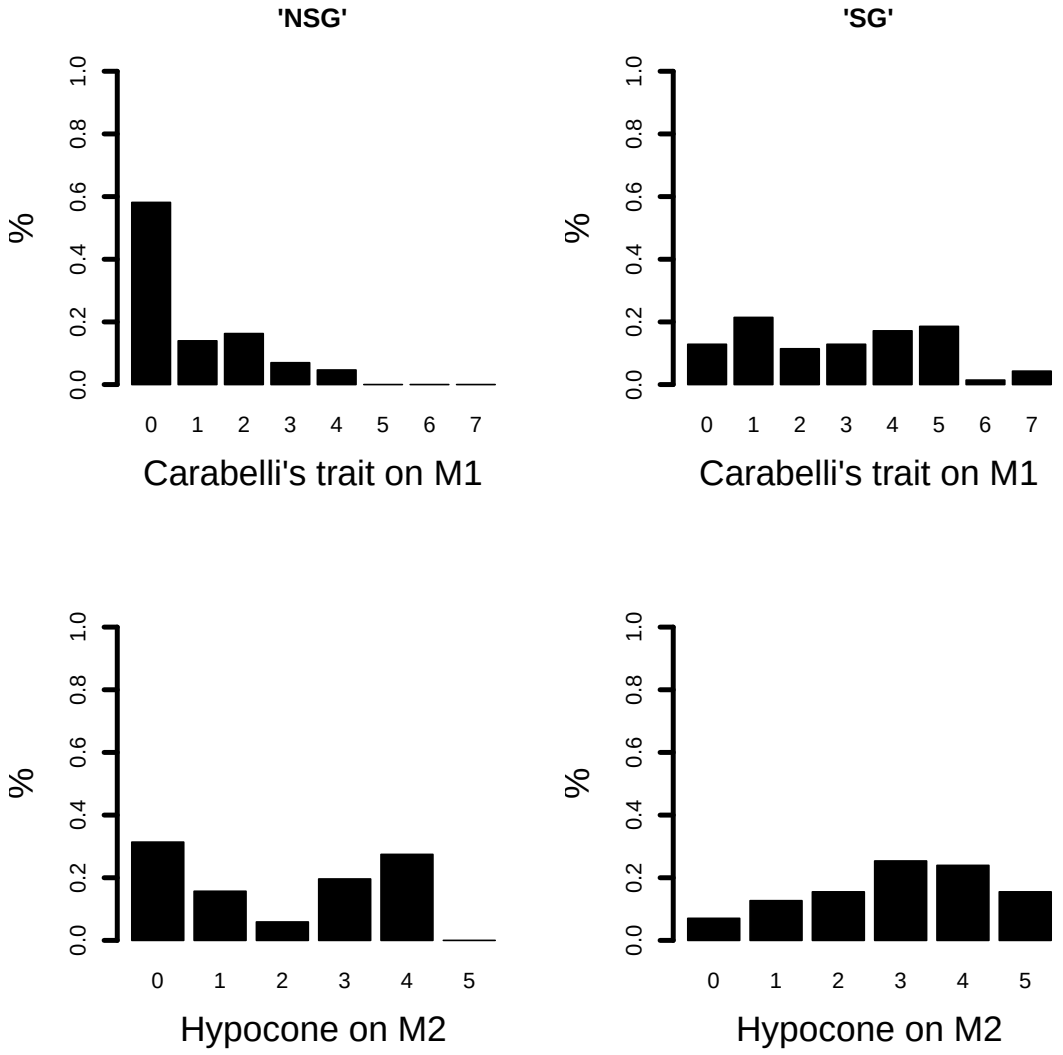

FIGURE 4.4: Differences between NSG (left column) and SG (right column) for Carabelli's trait on M^1 and hypocone on M^2. In this case, the increase in the mean of the SG cannot be due to a mathematical artifact, since the mode changes also; the most probable explanation is the action of a directional factor on the developmental response.

In these cusps the mean value increases in the SG but, unlike the former cases, the mode also increases in the SG. In particular for the Carabelli's trait on M^1, the whole distribution in the SG appears to be shifted toward higher grades, with a decrease in the "grade 0" and an increase in all the other grades. This evidence seems to support an explanation based on directional factors. Since dental development is an auto-organizing system, with a few starting inputs and a consequential cascade of events, with no apparent specific signals for different cusps, it is conceivable that the pattern of directional increase in the variability in the SG is produced by the same mechanism for

all cusps. For this reason, we cannot rely on an explanation based both on a non-directional and a directional factor. At the moment, a directional factor acting at the epigenetic level (gene expression and cellular parameters) seems to us to be the most probable explanation, since it can account for both types of distributions observed, whereas a non-directional factor fails to explain the distributions for Carabelli's trait on M^1 and hypocone on M^2.

Accepting a directional factor, which increases cusp development and number, appears to be in apparent contrast with results from other studies. McKee and Lunz (1990) reported a correlation between enamel hypoplasia occurrence and dental size reduction; however, this is not the case, since the ASU-DAS system, used in this study, takes into account the *relative* development of each trait in the tooth and not its absolute dimensions.
In another study, the expression of accessory cusps has been shown to be positively correlated with tooth size and negatively with cusp spacing (Hunter et al. 2010): thus, if cusp spacing doesn't change, smaller teeth should have less space for accessory cusps. Also, recent studies (Guatelli-Steinberg et al. 2013; Moormann et al. 2013) pointed out that, along the molar line, dental size reduction and cusp spacing are correlated with a negative allometry and cusp spacing decreases slower than tooth size. For this reason, a decrease in size on M^2 is not balanced by reduced cusp spacing and second molars usually have less accessory cusps. In our sample we didn't consider tooth size but it is likely that dental size is reduced in the hypoplastic sample. Therefore, the expectation should be to observe less accessory cusps in the SG.
However, the relationship between tooth size and cusp spacing for one tooth (for example M^1) in different individuals doesn't have to be the same of that observed on the molar line. Differences between M^1 and M^2 don't necessarily reflect differences between a large M^1 and a small one. For example, Salazar-Ciudad and Jernvall (2010) have suggested that differences along the molar line are explained best by cellular parameters, while individual differences between the same teeth are best explained by regulation of signalling during development. Thus, it is possible that, within the same tooth, differences in regulation between individuals can alter the allometry between cusp spacing and tooth size but differences along the tooth row (between metamers) don't change.

If we accept this hypothesis, then we might expect that the stressor considered acts not randomly on the variety of parameters affecting dental development. Among these parameters, regulation of signaling during cusp development seems to be the most important source of variation among individuals (Salazar-Ciudad and Jernvall, 2010). Cusps pattern is controlled by interactions between signaling molecules regulating epithelial and mesenchymal cell proliferation, such as FGF-4, BMP, and Shh (Jernvall et al., 1994; Vaahtokari et al., 1996). These interactions regulate the induction of new enamel knots, that is to say they produce the cusp pattern in a forming tooth. BMPs (Bone Morphogenetic Proteins), in particular, are associated with enamel knot apoptosis and its termination, for example inducing inhibitors of cell proliferation like *p21* (Jernvall et al., 1998) and *ectodin* (Kassai et al., 2005); studies on *ectodin* expression demonstrated that *ectodin*-deficient mice have extra teeth and extra cusps originated from a larger primary enamel knot (Kassai et al., 2005). Therefore, an environmental stressor could produce the pattern of increased cusp size and number simply inhibiting the production of BMPs, or BMP-induced molecules. Studies on gene expression during dental development are needed to confirm or reject this hypothesis.

As a final remark, we would like to consider the possible evolutionary significance of the kind of plasticity observed in dental development. It has been shown that the evolutionary history of mammalian dental pattern is characterized by repeated convergent or parallel evolution of new cusps such as the hypocone (Hunter and Jernvall, 1995; Jernvall, 2000; Jernvall et al., 1996), whereas cusp loss is an uncommon event. That is to say that the developmental response to environmental stress appears to be in the same direction as the evolutionary trend in mammalian dentition. There are several possible explanations. For example, the developmental response could reflect a developmental constraint biasing variation in a certain direction; a non-isotropic variation due to developmental reasons may result in a directionality imposed to evolution (Gould, 2002). Another intriguing possibility is that developmental plasticity in dental development plays an active role in evolution. Developmental plasticity is a topic that has received increasing attention in evolutionary studies (Aubret and Shine, 2009; Chapman et al., 2000; Emlen et al., 2007; Gibson and Hogness, 1996; Price et al., 2003; Price, 2006; Rutherford and Lindquist, 1998; Sollars et al., 2003; Stauffer and van Snik Gray, 2004; West-Eberhard, 2003). For example, plasticity in certain morphological traits could allow survival in difficult times; phenotypic changes, with the time, may accommodate and lead to a genetic

change in the same direction (Pigliucci and Murren, 2003; Pigliucci et al., 2006). Scholars are still debating this topic and there is no consensus (de Jong, 2005; Pigliucci et al., 2006), but it is certainly an interesting avenue for future investigations - also with special reference to dental evolution - in particular because nutritional stress as a result of environmental pressures is common in many species and may have played a significant role in human and mammalian evolutionary history.

Chapter 5

Future perspectives: the evolution of mammalian dentition

Mammalian evolutionary history is characterized by repeated convergent or parallel evolution of dental traits. The hypocone, for example, has evolved more than 20 times in different mammals linages (Hunter and Jernvall, 1995) and the protocone-like structure of tribosphenic and pseudotribosphenic molars has been recognized to be a homoplastic feature evolved more than once (Luo, 2007). This pattern of repeated evolution of similar traits can be explained by two different (but not alternative) models: (i) there is a constant selective pressure favoring individuals whose teeth have more cusps; (ii) there is a bias in development favoring variation in the direction of multicusped teeth. Some evidences suggest a role for the latter model. Jernvall and Salazar-Ciudad (2007) point out that "whereas in the most Mesozoic forms (for example, in rhyncosaurs and cynodonts) increased chewing efficiency was accomplished by increasing individual tooth number, later forms (tritylodonts, multituberculates, eutherian mammals) increase cusp numbers within individual teeth" (p. 210). In other words, the same evolutionary need (increased chewing efficiency) is satisfied through different developmental mechanisms (increase of tooth number VS increase of cusps number). In mammals, the mechanism is a reiteration of the same developmental module (the enamel knot) and its tendency to produces an increase in tooth complexity is called iterative sub- zoning (Jernvall and Salazar-Ciudad, 2007).
As we have seen in Chapter 4, in my research I found other clues suggesting a bias in dental morphology variation: my attempt was to investigate if

and how an environmental stressor could affect dental morphology (in human upper molars). Answering this question three major results came out: (i) there is a significant effect of environmental stressors like malnutrition and/or systemic diseases on the overall morphology of upper molars; (ii) the stressor generates a developmental response which increases the morphological variability of the stressed population; (iii) the increase of variability is directional, since stressed individuals have increased cusps dimensions and number. This bias in developmental variation in favor of more complicated teeth, recalls to mind the same bias in mammalian evolution: the evolution of a new cusp is a more frequent event than the loss of an already existing one.
These results open up new lines of research. In particular three main question arise:

1. does the bias towards dental complexity at the macroevolutionary level could stem from the bias in the same direction at the developmental level?
2. What is the role of environment in such a process? Has it an active role in leading this evolutionary trend?
3. In the case that the macroevolutionary trend is led by developmental processes, what is the role of selection in the evolution of tooth morphologies?

In this chapter I want to focus on some theoretical possibilities linked to these questions.

5.1 The role of environment

The bias in developmental response we observed in dentition could be simply representative of a developmental constraint causing a non isotropic variation of dental morphology; in this case the raw material on which natural selection acts would be biased and evolution would proceed necessarily in the direction imposed by development (Gould, 2002; Jernvall and Salazar-Ciudad, 2007): the role of the environment, in this case, would be null or marginal. But, paraphrasing the words of Waddington (1942) on ostrich callosities, the orthodox explanation, leaves entirely out of account the fact that new cusps may be produced by an environmental stimulus.
An alternative explanation, including developmental plasticity, would be

much more satisfactory. In particular, it is important to notice that one of the causes suggested to explain the pattern of increased variability, is malnutrition. And malnutrition and diet are already known to produce developmental responses, which, sometimes, can drive evolution. I suggest that the effects of malnutrition could have been an important and recurring trigger of morphological evolution.

5.1.1 Recurrence of malnutrition in evolution

Malnutrition, in his broader definition, is a state in which diet is insufficient or poorly balanced, or there is a defect in digestion or utilization of foods (Rush 1997); Simko et al. (1995) refers at malnutrition as to "inadequacies and imbalances in the diet, with respect to either the quality or the quantity of food consumed. This condition could result from [...] poor access to food as a result of poverty [and] environmental factors that affect food availability [...]" (p. 28). Diet is a direct link between an animal and its environment and environmental dynamics likely play an important role in dietary changes (Ungar et al. 2006); any time a population undergoes to a change in its habitat, a situation of malnutrition may occur: a colonization of a new habitat, or an abrupt change in the environment, can limit the available foods or can force individuals to exploit new resources and to establish new feeding regimes and feeding modes. Situations like these must have been common in human evolution and the origin of some taxa is often supposed to be correlated with dietary shifts.

For example Teaford and Ungar (2000) reviewed diet correlates in some cranio-dental features as tooth size, tooth shape, enamel structure, dental microwear and mandibular biomechanics. Comparing Miocene hominoids and Pliocene early hominids they suggest "a dietary shift at or near the stem of hominid evolution"(p. 13509). Also the radiation of *Paranthropus* and *Homo* is supposed to be correlated with the global cooling and drying occurred during the Late Pliocene (Ungar et al. 2006). The genus *Homo* responded to these changes evolving subsistence strategies to exploit a greater variety of foods (Ungar et al. 2006), while the genus *Paranthropus* (at least *P. boisei*) responded to the spread of grasslands with a specialization in consuming large quantity of low-quality vegetation (Cerling et al. 2011). Lastly, a shift to a high quality diet is supposed to be the trigger that allowed our ancestors to respond to selection for bigger brains, because a high quality

diet means a easy digestion and a shorter gut, making available more energetic resources for the other "expensive tissue" in our body, the brain (Aiello and Wheeler 1995).

5.1.2 The role of diet: an input for evolution

As said above, diet is a direct link between an animal and its environment (Ungar et al. 2006). Moreover, diet is essential to allow to an organism to develop and grow up: therefore, it is not surprising that diet may have a direct influence on the final morphology of an organism or in the expression of some genes through phenotypic and developmental plasticity. A number of studies demonstrated this relationship.

Beetle horns, for example, are known to be responsive to nutrition during their development. Nutrition during the pupal phase influence the development of horns, in particular horn's length, causing allometric changes in beetle's development (Emlen 1994). In *Onthophagus taurus*, a species of dung beetle, the extent of paternal effort to provide foods to their offspring establish the differentiation of the males in two morphs, one with horns, the other hornless (Hunt and Simmons 2000).

In fishes there are a lot of studies about trophic polymorphism (Ehlinger 1990; Wainwright et al. 1991). For example cichlids show a high phenotypic plasticity in facial morphology due to the diet they are fed (Meyer 1987); Stauffer and Van Snik Gray (2004) demonstrate how, at least in cichlids, trophic polymorphism is due to different biomechanical stress on the masticatory system during ontogeny, linked to the different mode of feeding.

Pattern of gene expression are demonstrated to be susceptible to nutrition. In bees, queens and sterile workers differentiate from genetically identical larvae thanks to a different nutrition that probably influences the expression of some genes through DNA methylation (Kucharski et al. 2008). In Drosophila gene expression of some genes is regulated by starvation (Zinke et al. 2002).

Diet influence the pattern of methylation also in mammals and the stable expression of some genes (Jaenisch and Bird 2003); nutrition is important also in gestational period and can influence the phenotype of the offspring, for example inducing changes in methylation in rats (Burdge et al. 2007).

In humans, it is known that babies respond to undernutrition changing the trajectory of their development; moreover their morphology and physiology is affected by the nutritional state of the mother (Bateson et al. 2004).

Somel et al. (2008) found that differences in diet can produce differences in gene expression. They replicated human and chimpanzee diets in laboratory mouses and, after two weeks, they analyzed differences in gene expression in the liver and in the brain. They found 4-8% of genes expressed in the liver changed their expression; these data are impressive if we consider that the difference between man and chimpanzee is about 15%.
In some of the cases listed above there are clues that plasticity due to the diet is linked to evolutionary changes. Emlen et al. (2007) proposes that the sensitivity of horn's dimensions to nutrition can explain the patterns of variation in horn allometry in and between species; this process played a central role in the differentiation of beetle horns, especially in the evolution of extreme or exaggerated horns. Stauffer and Van Snik Gray (2004) suggest that phenotypic plasticity aided in the rapid trophic radiation and subsequent speciation of the Old World cichlids. Somel et al. (2008) found that the genes expressed differently both between mice fed human and chimpanzee diets and between humans and chimpanzee evolve faster then the other genes expressed in the liver: for the authors this means that dietary changes "may have caused some adaptation in the human and chimpanzee genomes"(p. e1594).

5.1.3 Lamarckian revival

If we accept diet shift and malnutrition as a trigger for evolutionary change through mechanisms as genetic accommodation, in which the phenotype lead the evolutionary change and the genes follow it (Schwander and Leimar 2011), then the model of evolution we encounter is more similar to Lamarck's than it is to Darwin's. Merging the lists in Chapter 1 and Chapter 3:

1. Environmental conditions change;
   Example: a general drying up of climatic conditions causes a diminishing of forest and a spread of grassland.
2. The species adapt to the new conditions changing behaviour;
   Example: a species, which before fed of leaves in the forest, now must find some other resources to exploit, such as underground storage organs or grassland seeds.
3. New behaviours induce new phenotypes through plasticity (phenotypic accommodation);
   Example: with the new diet also nutrients and gene expression change and

there is a developmental disruption that produces new phenotypes, like new cusps in molars.

4. The new phenotypes can be genetically accommodated; In this perspective environment plays an indirect role and the new phenotypes arise in response to a change in behaviour that produces a developmental disruption and a plastic response.

5.2 Reconsidering the role of natural selection

Typically, the strong correspondence between tooth shape and dietary function has been interpreted as the result of an adaptation Butler (1983). However, the problem of how new cusps evolve through natural selection remain unsolved, since in their initial stages they does not occlude and thus they have no functional role.
If the first question rose at the beginning of this Chapter receives an affirmative answer and the bias towards dental complexity at the macroevolutionary level stems from the bias in the same direction at the developmental level, then the role of natural selection in shaping dental morphology must be reconsidered. Indeed, a complicated tooth would not arise for functional needs, but independently of them, due to developmental reason.
Also in the case that some sort of genetic accommodation occurred in dental evolution, it is possible to suppose that developmental dynamics prevailed on natural selection of the trait. Usually, selection on the new phenotype is the mechanism called in cause to advocate the genetic accommodation of the new traits; however, Masel (2004) demonstrated that, in a theoretical model, it is possible to have genetic assimilation in absence of selection. The requirements for this to happen are that (i) environment produces a phenocopy (a trait that can be induced both by genetic and environmental input) (ii) there is a selection for a developmental equilibrium, that is development must be complete in a determined time.
Dental development has both these requirements: indeed (i) new cusps can be obtained both environmentally that genetically (see Chapter 4) and (ii) tooth development must be completed before occlusion occurs, that is there is a selection to have tooth development complete in a certain time. Dental evolution seems to be the real counterpart of the in silico model proposed by Masel (2004). Both the cases examined require re-thinking the role of

natural selection in dental evolution. It is possible that, in some cases, a complicated dental morphology should be re-interpteted as an exaptation as meant by Vrba and Gould (1986): a causal process at a lower level (developmental network) that produces an exaptation at the higher level (the organism).

Appendix A

Supplementary information to Chapter 2

The next plots show the comparison between healthy and malnourished Nigerian children for each of deciduous tooth. In all the cases examined, the pattern of increased variability in asymmetric distributions is evident.

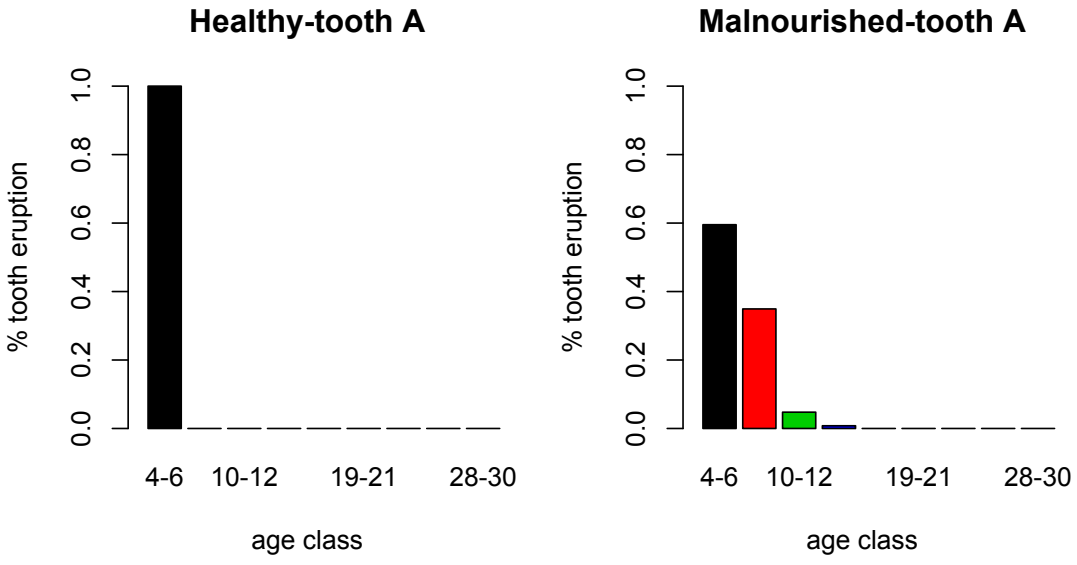

FIGURE A.1: Comparison between healthy and malnourished children for the mandibular deciduous tooth A

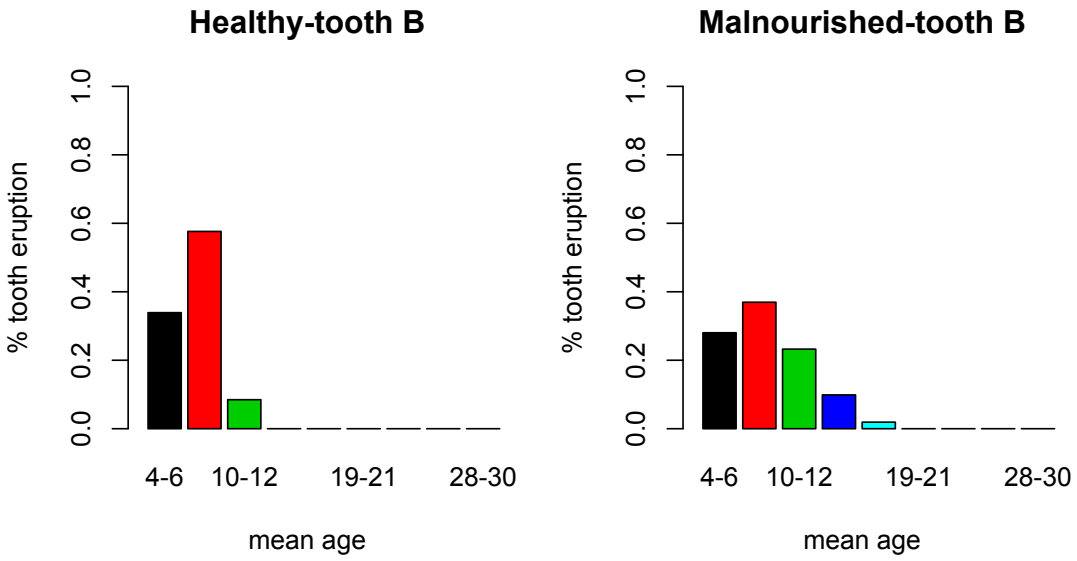

FIGURE A.2: Comparison between healthy and malnourished children for the mandibular deciduous tooth B

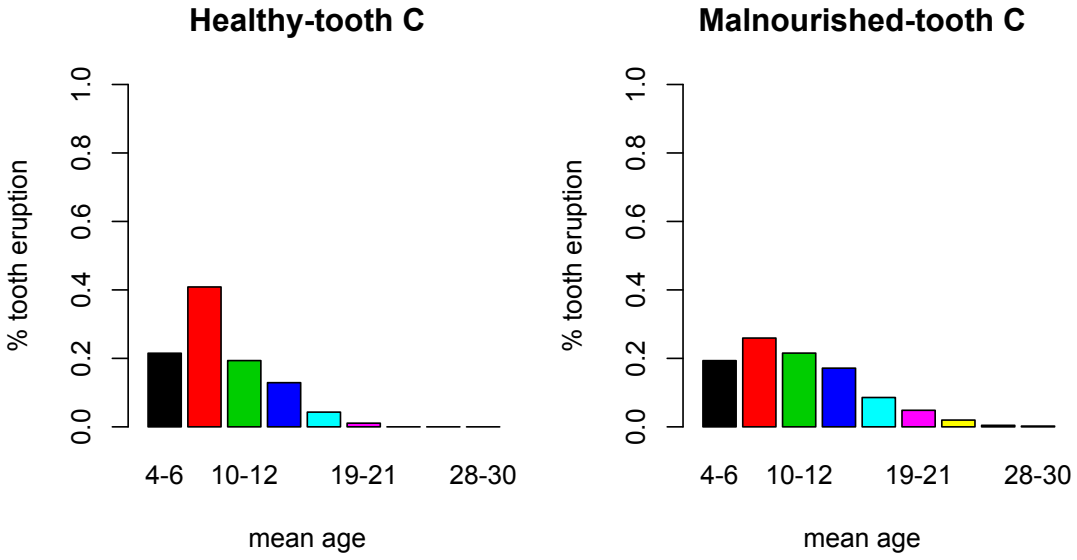

FIGURE A.3: Comparison between healthy and malnourished children for the mandibular deciduous tooth C

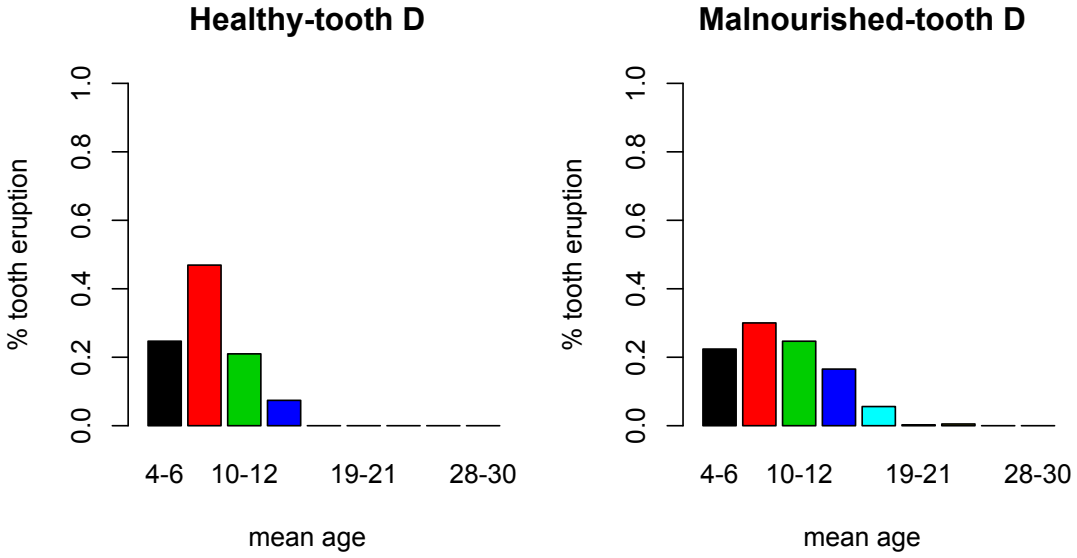

FIGURE A.4: Comparison between healthy and malnourished children for the mandibular deciduous tooth D

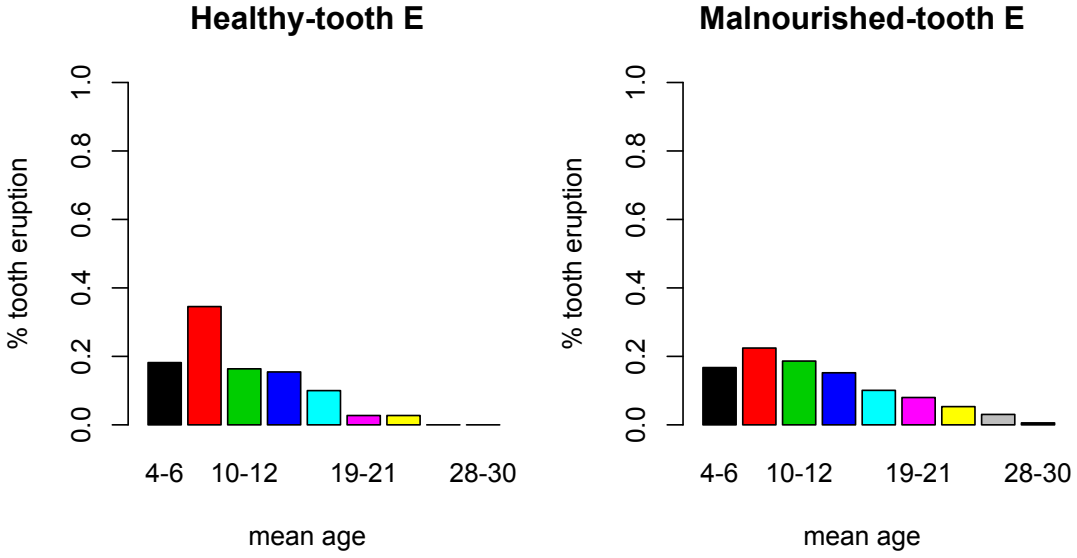

FIGURE A.5: Comparison between healthy and malnourished children for the mandibular deciduous tooth E

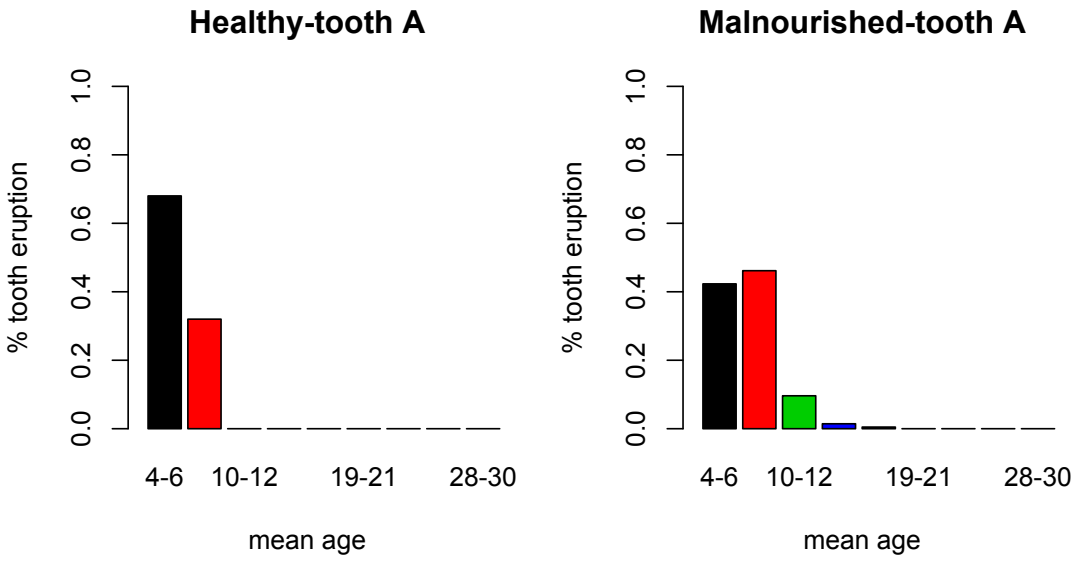

FIGURE A.6: Comparison between healthy and malnourished children for the maxillary deciduous tooth A

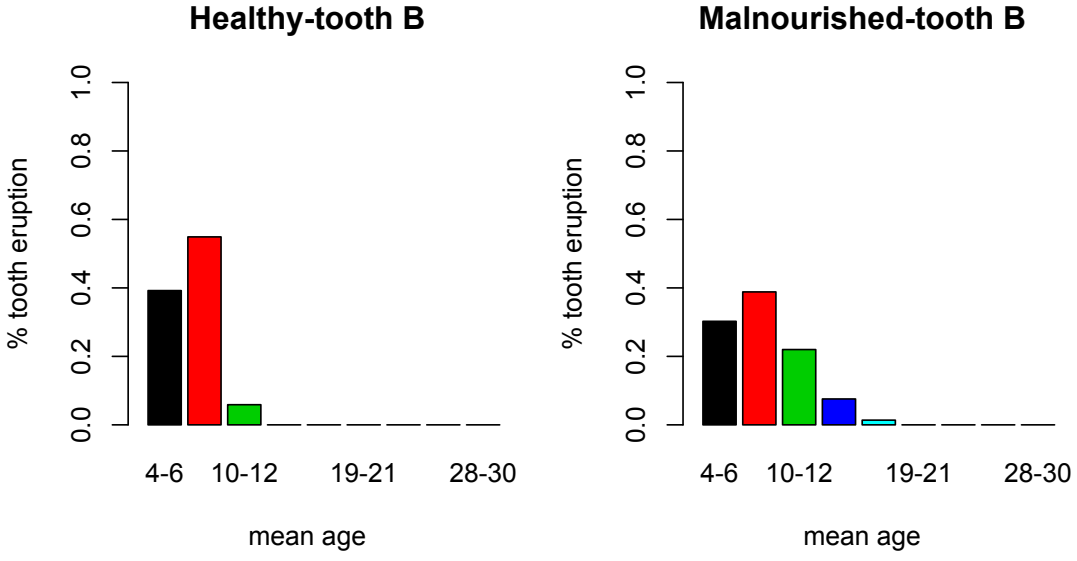

FIGURE A.7: Comparison between healthy and malnourished children for the maxillary deciduous tooth B

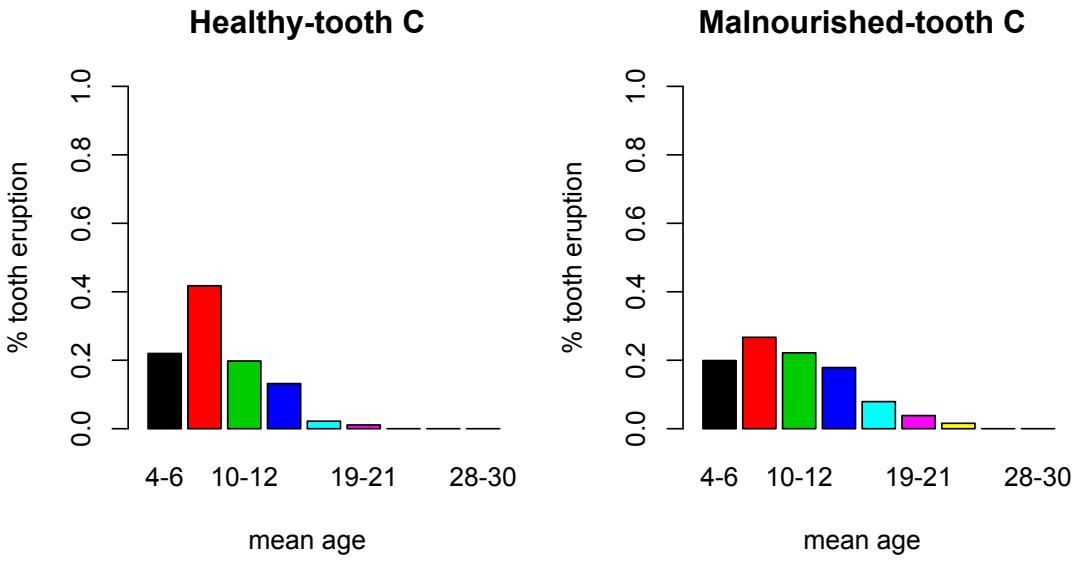

FIGURE A.8: Comparison between healthy and malnourished children for the maxillary deciduous tooth C

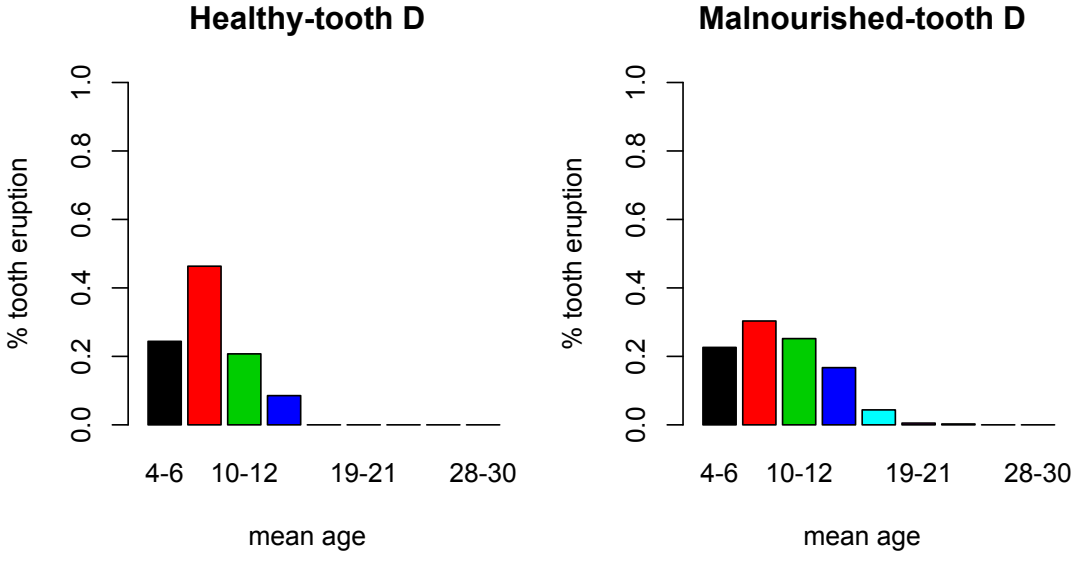

FIGURE A.9: Comparison between healthy and malnourished children for the maxillary deciduous tooth D

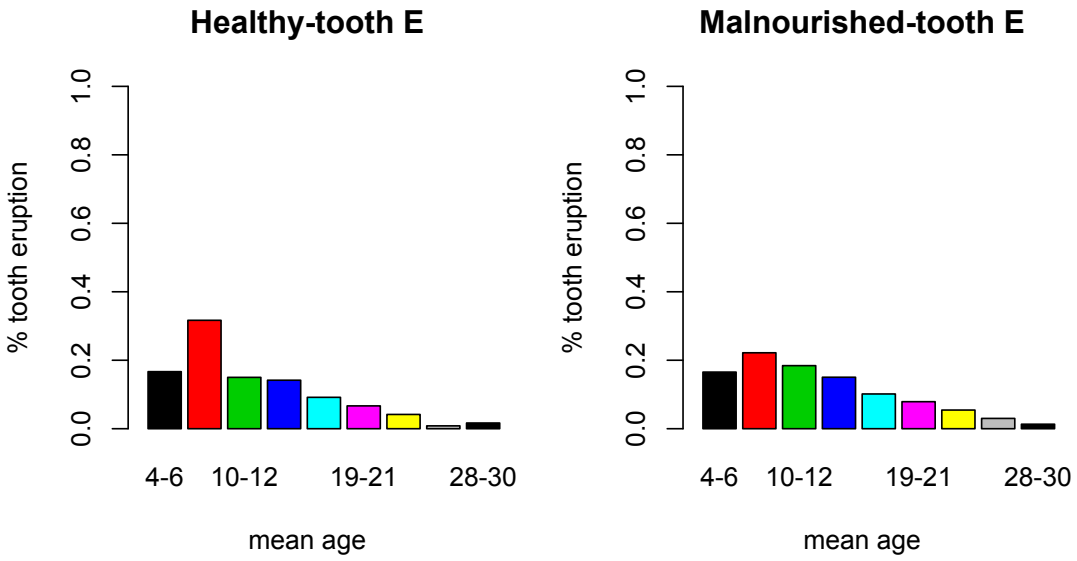

FIGURE A.10: Comparison between healthy and malnourished children for the maxillary deciduous tooth E

Appendix B

Supplementary information to Chapter 4

B.1 Frequencies of dental traits in the sample examined

Metacone	n	0	1	2	3	4	5
M^1	116	0	0	0	0	18.1	81.9
M^2	125	0	0	0.8	9.6	59.2	30.4
M^3	69	0	0	1.4	40.6	44.9	13.0

TABLE B.1: Relative frequencies of Metacone for the total sample, with sex and stress level pooled.

Hypocone	n	0	1	2	3	4	5
M^1	114	0	0	0	7.0	32.5	60.5
M^2	122	17.2	13.9	11.5	23.0	25.4	9.0
M^3	70	38.6	14.3	11.4	28.6	7.1	0

TABLE B.2: Relative frequencies of Hypocone for the total sample, with sex and stress level pooled.

Cusp 5	n	0	1	2	3	4	5
M^1	113	92.0	4.4	0.9	1.8	0.9	0
M^2	122	93.4	5.7	0	0.8	0	0
M^3	69	85.5	7.2	1.4	2.9	0	2.9

TABLE B.3: Relative frequencies of Cusp 5 for the total sample, with sex and stress level pooled.

Carabelli	n	0	1	2	3	4	5	6	7
M^1	113	30.1	18.6	13.3	10.6	12.4	11.5	0.9	2.7
M^2	122	80.3	13.1	4.1	1.6	0.8	0	0	0
M^3	67	85.1	4.5	7.5	0	1.5	1.5	0	0

TABLE B.4: Relative frequencies of Carabelli's trait for the total sample, with sex and stress level pooled.

Parastyle	n	0	1	2	3	4	5	6
M^1	116	100	0	0	0	0	0	0
M^2	125	99.2	0	0	0	0.8	0	0
M^3	69	98.6	0	0	1.4	0	0	0

TABLE B.5: Relative frequencies of Parastyle for the total sample, with sex and stress level pooled.

B.2 Univariate comparison between non stressed (NSG) and stressed (SG) groups

This section presents supplementary plots to those presented in Chapter 4. On the left there are the distributions of NSG and on the right those of SG; in the figure captions is also reported the $p - value$ obtained from the Wilcoxon test. Threshold was set to $p = 0.05$; after Bonferroni's correction, the new threshold is $p = 0.00333$

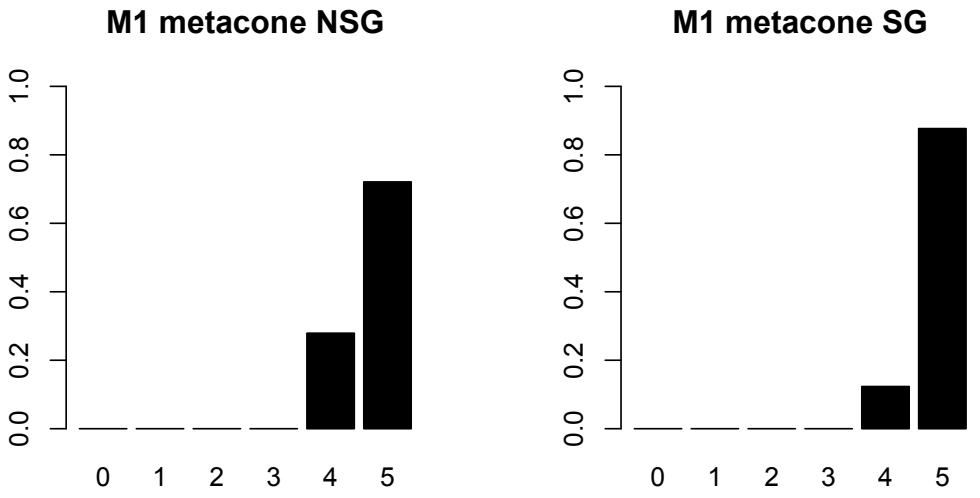

FIGURE B.1: Differences between NSG (left column) and SG (right column) for Metacone on M^1. $p - value = 0.03651$ (Wilcoxon test)

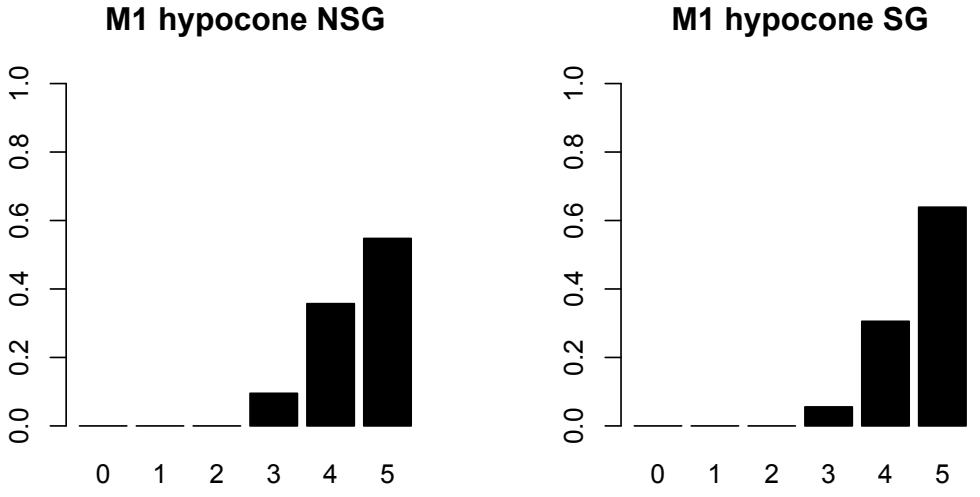

FIGURE B.2: Differences between NSG (left column) and SG (right column) for Hypocone on M^1. $p - value = 0.30211$ (Wilcoxon test)

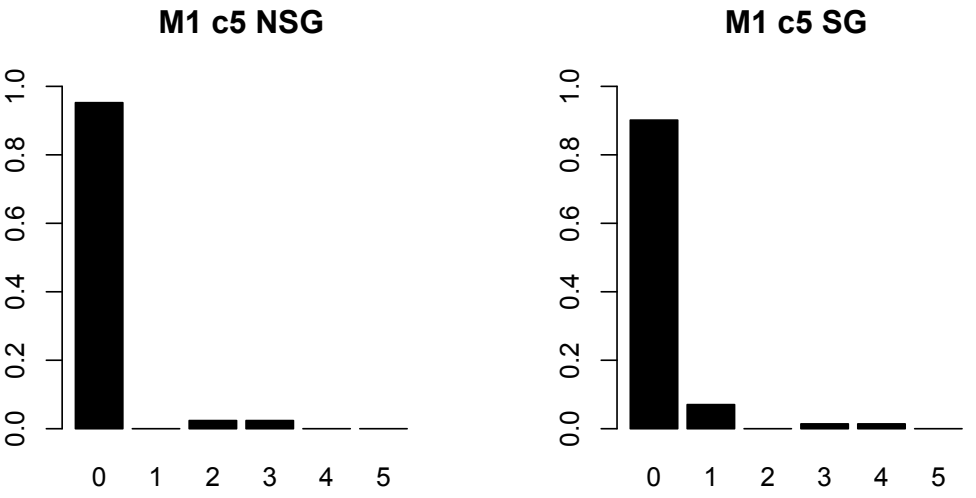

FIGURE B.3: Differences between NSG (left column) and SG (right column) for cusp 5 on M^1. $p - value = 0.36212$ (Wilcoxon test)

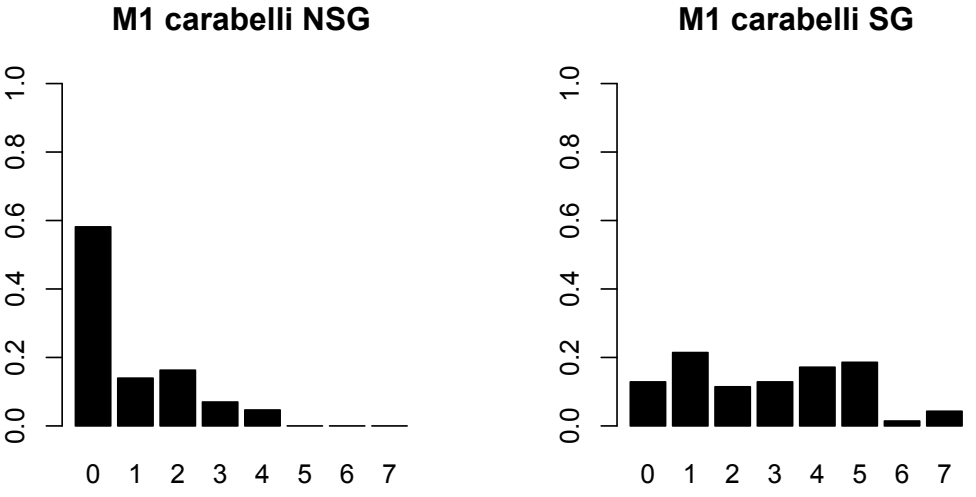

FIGURE B.4: Differences between NSG (left column) and SG (right column) for Carabelli's on M^1. $p - value = 0.00000$ (Wilcoxon test)

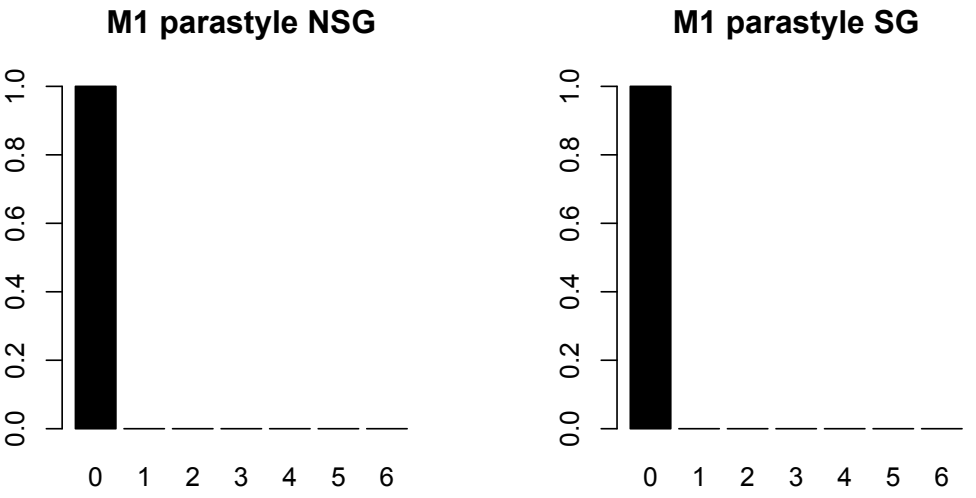

FIGURE B.5: Differences between NSG (left column) and SG (right column) for peristyle on M^1. $p - value = na$ (Wilcoxon test)

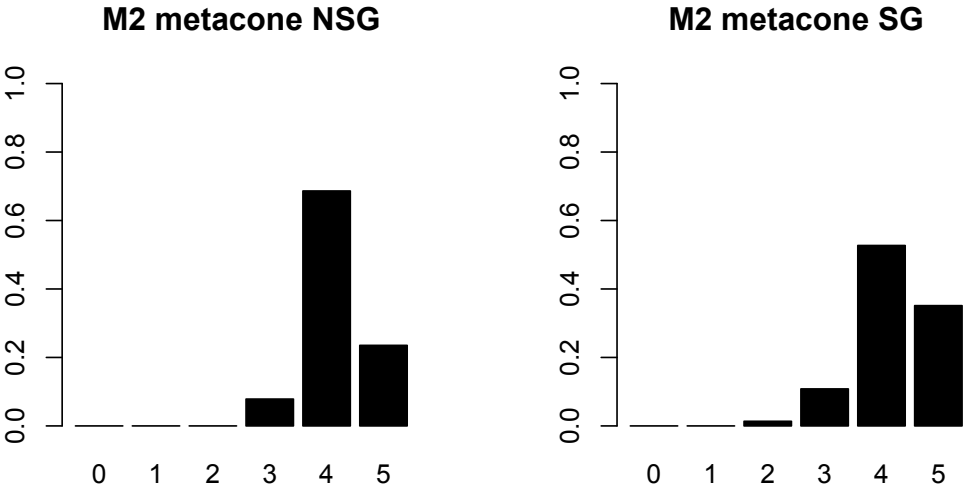

FIGURE B.6: Differences between NSG (left column) and SG (right column) for Metacone on M^2. $p - value = 0.43094$ (Wilcoxon test)

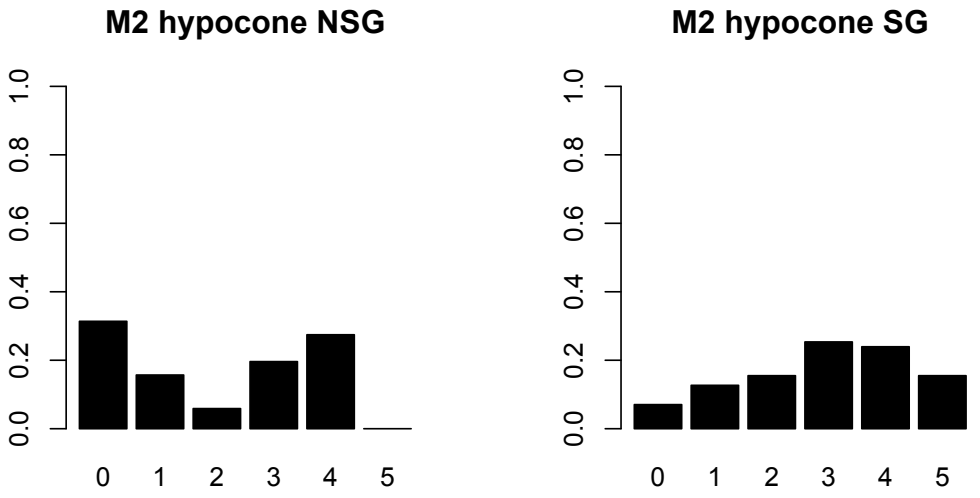

FIGURE B.7: Differences between NSG (left column) and SG (right column) for Hypocone on M^2. $p - value = 0.00236$ (Wilcoxon test)

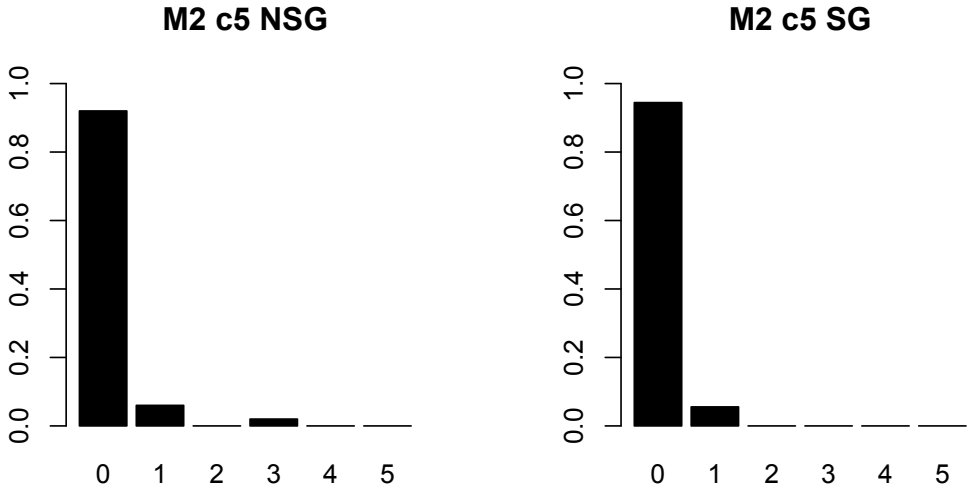

FIGURE B.8: Differences between NSG (left column) and SG (right column) for cusp 5 on M^2. $p - value = 0.58074$ (Wilcoxon test)

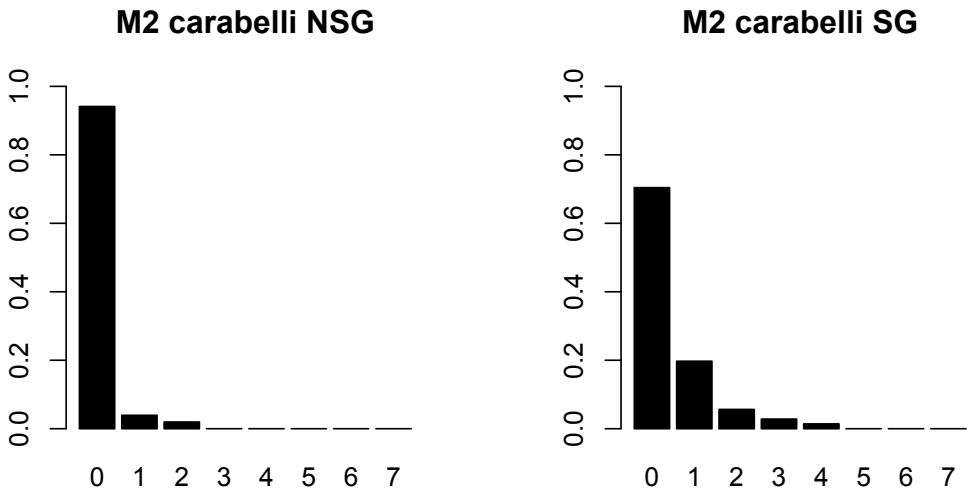

FIGURE B.9: Differences between NSG (left column) and SG (right column) for Carabelli's on M^2. $p - value = 0.00126$ (Wilcoxon test)

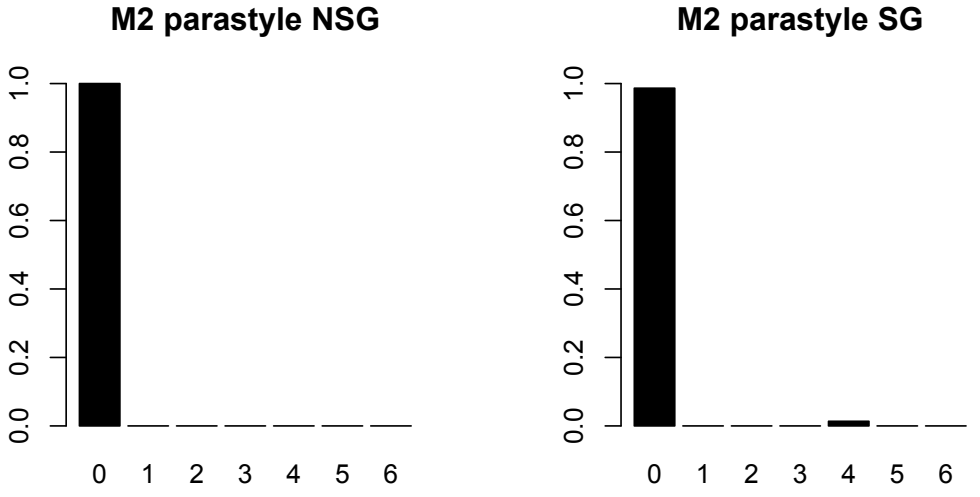

FIGURE B.10: Differences between NSG (left column) and SG (right column) for peristyle on M^2. $p - value = 0.41570$ (Wilcoxon test)

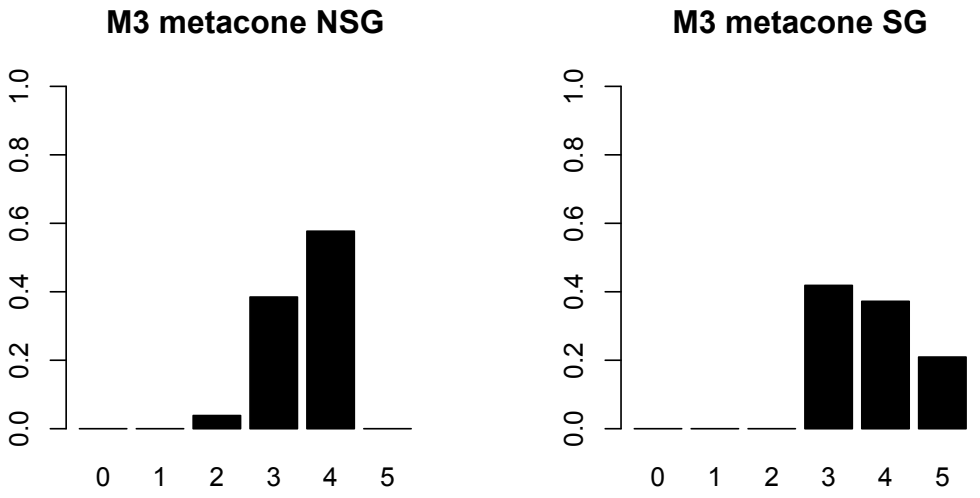

FIGURE B.11: Differences between NSG (left column) and SG (right column) for Metacone on M^3. $p - value = 0.28900$ (Wilcoxon test)

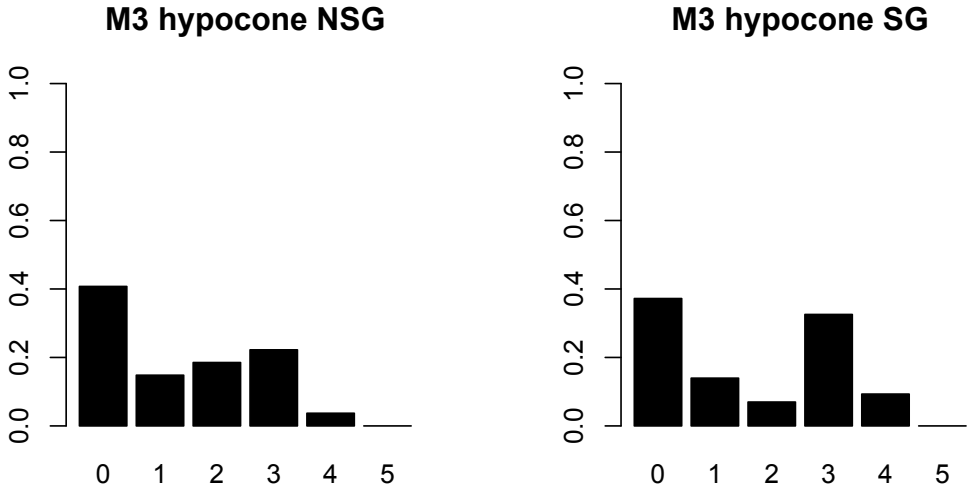

FIGURE B.12: Differences between NSG (left column) and SG (right column) for Hypocone on M^3. $p - value = 0.40862$ (Wilcoxon test)

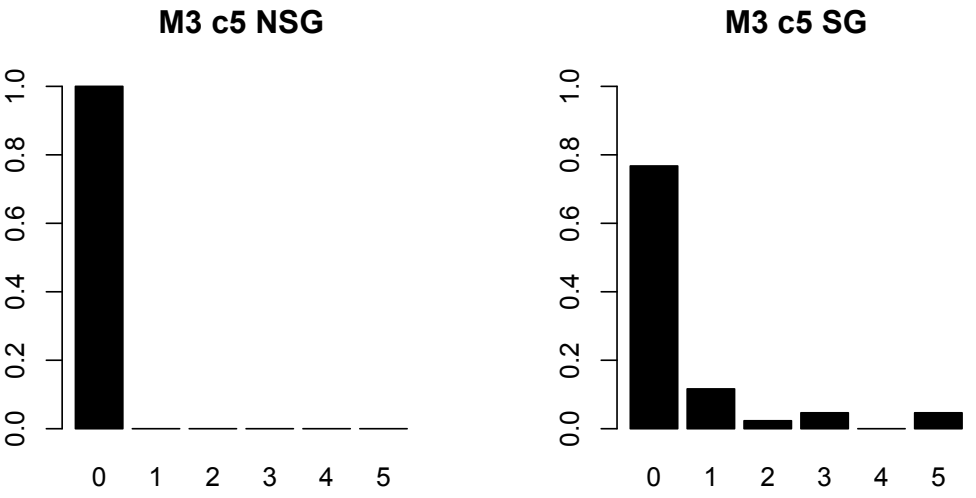

FIGURE B.13: Differences between NSG (left column) and SG (right column) for cusp 5 on M^3. $p - value = 0.00878$ (Wilcoxon test)

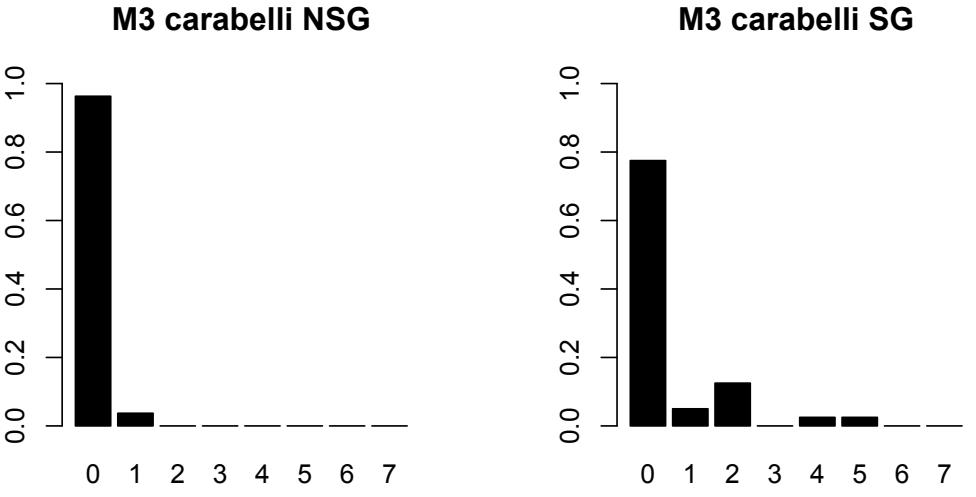

FIGURE B.14: Differences between NSG (left column) and SG (right column) for Carabelli's on M^3. $p - value = 0.03107$ (Wilcoxon test)

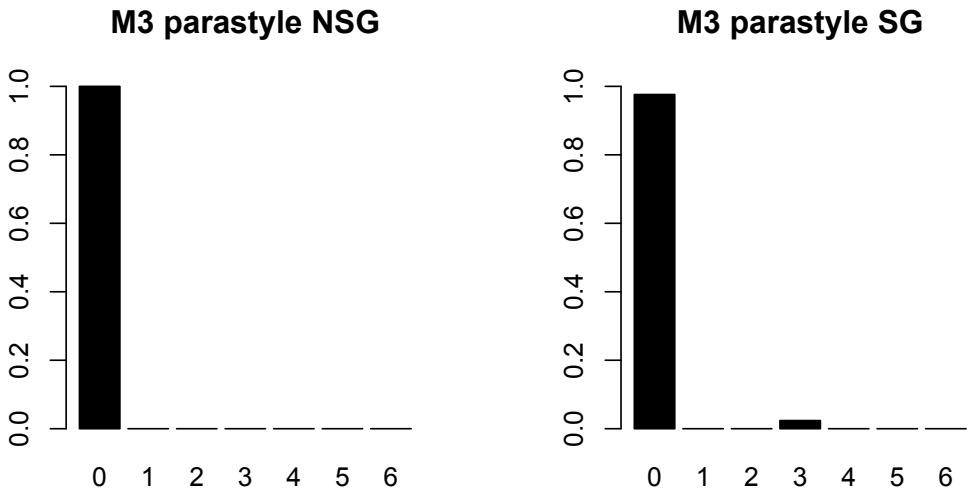

FIGURE B.15: Differences between NSG (left column) and SG (right column) for peristyle on M^3. $p - value = 0.44006$ (Wilcoxon test)

B.3 Kernell's density plots

For a better understanding of the differences in variability of the traits in the multivariate analysis, in this section Kernell's density plot are presented for each of the three principal components obtained for the three molars pooled together. In each plot is possible to see how variability in the stressed group is higher and biased in one direction.

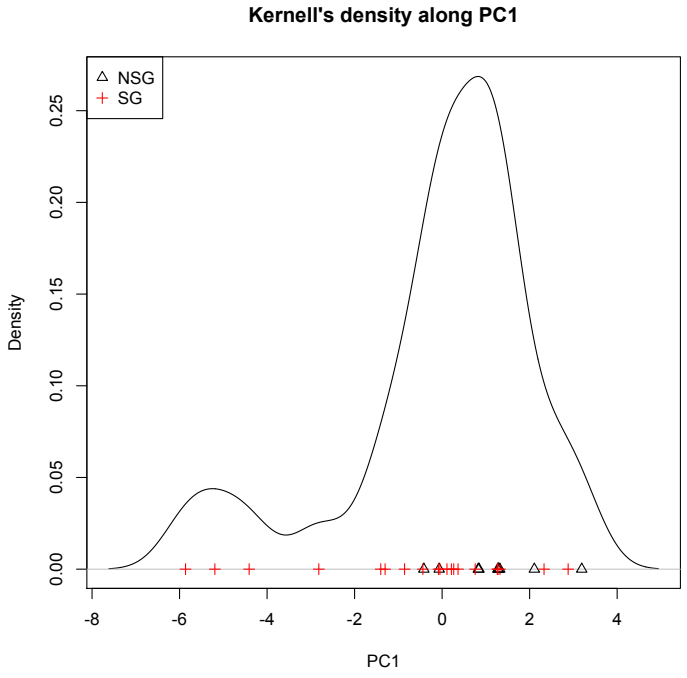

FIGURE B.16: Kernell density plot for PC1. PC1 varies between -3.19 and 0.42 in NSG and between -2.88 and 5.86 in the SG

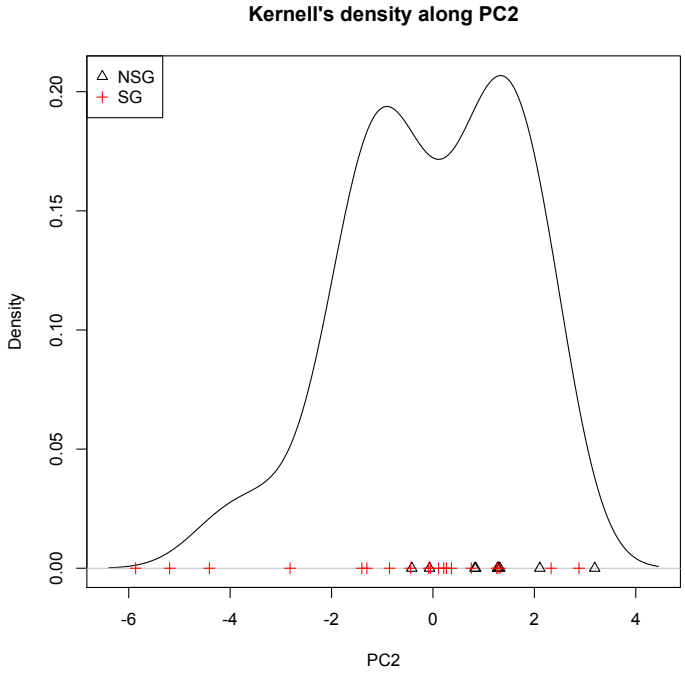

FIGURE B.17: Kernell density plot for PC2. PC2 varies between -1.62 and 2.10 in NSG and between -4.12 and 2.17 in the SG

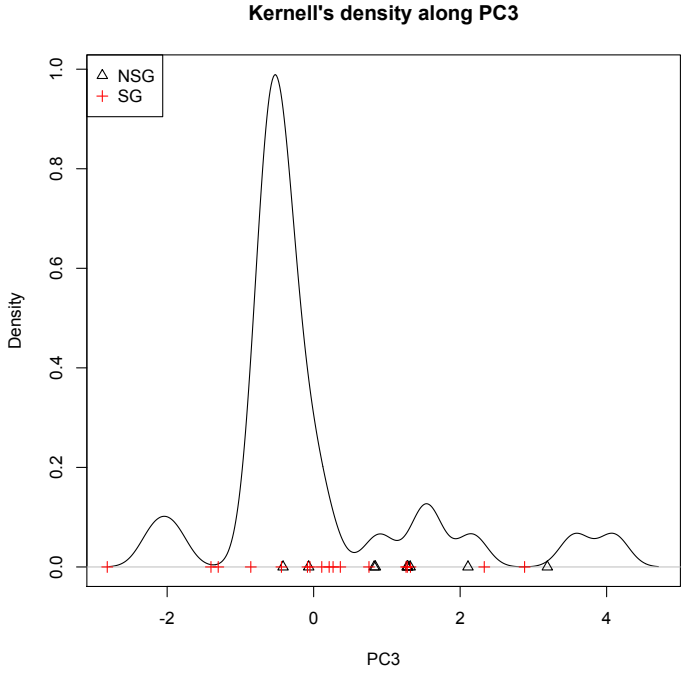

FIGURE B.18: Kernell density plot for PC3. PC3 varies between -070 and 0.17 in NSG and between -2.18 and 4.09 in the SG

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