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1 **The *flexible stem hypothesis*: evidence from genetic data**

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21 **Abstract: Phenotypic plasticity, the ability of a given genotype to produce**
22 **different phenotypes in response to distinct environmental conditions, is**
23 **widely observed in the wild. It is believed to facilitate evolution and, under**
24 **the “flexible stem hypothesis”, it is thought that an ancestral plastic species**
25 **can be at the origin of sister lineages with divergent phenotypes fixed by**
26 **genetic assimilation of alternative morphs. We review here the genetic**
27 **mechanisms underlying such phenomenon. We show several examples in**
28 **which the same gene shows transcriptional plasticity in response to**
29 **environmental factors and divergence of expression within or between**
30 **species. Thus, the same gene is involved both in the plasticity of a trait and**
31 **in the evolution of that trait. In a few cases, it can be traced down to *cis*-**
32 **regulatory variation in this gene and, in one case, in the very same**
33 **regulatory sequence whose activity is modulated by the environment.**
34 **These data are compatible with the “flexible stem hypothesis” and also**
35 **suggest that the evolution of the plasticity of a trait and the evolution of the**
36 **trait are not completely uncoupled as they often involve the same *locus*.**
37 **Furthermore, the “flexible stem hypothesis” implies that it is possible to**
38 **canalize initially plastic phenotypes. Several studies have shown that it was**
39 **possible through modification of chromatin regulation or hormonal**
40 **signalling/response. Further studies of phenotypic plasticity in an**
41 **evolutionary framework are needed to see how much the findings**
42 **described in this review can be generalized.**

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44 **Key words: Phenotypic plasticity, Evolution, Gene expression, Review**

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46 In developmental biology, the environment has long been thought to fulfil
47 essentially a permissive role, simply providing the required conditions for
48 normal development to occur. Thus, most developmental biology laboratories
49 still use standardized environmental conditions and focus on genetic factors to
50 dissect developmental mechanisms. However, in many cases the environment
51 has an instructive role, directing the development to particular alternative path
52 (Gilbert 2001). Phenotypic plasticity is a major concept which describes "*the*
53 *property of a given genotype to produce different phenotypes in response to*
54 *distinct environmental conditions*" (Pigliucci 2001). It is widely observed in the
55 wild, and this concept was formulated by Bradshaw in the sixties (Bradshaw
56 1965). Phenotypic plasticity can be an adaptation to fluctuating environments
57 such as seasonal variations, presence/absence of predators, or population
58 density. It can also be non adaptive and Schmalhausen used the term "*morphose*"
59 to describe aberrant phenotypes induced by extreme environmental conditions
60 (Schmalhausen 1949). A major tool to illustrate phenotypic plasticity is the
61 reaction norm, first drawn by Woltereck in 1909 (Woltereck 1909), which
62 represents the value of a quantitative phenotype produced by a given genotype
63 in function of an environmental parameter. Note that "*genotype*" and "*phenotype*"
64 were defined only shortly after by Johannsen (1911) who reinterpreted the
65 reaction norms of Woltereck (Johannsen 1911). Indeed, Woltereck observed
66 that different pure lines could produce different morphologies in a given
67 environment but the same morphology in another environment. He interpreted
68 this as inconsistent with the existence of constant differences between the
69 genotypes. Because he did not distinguish between genotypes and phenotypes,

70 he thought that the different pure lines had the same genotype in a given
71 environment (when they had the same phenotype) and different genotypes in
72 another environment (when they had different phenotypes). When phenotypic
73 plasticity leads to discrete morphs, these morphs are called polyphenisms.
74 Phenotypic plasticity can generate, within a single species, morphological
75 differences of a magnitude usually observed between distantly related species.
76 As selection operates on phenotypes, this direct input of the environment on the
77 expression of the genotype must be taken into account to understand
78 evolutionary processes. This was clearly formulated by Schmalhausen (1949)
79 (Schmalhausen 1949): *“Darwin has previously noted the fundamental importance*
80 *of inherited variability (mutations) for the evolution of organisms. However,*
81 *mutations have different expressions under diverse conditions. If natural selection*
82 *is the basic factor in evolution, then the concrete expression of individual*
83 *characteristics under given environmental conditions is of decisive importance in*
84 *this process»*. Thus, the role of phenotypic plasticity in evolution has been
85 actively discussed (Pfennig et al. 2010; Moczek et al. 2011; Laland et al. 2014,
86 2015; Schneider and Meyer 2017). The issue is complex and certain empirical
87 results suggest that it is important to distinguish between adaptive and non-
88 adaptive plasticity as they may have opposite effects on genetic divergence
89 (Ghalambor et al. 2015).

90 The idea that an ancestral plastic species could have been at the origin of
91 divergent lineages with pronounced phenotypic differences has been proposed
92 by Mary-Jane West-Eberhard as the *“flexible stem hypothesis”* (West-Eberhard
93 2003). It is intimately linked to the process of genetic assimilation discovered by
94 Conrad Waddington more than 50 years ago (Waddington 1952, 1956, 1959). In

his experiments on *Drosophila*, Waddington showed that a phenotype initially induced by the environment can be fixed by selection and become constitutively expressed even in the absence of the initial environmental factor. Most of these experiments were based on developmental defects induced by stressors such as heatshock (crossveinless phenotypes on wings) (Waddington 1952) or ether vapour (*bithorax* phenocopies) (Waddington 1956). However, they could be generalized to adaptive phenotypes and had therefore major evolutionary implications. Indeed, in another Waddington experiment, the morphological change induced by the environment, *i.e.* size increase of the anal papilla of larvae in response to high salt concentration, was likely adaptive and was also genetically assimilated (Waddington 1959). Importantly, genetic assimilation is a genuine evolutionary process based on selection of genetic variation existing in the population submitted to environmental changes. Indeed, Waddington experiments were later repeated by Bateman on outbred or isogenized stocks and it was shown that genetic assimilation did not happen in the absence of genetic variation in the population (Dworkin 2005). This genetic variation is cryptic in the original environment in which it accumulates as it has no phenotypic consequences. This genetic variation translates into phenotypic variation in the new environment, which allows genetic assimilation by selection. Thus, a lineage characterized by a constitutively expressed morph could evolve from a plastic ancestor by genetic assimilation of a phenotype initially induced by environmental change.

This has lead Mary Jane West-Eberhard to propose that genes act more as followers than as leaders during evolution (West-Eberhard 2005). Although very stimulating, this idea cannot be generalized to the evolution of all traits. Indeed,

in many examples, genetic polymorphisms have played a major role in the origin of a phenotype later fixed during evolution (Schwander and Leimar 2011). For example, a particular allele of the pigmentation gene *tan* leading to lighter pigmentation is segregating in *Drosophila americana* and has been fixed in its sister species *Drosophila novamexicana* which has a derived pale phenotype (Wittkopp et al. 2009). However, although the “*plasticity first hypothesis*” does not apply to all traits, it is worth investigating it empirically. The flexible stem model of adaptive radiation predicts that the pattern of phenotypic divergence between derived lineages should mirror the pattern of developmental plasticity in their common ancestor (Wund et al. 2008). Several studies have provided empirical morphological data strongly suggesting that it could occur in nature (Wund et al. 2008). For example, the pharyngeal jaws of midas cichlids show a morphological plasticity in response to the hardness of the diet which parallels the morphological diversity observed between specialised species (Muschick et al. 2011). Similarly, stress-induced changes in mandible shape in *Sorex* shrews correspond to traits showing divergence between species (Badyaev and Foresman 2000).

What we would like to review and discuss in this article is the underlying genetic bases of the flexible stem hypothesis. Indeed, under this hypothesis, it is expected that the genetic mechanisms that generate the plastic phenotypes should be the same as those that generate the phenotypic divergence. The idea that phenotypic plasticity is under genetic control was initially developed by Bradshaw (Bradshaw 1965) and a recurrent question was the existence and the nature of “*plasticity genes*” (Via et al. 1995). However, Via considered that modifications of reaction norms was an indirect consequence of the selection on

the mean phenotype in each separate environment and that no plasticity genes were required (Via 1993). Scheiner, Schlichting and Pigliucci objected to Via that the plasticity of a trait could evolve independently of the mean of the trait because the plasticity and the mean of the trait are under the control of different genes (Scheiner 1993; Schlichting and Pigliucci 1993). This debate settled down in the mid nineties in favour of the existence of plasticity genes. Two kinds of genetic mechanisms involved in phenotypic plasticity were proposed (Via et al. 1995). The first one, "*allelic sensitivity*", refers to alleles expressed differently in distinct environment. This could correspond to a single gene showing transcriptional plasticity or to an enzyme active in only some environmental conditions for example. The second genetic mechanism, "*gene regulation*", corresponds to a regulatory switch under environmental control, which would lead to the activation of a panel of target genes depending on the environment. Obviously, the two mechanisms may blur as the regulatory *locus* influences the amount of products of its targets, thus mediating allelic sensitivity (Via et al. 1995). Clearly, a great deal of information could be gained by genetic dissection of phenotypic plasticity in a variety of organisms.

A few empirical studies have identified genes involved in the phenotypic plasticity of particular traits and in several cases, data are available regarding genes involved in the variation within or between species of the traits of interest. Thus, it is possible to analyse the genetic changes that underlie genetic assimilation of initially plastic traits. Furthermore, several studies have dissected the mechanisms involved in phenotypic plasticity and environmental canalization. The typical questions we would like to address are the following: What are the relationships between genes involved in phenotypic plasticity of a

170 trait and genes involved in the evolution of this trait? What kind of mutations are
171 selected during the evolution of these traits? Are they regulatory or coding
172 mutations? Do they target *cis*-regulatory sequences of a modulated gene or *trans*-
173 acting factors? How is it possible to make independent of the environment an
174 initially plastic trait? We present here several examples of phenotypic plasticity,
175 chosen in animals or plants, whose genetic bases have been addressed through
176 candidate gene approach or global transcriptome analyses. Most of these
177 analyses are performed in an evolutionary context (either artificial or natural) or
178 can be complemented by other studies on the evolution of the particular traits of
179 interest in closely related species. Furthermore, we describe studies dissecting
180 the mechanisms involved in phenotypic plasticity and environmental
181 canalisation. Modulation of such mechanisms must be involved in the
182 environmental canalization of initially plastic traits involved in the flexible stem
183 hypothesis.

185 **Ether-induced *Ultrabithorax* phenocopies in *Drosophila***

186 Treatment of *Drosophila* embryos with ether vapor leads to phenocopies of the
187 *bithorax* mutant in adults (Waddington 1956). This corresponds to partial
188 homeotic transformation of the third thoracic segment into the second thoracic
189 segment. In *Drosophila*, like in all dipterans, the dorsal appendages of the third
190 thoracic segments are not wings but little round structures named halteres. In
191 *bithorax* phenocopies, halteres are partially transformed into wings and the
192 postnotum is partially transformed into an additional mesonotum (Figure 1).
193 Waddington showed that this ether-induced phenotype can be genetically
194 assimilated (Waddington 1956). By selection after ether treatment at each

generation, he could progressively increase the proportion of individual showing the *bithorax* phenocopy. At the eighth generation, this phenotype was observed in some individuals even in the absence of ether treatment (Figure 1). The *bithorax* phenotype was therefore genetically assimilated. Waddington experiments were repeated by Gibson and Hogness (Gibson and Hogness 1996). They analysed the expression of the homeotic gene *Ultrabithorax (Ubx)* which is expressed in haltere but not wing imaginal disc and is responsible for the different identities of these organs. They showed that ether-induced *bithorax* phenocopies are caused by clonal loss of expression of *Ubx* in the haltere imaginal disc. Furthermore, using polymorphism in *Ubx*, they could show that the frequency of particular alleles of *Ubx* increased during genetic assimilation of the *bithorax* phenotype. The *Ubx* polymorphism was not the only source of genetic variation affecting sensitivity to ether, but it had a major effect. Thus, *Ubx* expression is sensitive to ether and during genetic assimilation of this phenotype, artificial selection targeted the *Ubx* locus itself.

Transcriptional response to osmotic challenge in killifish:

The Atlantic killifish (*Fundulus heteroclitus*) can be found along a steep salinity gradient in Atlantic coast estuaries and can quickly adjust to extremes in environmental salinity (Figure 2). Thus, this species is able to occupy an extremely wide osmotic niche. Hypo-osmotic challenge induces a change of cellular morphology of the apical surface of the gill epithelium (Whitehead et al. 2011) (Figure 2). In sea water, the apical surface of the gill epithelium is made of pavement cells interspersed with mitochondrion-rich cells possessing apical crypt with small surface area (Whitehead et al. 2011). Exposure to fresh water

leads to enlargement of the apical surface of mitochondrion-rich cells. This modification occurs within a few days, but its speed and the precise morphology of the gill epithelium differs between populations originating from different point of the salinity gradient. Analysis of the transcriptome of gills in fish submitted to hypo-osmotic challenge allowed to identify sets of genes whose expression was modulated by salinity (Whitehead et al. 2011). It was found that genes showing transcriptional plasticity during osmotic challenge are more likely to show divergence of expression between populations living in different environment (Whitehead et al. 2011, 2012). Indeed, many of the genes modulated during hypo-osmotic challenge show adaptive divergence in expression in populations living exclusively in freshwater habitat. Among these genes, many have a well known role in osmotic regulation, for example Aquaporin 3 (AQP3), the sodium/potassium transporting ATPase ATP1A1 or versican (VCAN) (Whitehead et al. 2012).

Plasticity of wing eyespots in the butterfly *Bicyclus anynana*

The African satyrine butterfly *Bicyclus anynana* shows seasonal polyphenisms. In the wet season morph, conspicuous eyespots made of concentric rings of differently pigmented scales are observed on the ventral wings. In the dry season morph, the ventral eyespots are absent. Only a reduced version of the white spot in the centre of the eyespot is visible. The wet and dry season morphs can be produced in the laboratory by rearing the larvae at 27°C or 17°C respectively (Brakefield et al. 1996) (Figure 3).

Many developmental regulatory genes are expressed in developing eyespots of *Bicyclus anynana*, among which *Distal-less (Dll)* which is expressed in the future

245 centre of the eyespot (Brakefield et al. 1996; Shirai et al. 2012). This is not
246 simply a correlation as it was recently shown by gain and loss of function
247 experiments using transgenic *Bicyclus anynana* that *Dll* is involved in eyespot
248 formation (Monteiro et al. 2013). Indeed, overexpression of *Dll* increases eyespot
249 size and induces ectopic eyespots. Conversely, decreasing *Dll* expression reduces
250 eyespot size. Furthermore, the expression of *Dll* in presumptive eyespots is
251 modulated by temperature: it is stronger and wider in pupae grown at 27°C that
252 will produce the wet season morph (Brakefield et al. 1996). Thus, *Dll* is one of
253 the genes involved in eyespot plasticity.

254 It is possible to fix alternative morphs by artificial selection performed at
255 intermediate temperature. This lead to a LOW line and a HIGH line showing
256 almost non plastic dry season and wet season phenotypes, respectively
257 (Brakefield et al. 1996) (Figure 3). Thus, the wet or dry season morphs can be
258 genetically assimilated and become virtually independent of the rearing
259 temperature. This correlates with a modification of *Dll* expression, which is
260 stronger and wider in pupae of the HIGH line than in pupae of the LOW line
261 grown in the same environmental conditions. Thus, genetic assimilation of the
262 alternative seasonal morphs has involved modulation of *Dll* expression. Whether
263 this genetic assimilation was caused by selection at the *Dll* locus itself (*cis*
264 variation) or by *trans*-acting factors was not tested in this experiment. However,
265 another selection experiment targeting the size of dorsal eyespots (which
266 however are not plastic), showed that natural genetic variation in *Dll* was linked
267 to eyespot size variation in *Bicyclus anynana* (Beldade et al. 2002). Thus,
268 variation at the *Dll* locus with significant impact on eyespot patterning exists in
269 *Bicyclus anynana*.

Polyphenism of cuticular teeth in diplogastrid nematods

The diplogastrid nematod *Pristionchus pacificus* displays two alternative tooth morphs, stenostomatous (St) or eurystomatous (Eu), that are produced after an irreversible decision during larval development (Bento et al. 2010) (Figure 4). Eu morph is adapted to predation on other nematods, whereas St morph is adapted to bacterial diet. The ratio of individuals with St or Eu teeth varies between strains but is also influenced by environmental factors such as crowding and starvation (Bento et al. 2010; Ragsdale et al. 2013). The high expression of the gene *eud-1*, encoding a sulfatase, is necessary and sufficient to produce the Eu phenotype in *Pristionchus pacificus* (Ragsdale et al. 2013). Loss of function mutations of *eud-1* are dominant and lead to the St phenotype. Difference of the Eu/St ratio between wild isolates of *Pristionchus pacificus* correlates with difference in *eud-1* expression. Furthermore, *eud-1* over-expression of is sufficient to transform high St strains into Eu ones. The role of *eud-1* in tooth dimorphism extends to the other diplogastrid species *Pristionchus exspectatus* (Ragsdale et al. 2013). Indeed, through hybrid crosses between various *P. pacificus* lines and a *P. exspectatus* St line, it was shown that the St phenotype of the *P. exspectatus* line was caused by a low expression of *eud-1*. As transformation of *P. pacificus eud-1* mutant with the *eud-1* allele of the *P. exspectatus* St line leads to a Eu phenotype, the *eud-1* allele of *P. exspectatus* is functional and differences of *eud-1* expression between *P. pacificus* and *P. exspectatus* are caused by differences in *trans*-acting factors. Thus, divergence in the ratio of tooth morphs might have involved several components of the *eud-1* network.

It is thought that the stem species in diplogastrid nematods was polyphenic (Kiontke and Fitch 2010). Many descendant species such as *Pristionchus pacificus* have kept both morphs, but others have become exclusively Eu or St. Interestingly, recent morphometric analysis of diplogastrid mouth structures in a more global evolutionary framework revealed a rapid process of diversification associated with developmental plasticity (Susoy et al. 2015): The gain of dimorphic mouth structures was associated with an increase rate of evolution and the appearance of numerous new species with diversified mouth parts.

Modulation of leaf margin dissection by temperature in *Capsella*

The two brassicaceae sister species *Capsella rubella* and *Capsella grandiflora* show differences in leaf margin dissection (Sicard et al. 2014). Leaf margin dissection is more pronounced in *Capsella rubella* and is increased at low temperature (16°C versus 22°C). It was shown that the major locus responsible for the difference in leaf margin dissection between *C. rubella* and *C. grandiflora* is the homeobox gene *RCO-A* which is expressed in sinuses between growing lobes of developing leaves in *C. rubella* (Sicard et al. 2014). This difference of expression level is caused by insertions in the *C. rubella* *RCO-A* promoter. The effect of the *RCO-A* region on leaf margin dissection is visible in nearly isogenic lines differing only in the region of *RCO-A*, *NILgg* (carrying the *RCO-A* allele of *C. grandiflora*) and *NILrr* (carrying the *RCO-A* allele of *C. rubella*) (Figure 5). In addition, *RCO-A* expression is strongly modulated by temperature. Expression of the *C. grandiflora* *RCO-A* allele is extremely weak at 22°C and the leaves show no dissection. This allele is up-regulated more than 150 folds at 16°C compared to 22°C, which leads to leaf margin dissection. The *C. rubella* *RCO-A* allele is more

expressed than the *C. grandiflora* one at 22°C and is upregulated less than 5 folds between 22°C and 16°C. Consequently, it leads to leaf margin dissection at both temperatures in *C. rubella*.

In another brassicae species, *Arabidopsis thaliana*, the *RCO-A* locus is deleted and there is no leaf margin dissection. Insertion of transgenes carrying *Capsella rubella* *RCO-A* gene in *Arabidopsis thaliana* is sufficient to induce leaf margin dissection (Sicard et al. 2014).

Thus, the same *locus* is directly involved in phenotypic plasticity and species divergence regarding leaf margin dissection. However, as the activity of the regulatory sequences of *RCO-A* responsible for the difference of expression between the two *Capsella* species was not studied with a reporter gene in transgenic lines grown at different temperatures, it is not known whether they also mediate the effect of the environment.

Thermal plasticity of abdominal pigmentation in *Drosophila melanogaster* females

Cold induces melanism in many insects (Gibert et al. 2000; Michie et al. 2010; Fedorka et al. 2013). This is thought to be beneficial as dark pigmentation increases body temperature. However, pigmentation is highly pleiotropic and is also correlated to many other traits such as longevity, fecundity or immunity (Wittkopp and Beldade 2009; Fedorka et al. 2013; Kutch et al. 2014; Rajpurohit et al. 2016). In *Drosophila melanogaster* females, abdominal pigmentation is strongly modulated by temperature (Figure 6). This is particularly pronounced in the more posterior segments (A5, A6 and A7) (Gibert et al. 2000). Correspondingly, the expression of several genes encoding the enzymes of the

345 cuticular pigment synthesis pathway in the abdominal epidermes of late pupae
346 and young adults is modulated by temperature (Gibert et al. 2016, 2017).
347 However, among them, the gene *tan* stands out, being expressed seven times
348 more in the abdominal epidermis of young females grown at 18°C than 29°C. The
349 activity of *tan* increases melanin production and genetic experiments showed
350 that temperature modulation of *tan* expression plays an essential role in thermal
351 plasticity of female abdominal pigmentation (Gibert et al. 2016). Furthermore,
352 temperature modulates the activity of the *tan* abdominal enhancer (*t_MSE*) and
353 the level of the active histone mark H3K4me3 on *tan* promoter. Interestingly, *tan*
354 is well known for its involvement in pigmentation variation and divergence in
355 the *Drosophila melanogaster* species subgroup. In *Drosophila melanogaster*,
356 independent genome wide association studies performed on European, African
357 and American populations have identified single nucleotide polymorphisms in
358 the *t_MSE* involved in variation of female abdominal pigmentation (Bastide et al.
359 2013; Dembeck et al. 2015; Endler et al. 2015). In *Drosophila erecta*, female
360 dimorphic pigmentation is caused by genetic variation in the *t_MSE* maintained
361 by balancing selection (Yassin et al. 2016). In *Drosophila santomea*, several
362 independent loss of function mutations located in the *t_MSE* have lead to the light
363 phenotype of this species, whereas in the sister species *Drosophila yakuba* a
364 functional *t-MSE* enhancer is associated with dark abdominal pigmentation
365 (Jeong et al. 2008). Thus, in the *Drosophila melanogaster* species subgroup,
366 plasticity and variation between species of abdominal pigmentation work
367 through modulation of the same gene and involves genetic changes in the very
368 same regulatory sequence, *t_MSE*, that also mediates the effect of temperature on
369 *tan* expression in *Drosophila melanogaster*.

370

371 **Discussion**

372 These examples demonstrate that several genes showing transcriptional
373 plasticity in response to environmental changes are differentially expressed
374 within and/or between species and contribute to phenotypic variation and/or
375 divergence. Thus, the same genes respond to the environment and are involved
376 in evolution. It might seem predictable that the same genes are involved in the
377 plasticity of a trait and the variation/evolution of this trait. However, this is not
378 so obvious. Indeed, the same phenotypes can sometimes be produced by
379 different mechanisms. For example, *Drosophila* wing size can be modulated by
380 both temperature and clinal genetic variation. However, this occurs through
381 distinct mechanisms: temperature modulates cell size (Azevedo et al. 2002),
382 whereas clinal genetic variation primarily affects cell number (James et al. 1995).
383 Similar pigmentation phenotypes can be produced by modulating the expression
384 of different pigmentation genes (Wittkopp et al. 2003). For example, melanin
385 production can be increased by up-regulating *tan* or down-regulating *ebony*, that
386 encode enzymes with antagonist activities (True et al. 2005).

387 In the case of *RCO-A* and *tan*, the variation or divergence in expression is caused
388 by mutations in the *cis*-regulatory sequences of the genes. This is also likely the
389 case for *Ubx* during genetic assimilation of the *bithorax* phenotype. Remarkably,
390 for *tan*, the same *cis*-regulatory sequence mediates, at least partly, the effect of
391 temperature. It is generally thought that *cis*-regulatory mutations are less
392 pleiotropic than mutations in *trans*-acting factors (Prud'homme et al. 2007).
393 Thus, targeting *tan*, a gene encoding a pigmentation enzyme, or *RCO-A*, whose
394 expression is limited to the sinuses between growing leaf lobes, limit potential

395 deleterious effects. The recurrent involvement of *RCO-A* and *tan* in evolution lead
396 to their characterization as evolutionary hotspots (Martin and Orgogozo 2013;
397 Sicard et al. 2014). Why are these two genes targets of recurrent evolution ? For
398 a given allele, temperature sensitivity of *RCO-A* and *tan* expression leads to
399 different phenotypes in different environments. Reciprocally, different alleles
400 will induce different phenotypes in the same environment. It follows that only
401 some genotypes will produce an optimal phenotype in a given environment. This
402 may lead to selection of different alleles of these genes in different environments
403 to reach an optimal phenotype. This is in particular the case if the phenotype
404 produced by the plastic response is not well adjusted to the environment.
405 Genetic variation will be selected to reduce or to increase the effect of the
406 environment. As the environment fluctuates spatially and temporally, adjusting
407 selection increases genetic variation with functional consequences at these *loci*.
408 This higher genetic variation facilitates the involvement of those *loci* in
409 evolution.

410 In the other cases presented in this review, the respective contributions of *cis*
411 and *trans* variations are not known (*eud-1* in *P. pacificus*, genes modulated by
412 osmotic challenge in *Fundulus*, *Dll* in *Bicyclus anynana*) but the recent
413 identification of *trans*-regulators of *eud-1* is a step towards answering this
414 question (Serobyanyan et al. 2016). However, what is clear is that evolution has
415 targeted components of the gene networks regulating the transcriptionally
416 plastic genes if not these genes themselves. In most cases described in this review,
417 the gene showing transcriptional plasticity also exhibits genetic variation that
418 provides substrate for evolution through selection or simply drift. The fact that
419 the same networks, or even the same genes, are involved in plasticity and

420 evolution contradicts somehow Scheiner's ideas on the decoupling of the genetic
421 processes controlling the plasticity and the mean of a trait (Scheiner 1993).
422 In addition, the "flexible stem hypothesis" implies that it is mechanistically
423 possible for a trait initially plastic to undergo environmental canalization and
424 become independent of the environment. Several studies have dissected the
425 mechanisms behind phenotypic plasticity and environmental canalization. Two
426 major mechanisms have been identified: regulation of chromatin structure and
427 hormonal signalling/response. It is known that environmental conditions can
428 modulate DNA methylation (Kucharski et al. 2008), histone post-translational
429 modifications (Simola et al. 2016; Gibert et al. 2016) or chromatin compaction
430 (Leung et al. 2014). Thus, stabilization of chromatin structure in an initially
431 plastic species, could lead to production of a constant phenotype independent of
432 the environment. For example, artificial down-regulation of DNA methylation in
433 honey-bees lead to the production of a queen phenotype from larvae that should
434 have developed as workers because on the diet they fed on (Kucharski et al.
435 2008). Some alleles might carry variation in their *cis*-regulatory sequences that
436 promotes the formation of a particular chromatin structure independently of the
437 environment. This might be the case for *tan* or *Ubx* and selection on this
438 variation might contribute to modify the sensitivity of their expression to the
439 environment. Regarding, hormonal regulation, it is known that differences of
440 plasticity between genotypes could be linked to differences of hormonal
441 signalling or response (Suzuki and Nijhout 2006). In *Manduca sexta* larvae,
442 artificial selection on temperature induced pigmentation variation lead to the
443 production of a polyphenic and a monophenic lines differing by their juvenile
444 hormone titer and response (Suzuki and Nijhout 2006). Interestingly a similar

445 process applies to differences of plasticity between organs. In *Drosophila*, the
446 size of late larval brain and adult male genitalia is buffered against nutrient
447 restriction, whereas the size of other organs decreases under these conditions.
448 The buffering of genitalia size is caused by repression of *FOXO* expression in the
449 genital disc which makes it insensitive to the nutritional information provided by
450 insulin signalling (Tang et al. 2011). The buffering of late larval brain size is
451 caused by cell-type specific expression of *Alk* and its ligand *Jelly Belly* which
452 renders neuroblasts insensitive to amino acid and insulin sensing (Cheng et al.
453 2011). Thus, by modifying the expression of key genes involved in hormonal
454 signalling or response it is possible to reduce or even abolish the influence of the
455 environment on a given trait. These studies illustrate what kind of mechanisms
456 might be involved in the genetic assimilation taking place during the formation
457 of a monophenic lineage under the “flexible stem hypothesis”.

458 The examples described in this review are compatible with the “flexible stem
459 hypothesis” (West-Eberhard 2003), which postulate ancestral plasticity followed
460 by genetic assimilation of alternative morphs in derived lineages. Analysis of
461 phenotypic plasticity of nematode feeding structures in an evolutionary
462 framework supports indeed that plasticity was ancestral and that one morph
463 was stabilized (genetically assimilated) in particular lineages whereas others
464 have kept plasticity (Kiontke and Fitch 2010). A definite demonstration of the
465 “flexible stem hypothesis” would be to follow allelic frequencies during genetic
466 assimilation of a phenotype of interest. This was done in only one of the
467 examples, but for a deleterious phenotype normally not observed in the wild, *i.e.*
468 the *bithorax* phenocopies (Gibson and Hogness 1996). Thus, more studies on the
469 genetic bases of phenotypic plasticity in an evolutionary conceptual framework

are needed. In particular, it would be interesting to know whether the example of *tan* and its t_{MSE} is just a particular case or whether it is predictive of a more systematic involvement of the same regulatory sequences in phenotypic plasticity and species variation and divergence.

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- 659

Figure legends:

Figure 1: Above: wild-type *Drosophila melanogaster* female. Bellow: female *Drosophila* showing a *bithorax* phenotype obtained after genetic assimilation of the *bithorax* phenocopy initially induced by ether treatment of the embryo (modified after (Waddington 1956)). The post-notum is replaced by an additional meso-notum and halteres are replaced by rudimentary wings.

Figure 2: Left: the network of rivers flowing into the Chesapeake-bay, in north-east America, constitutes a range of water habitat with different salinity going from pure sea water (dark blue) to pure fresh water (red), modified after (Whitehead et al. 2011). Top right, populations of Atlantic killifish (*Fundulus heteroclitus*) are locally adapted to particular salinities but are also able to quickly adjust to osmotic challenges. Bottom right: modification of gill epithelium after transfer from sea water to fresh water (after (Whitehead et al. 2012)). In sea water (32 ppt), the apical surface of the gill epithelium is made of pavement cells (red asterisk) interspersed with mitochondrion-rich cells possessing apical crypt with small surface area (green arrowhead). Exposure to fresh water (0.1 ppt) leads to an enlargement of the apical surface of mitochondrion-rich cells (green arrowhead).

Figure 3: Top: natural populations of the satyrine butterfly *Bicyclus anynana* show two seasonal morphs, the dry season morph (left) and the wet season morph (right) that can be produced in the lab by rearing the larvae at 17°C or 27°C, respectively. Middle and bottom: the dry season and the wet season

morphs can be fixed by artificial selection leading to a LOW line (middle) or a HIGH line (bottom) with very limited plasticity (after (Brakefield et al. 1996)).

Figure 4: The diplogastrid nematode *Pristionchus pacificus* displays two alternative tooth morphs: a wide one, eurystomatous (Eu, left) or a narrow one stenostomatous (St, right), that are produced after an irreversible decision during larval development (after (Kiontke and Fitch 2010)). The ratio of these two morphs is variable between strains and is modulated by environmental factors such as crowding and starvation.

Figure 5: *Capsella grandiflora* and *Capsella rubella* differ regarding the dissection of their leaf margins. This difference was mapped to the *RCO-A locus*. Above nearly isogenic lines (NIL) differing only in the *RCO-A locus* region. The line with the *C. grandiflora* allele (*NILgg*) (left) does not show any leaf margin dissection at 22°C contrary to the line with the *C. rubella* allele (*NILrr*) (right). Lower temperature (16°C) induces (*NILgg*) or increases (*NILrr*) leaf margin dissection.

Figure 6: Abdominal cuticles of females *Drosophila melanogaster* from an inbred line (*w¹¹¹⁸*) grown at 18°C (left) or 29°C (right). Female abdominal pigmentation is sensitive to temperature during development in particular in the most posterior segments (A5, A6 and A7). Anterior is up, ventral is left (after (Gibert et al. 2016)).

709

32 ppt, Pre-transfer

0.1 ppt, 1 day post transfer

710

711

712

713

714

Eurystomatous (Eu)

Stenostomatous (St)

715

NILgg

NILrr

22°C

16°C

716

717

718

719

720