Conservation of the segmented germband stage: robustness or pleiotropy?

Frietson Galis, Tom J.M. van Dooren and Johan A.J. Metz

Gene expression patterns of the segment polarity genes in the extended and segmented germband stage are remarkably conserved among insects. To explain the conservation of these stages, two hypotheses have been proposed. One hypothesis states that the conservation reflects a high interactivity between modules, so that mutations would have several pleiotropic effects in other parts of the body, resulting in stabilizing selection against mutational variation. The other hypothesis states that the conservation is caused by robustness of the segment polarity network against mutational changes. When evaluating the empirical evidence for these hypotheses, we found strong support for pleiotropy and little evidence supporting robustness of the segment polarity network. This points to a key role for stabilizing selection in the conservation of these stages. Finally, we discuss the implications for robustness of organizers and long-term conservation in general.

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Frietson Galis*
Tom J.M. van Dooren
Johan A.J. Metz
Institute of Evolutionary and Ecological Sciences, Leiden University, PO Box 9516, 2300RA Leiden University, The Netherlands.
*e-mail: galis@rulsfb.leidenuniv.nl

Morphogenetic patterning during early embryonic stages, including the process of segmentation, has diverged markedly among insects. The diversity in morphogenetic patterning is reflected in the diverse expression patterns of the genes involved (e.g. gap and pair-rule genes) [1–5]. However, at the end of gastrulation, developmental trajectories converge on a highly conserved stage that coincides with early organogenesis: the segmented germband stage [2,5–8] (Fig. 1). The conservation of morphological patterns corresponds with that of gene expression: both the striped expression patterns of the segment polarity genes and the co-linear pattern of the Hox genes are remarkably conserved [2–4,9].

Sander [7] and Raff [10] hypothesize that high connectivity between modules (Fig. 2) is the major cause of conservation in the segmented germband stage. This high connectivity causes mutations to have multiple pleiotropic effects that become amplified as development proceeds. Because pleiotropic effects during embryogenesis are generally disadvantageous [11,12], strong stabilizing selection against mutational variation ensues. In this scenario, conservation is a consequence of consistently strong selection against mutations via their pleiotropic effects. The strong 'connectedness' of modules implies an easily destabilized network of inductive events, with low effective robustness and low effective modularity (Fig. 2). Although this hypothesis was proposed for conservation of the segmented germband stage, it is natural to include the earlier extended germband stage (Fig. 1) when evaluating its explanatory power, as the characteristic gene expression patterns of the segment polarity and Hox genes are then already present.

von Dassow and Munroe [13] also assume that conservation is associated with network characteristics. While referring to the model of von Dassow *et al*. [14] on the robustness of the segment polarity gene network, they hypothesize that this network is causally involved in conservation of the expression pattern of the segment polarity genes in the ectoderm. In robust gene networks, by definition, developmental noise and mutations do not lead to clear phenotypic effects because gene interactions tend to neutralize perturbations and, in particular, make mutations recessive [15,16]. According to von Dassow *et al*. [14], robustness should buffer the network both against changes of the input at the start of the network (i.e. changes in the signals from the preceding stage) and against changes in the input during running of the network. Together, these two articles [13,14] suggest that conservation of the network occurs despite accumulation of genetic changes because these changes have little phenotypic effect and mainly lead to hidden variation.

The robustness hypothesis was only proposed for the segment polarity gene network involved in the striped expression pattern that occurs in the

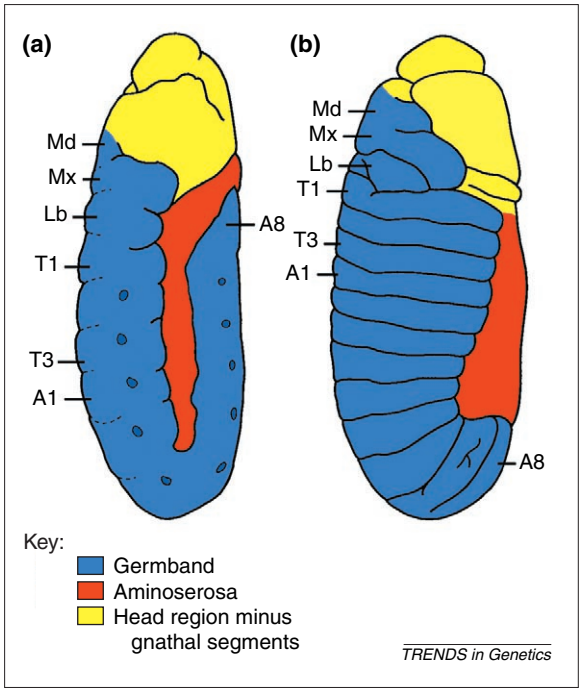

Fig. 1. Extended (a) and segmented (b) germband stages in *Drosophila*. The germband (blue) refers to the part of the embryo that will give rise to the metameric regions: gnathal segments of the head region (Md, mandible; Mx, maxilla; Lb, labium), thoracic segments (T1–3) and abdominal segments (A1–8). The amnioserosa (red) is an extra-embryonic membrane. The extended germband stage starts ~6.5 h after fertilization and the segmented germband stage ends at ~10.5 h after fertilization.

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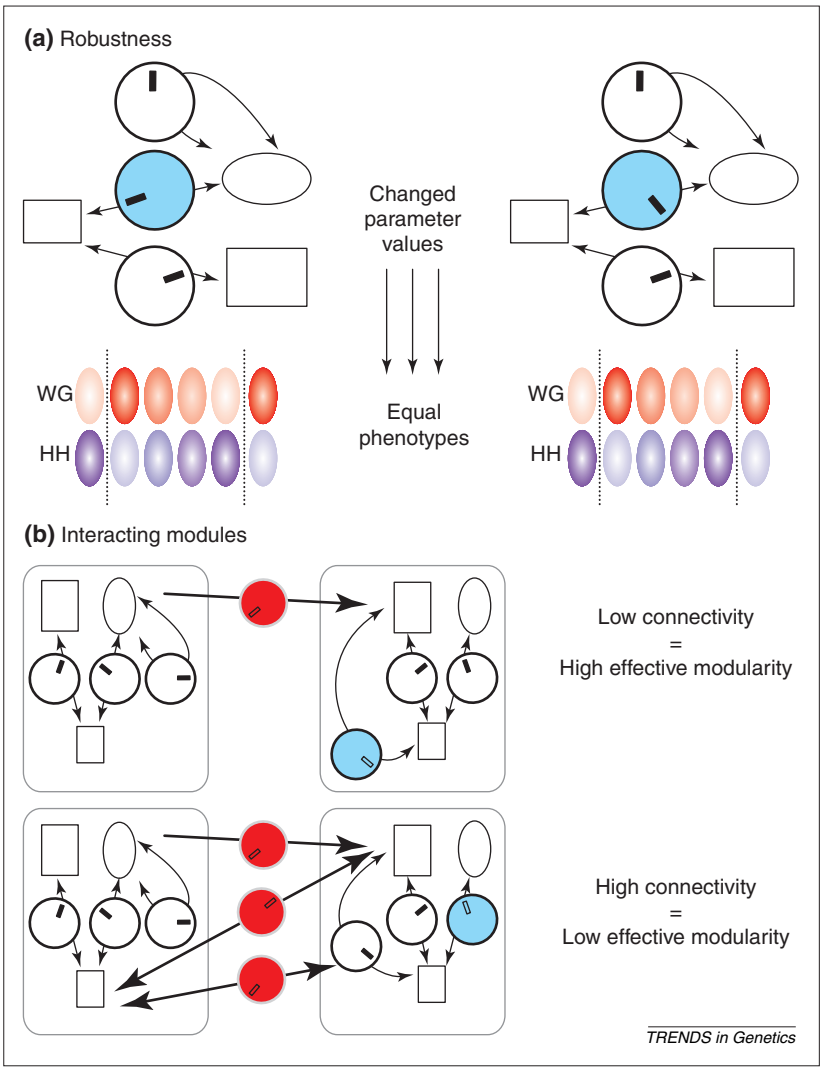

Fig. 2. Robustness and effective modularity. (a) Robustness. When a parameter is changed in a robust genetic network, the resulting phenotype does not change [in this case illustrated with the concentration of WINGLESS (WG) and HEDGEHOG (HH) in the cells of the ectoderm]. (b) Effective modularity. Modules are discernible and discrete units within large genetic networks that have some autonomy and a clear physical location [10]. These modules can differ in the amount of connectedness. All input to robust elements of a module can be ignored as it will have no discernible effect. A large proportion of robust components in a module therefore reduce potential connectivity. Low connectivity, with few connections having small effects, implies high effective modularity. High connectivity implies low effective modularity.

epidermis during the extended and segmented germband stage (Table 1). von Dassow *et al.* [14] are the first to model this network and its robustness, which is valuable for its own sake. However, the model needs to be evaluated with respect to influences of genes directly regulating the network. If the network is robust to changes in its input, mutations in genes regulating the network (e.g. *eve*, *ftz*, *slp*, *tsh*, *Dpresenilin*, *hid* and genes of the Notch pathway) should not affect its activity. Otherwise, the network is not robust in reality, and the predicted robustness hinges on the specific modularity assumptions made by von Dassow *et al.*

During the extended and segmented germband stages, the segment polarity network acts as an organizer that is central to many patterning events (Box 1). These stages are conserved as a whole. Therefore, the robustness hypothesis should be extended to the organization of these stages in full, otherwise it can never explain evolutionary conservation.

Table 1. Constituents of the segment polarity gene network modeled by von Dassow *et al.* [14]

ci	cubitus interruptus (*CI*)
en	engrailed (*EN*)
hh	hedgehog (*HH*)
ptc	patched (*PTC*)
wg	wingless (*WG*)
PH	patched–hedgehog complex
CN	repressor fragment of *ci*

The two hypotheses become diametrically opposed when extended to the overall conservation of the two stages. The pleiotropy hypothesis points to a low modularity as the cause of the conservation, whereas the robustness hypothesis assumes that a high modularity for inductive interactions between segments and germ layers is the cause (Fig. 2). Therefore, they imply strikingly different roles for modularity in evolution: constraining versus facilitating evolutionary change. Furthermore, they lead to different predictions regarding mutations affecting segment polarity gene activity in the extended and segmented germband stages (Table 2). In the following sections, we test the explanatory power of the robustness and pleiotropy hypotheses for the overall conservation of these stages, placing special emphasis on the segment polarity gene network in the ectoderm.

Mutations acting on the extended and segmented germband stages

The occurrence of mutants

Mutant screens with sensitized genetic backgrounds have probably uncovered the majority of genes that affect segmentation. For instance, Müller *et al.* [17] conducted a translocation screen for zygotically expressed genes in *Drosophila* that covered >99% of the genome. They found that nearly all zygotically expressed genes that regulate *wingless* (*wg*) expression in the ectoderm had already been identified. Moreover, nearly all these documented mutations appear to have a phenotypic effect.

Spontaneous mutations with a phenotypic effect on the extended and segmented germband stages have long been documented. Mutations that cause homeotic changes have received special attention [18]. More recently, other spontaneous mutations with an effect during these stages were recovered in laboratories, including mutants of the *engrailed* [19] and *cubitus interruptus* [20] genes that were modelled by von Dassow *et al.* [14], and mutants of other genes that they disregarded [21,22].

There is an abundance of induced mutations that have an effect on the extended and segmented germband stages, including an effect on the segment polarity network in the ectoderm. Some of these mutations (e.g. mutations of the pair-rule genes) change the initial input of the segment polarity gene network in the ectoderm and this usually leads to

Box 1. Cascading pleiotropic effects

Signaling of segment polarity genes in the ectoderm acts as an organizer that affects many processes; for example, the segregation and early differentiation of neuroblasts, epidermoblasts, sensory precursor cells, salivary precursor cells, imaginal discs and tracheal precursor cells in the ectoderm [a–c]. The downstream effects involve a signaling cascade that crosses segmental and germ-layer boundaries. For instance, subdivision of the mesoderm into the primordia of heart, fat body and visceral mesoderm, as well as differentiation of the somatic mesoderm, are regulated by segment polarity genes with signals coming from both ectoderm and mesoderm [d–f]. In turn, signaling from the mesoderm is crucial for the local differentiation of the non-segmented endoderm and ectodermal gut [f–h]. Signaling from the gut influences the patterning of the visceral mesoderm [g,i]. Patterning of the visceral mesoderm also involves signaling across segmental borders; for example, in the anterior migration of caudal precursor cells of the longitudinal musculature around the gut. Any abnormality in the trunk visceral mesoderm disrupts this migration [j]. The migration of somatic muscle precursors also involves complex signaling between germlayers, and the activity of segment polarity genes around the parasegmental border in the ectoderm plays a key role in this [k,l]. Signaling across germline and segmental borders must also be involved in the differentiation and migration of the nervous system and trachaeae. The complex branching pattern of the tracheal network is established by migrating precursors [m]. Again, segment polarity genes, especially *hh*, appear to play a central role [n]. Furthermore, signaling between the ectoderm and the extra-embryonal layer (amnioserosa) is crucial for two essential morphogenetic processes, germband retraction and dorsal closure [o,p]. The *wg* pathway influences this patterning through interaction with the JNK (c-Jun N-terminal kinase) pathway [p]. Finally, cell death probably plays an important role in the cascade of pleiotropic effects. Programmed cell death occurs at a relatively high rate during the extended and segmented germband stages, and activity of the segment polarity genes (in particular *Wg*) plays an important role in the patterning of cell death [q].

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severe abnormalities in the expression of striped segment polarity genes [17,23–26]. In addition, many mutations (e.g. mutations in the Notch and JNK pathways; see examples in Refs [23,27–32]) affect the input during the active phase of the network in the ectoderm and have similarly drastic abnormalities as a result. For a few mutations, no or few phenotypic effects are reported, owing to overlapping gene functions. This occurs in *invected/engrailed*, *fz/dfz(2)* and *cubitus interruptus/teashirt* mutations [17,33,34]. The robustness provided by gene redundancy differs from that considered in the model of von Dassow *et al.*

One can argue that most of these mutants correspond to loss of function and, thus, fall out of the scope of the robustness modelled by von Dassow [14]. However, severe effects are also observed in hypomorphic mutations that only reduce gene function. Many hypomorphic mutations (e.g. mutations of *en*, *wg*, *ptc*, *arm*, *dsh*, *porc*, *eve*) disrupt the normal signaling of segment polarity genes in the ectoderm and elsewhere, often with lethal phenotypic effects [24,25,27,35–39] (Figs 3,4). Comparisons of hypomorphic and other mutants show that cells are sensitive in their response to different concentrations of *wg* (e.g. in ectoderm and imaginal discs [23,27,40]) and *hh* [41,42]. This lack of robustness ensures that small differences in gene dosage can lead to severe effects throughout the embryo. In addition, cell responses appear sensitive to the dosage of other segment polarity genes and of genes affecting their expression (e.g. *gsb*, *arm*, *ptc*, *nkd*, and *hid*) [23,36,43,44]. The altered expression patterns of segment polarity genes usually maintain a striped character in a segmentally iterated fashion.

Table 2. Predictions of the extended robustness and pleiotropy hypotheses

	Effects of mutations	
	Pleiotropy hypothesis	**Robustness hypothesis**
Genetic mutational variation	Visible at the phenotypic level	Hidden
Direct phenotypic effects	Potentially large	Small
Dominance of direct effects	Haploinsufficiency possible	Recessivity or near recessivity
Pleiotropic effects	Many	Few

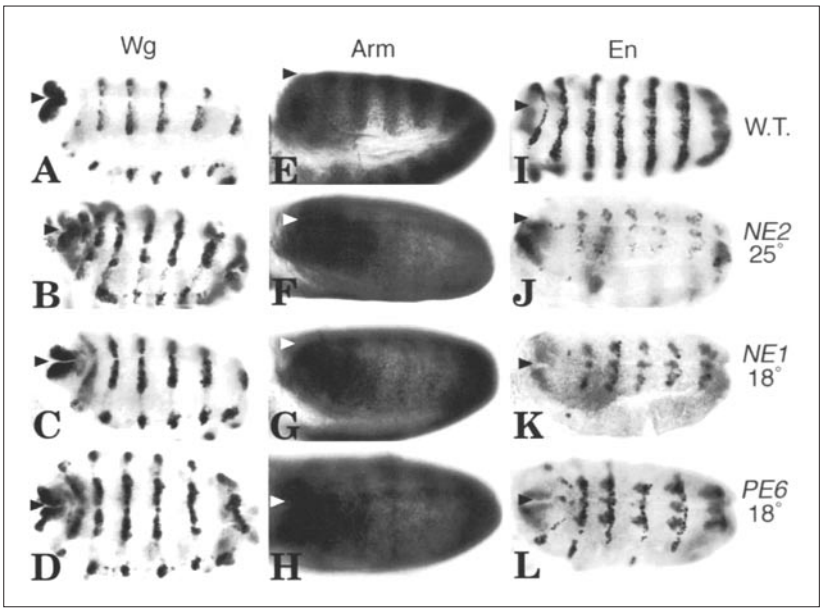

Fig. 3. Null ($wg^{-/-}$), reduced [Df(2)DE] and partial (NE2) function mutations of the *wg* gene lead to abnormalities in the larval ectoderm. Expression of *wg* in the ectoderm (A–D), and cuticular pattern in the ventral (E–H) and dorsal (I–L) larval epidermis (W.T. denotes wild type). In Df(2)DE mutants, *wg* expression is reduced, and in NE2 mutants, *wg* transport is hampered (reproduced, with permission, from Ref. [27]).

However, changes in the position, shape and intensity of stripes lead to dramatic phenotypic effects, so that the system does not appear robust.

Although most mutations affecting the extended and segmented germband stages are virtually recessive, dominant mutations do occur. Examples are *cubitus interruptus* [20], *fused* (segment polarity gene), *krüppel*, *lethal myospheroid*, *notch*, *delta*, *deformed* [12] and *Antp* [45]. Also, most recessive lethal mutations are not innocuous in heterozygous conditions [11]; in most investigated cases, they are associated with a reduced viability and an altered developmental rate.

Pleiotropy

Although some robustness can occur, most mutations affecting the extended and segmented germband stages have dramatic pleiotropic effects. Mutations of segment polarity and other genes produce disturbances in many parts of the embryo [9,11,12,29,39,45,46]. Interestingly, pleiotropic effects are also found for hypomorphic mutations, in the ectoderm and elsewhere. For instance, in addition to a disturbed striped segment polarity expression, hypomorphic *wg* mutants have defects in thorax, antennae and wings, and in polarity in general [27,38,47] (Fig. 4). It is not surprising that pleiotropy is widespread. The segment polarity genes are, together with *hox* and other genes, involved in many functions during this stage: the specification and early differentiation of virtually all organ primordia and the patterning of drastic morphogenetic events (e.g. germband retraction, dorsal closure and head involution). In addition, changes in the activity of these genes have important pleiotropic effects because of downstream cascading effects, especially in relation to the organizer activity of the segment polarity genes in the ectoderm (Box 1). The overall picture agrees with the high connectivity that underlies the pleiotropy hypothesis. The downstream cascading effects show that the interactions across compartment boundaries are considerable and that the overall level of effective modularity is, therefore, low.

Fig. 4. Hypomorphic Wg-1 mutant showing a failure in the development of antennae, wings, halteres and thorax (half of the thorax is missing, scutellum missing and hairs deranged (reproduced, with permission, from Ref. [28]).

Transheterozygotes

Pleiotropic effects of mutations in the conserved early embryonic stages will no doubt lead to severe and rapid selection against most mutations. Selection is usually expected to be slow against recessive mutations. However, the picture changes if we also account for haplo-insufficiency when different recessive mutations on the same locus are combined. Kornberg [46] found that, for 58 mutations at the engrailed locus, virtually all combinations of two mutations in heterozygous conditions (transheterozygotes) were lethal and all combinations had phenotypic effects. Similar transheterozygous effects were found for *wg*, *gsb* and *Antp* [24,43,45]. In addition, many mutations show severe and usually lethal haploinsufficient effects in combination with mutations of different genes in the same or interacting pathways (e.g. *hh* [42], *wg*, *dsh* [24], *ptc*, *nkd* [23], *dTCF*, *puckered*, *hid* and *Dpresenilin* [44]). This combined haploinsufficiency will severely constrain the accumulation of recessive mutations.

Towards an integration

Which genetic network is the robust one?

We agree with von Dassow *et al.* [14] that robustness could have an important evolutionary role in allowing evolvability of stages preceding the conserved stages. The evolutionary diversification among insects of the gap and pair-rule genes would, indeed, have been facilitated by robustness of the network that patterns the conserved stripes of *wg* and *en*/*hh* signaling cells. However, the appearance of the segment polarity stripes precedes the signaling network modeled by von Dassow *et al.* [14]. In the initial phase of the conserved striped pattern, *wg* is not yet dependent on *en* and involves the activity of pair-rule genes [17]. It is possible that robustness resides in this earlier, more evolutionarily diversified, signaling network, allowing evolutionary change of the preceding phase. Otherwise, it is difficult to indicate any particular

phase of the gene network that could be characterized as robust. The signaling of the segment polarity genes during the time of the striped expression seems to be in a state of flux. For example, after the initial phase, *en* expression depends on *wg* expression in the ectoderm, then *en* and *wg* require expression of each other, then *en* is no longer dependent on *wg*, and finally both are independent of each other [17,23,25,48,49]. Adding to such dynamics are the many spatial differences in expression; for example, interaction of the segment polarity genes differs in the dorsal and ventral ectoderm [23,27,50] and in the mid-lateral gap that appears in the *wg*-expressing stripe in the epidermis [48]. The upstream and downstream interactions of the segment polarity genes, thus, show a surprisingly dynamic pattern in both space and time.

Low modularity of organizers and robustness

Despite the observed dynamic pattern, we agree with von Dassow *et al.* [14] that the input of the segment polarity gene network should be as robust as possible to changes. Yet, we expect that small changes in the connectivity of the segment polarity genes will have major effects on the outcome. The *wg* and *en*/*hh* signaling cells along the parasegmental boundary function as an organizer (Box 1). In organizers, small changes in output cause a cascade of effects because they organize a large part of the patterning in embryos. This is also true for any organizer during the earlier, more diversified stages of cleavage and gastrulation. Therefore, organizers are not independent modules as they affect many processes in different parts of the embryo. The chance of a feedback effect on the input of the organizer is, for this reason, considerable, further lowering the independence of modules and, in addition, affecting robustness.

Robustness and long-term conservation

Stabilizing selection is expected to lead to robustness to protect optimized traits against developmental noise and mutations [16,51–54]. This robustness can produce short-term conservation. However, during periods with drastic environmental changes that lead to strong directional selection, the robustness of genetic interactions can never be sufficiently high to prevent change. A robust gene network is characterized by selective neutrality of mutants, causing genetic drift within the set of morphogenetically equivalent genotypes and, thus, the accumulation of hidden variation [54]. When there is selection for robustness, the neutral set is expected to grow larger. However, after a while, through the combined effects of drift and constraints on the maximum achievable robustness, mutations come within reach that lead to a loss of robustness (i.e. the genetic composition of the population reaches the boundary of the neutral set) [54]. This process is facilitated by the fact that neutral sets in genotypic sequence spaces tend to have large boundaries relative to their interior [55,56], so that the proportion of genotypes close to the boundary is large.

Further evaluation

The next step in investigating the support for both hypotheses should be a comparison between the segmented germband stage and earlier or later stages, in which the vulnerability to mutations and the amount of genotypic variation without phenotypic effects are assessed. We recently analyzed teratological studies in vertebrates for reported phenocopies of mutational change during other developmental stages and the pharyngula stage [57] – the vertebrate phylotypic stage that is comparable to the germband stage in insects. Our study supports the validity of Sander's [7] and Raff's [10] hypotheses for conservation of the vertebrate phylotypic stage. If a similar pattern turned up in insects, this would further underpin the important role of pleiotropy and stabilizing selection in evolutionary conservation.

Conclusion

We found little evidence for robustness of gene networks towards mutational change acting on the extended and segmented germband stages, and, more specifically, on the segment polarity gene network in the ectoderm. The phenotypic effects of even weakly hypomorphic mutations are in agreement with the observation of Lande *et al.* [58] that mutations of small, nearly additive effects are usually expressed relatively late in development, whereas lethal mutations are usually expressed early (see also Refs [11,12]). The organizer function of segment polarity and other genes causes mutations in these genes to result in a cascade of pleiotropic effects. In addition, many auto-regulatory and cross-regulatory interactions provide feedback on the input of the segment polarity gene network. As a result, the segment polarity gene network shows relatively low effective modularity and robustness. The feedback that modulates the input of the network is absent in the model of von Dassow *et al.*, and this probably explains the discrepancy between the predicted and observed robustness of the network (and, in extended form, of the overall organization of the stages). This discrepancy can further be explained by the crucial importance of concentration differences (of gene products) for patterning, which increase the sensitivity of the system to changes even when striped patterns are still generated. Why has more robustness not evolved? Perhaps, within these stages, the total number of interactions involved in morphogenetic patterning is too limited to organize the pattern in an independent, modular way, thereby allowing greater robustness.

Even if the segmented gene network were robust, it would not provide long-term conservation for two reasons. First, the organizer function of the segment polarity genes implies large consequences for small changes. This makes it almost impossible to avoid phenotypic effects. Second, robustness that is achieved during periods of ecological stasis loses its effectiveness in periods with strong environmental changes and directional selection. Drift during

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Hans Metz is also affiliated to the International Institute for Applied Systems Analysis, Adaptive Dynamics Network, A-2361 Laxenburg, Austria.

episodes of stabilizing selection accumulates hidden genetic variation, which enables fast evolutionary change as soon as selection becomes directional.

The severe phenotypic effects of most investigated mutations indicate that there is no absence of genetic variation with phenotypic effects. Hence, strong stabilizing selection appears to be the major force in conservation of the extended and segmented germband stages. The documented pleiotropic effects of mutations of these stages are in agreement with the hypotheses of Sander [7] and Raff [10], stating that negative pleiotropic effects of mutational changes resulting from global interactions are constraining evolutionary change. A considerable part of the pleiotropic effects is caused by cascading interactions, indicating a low effective modularity (Box 1). It thus appears that low effective modularity constrains evolutionary change.

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