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OPINION PAPER

A criticism of the value of midparent in polyploidization

A. Gianinetti

Consiglio per la ricerca e la sperimentazione in agricoltura, Genomics Research Centre, via S. Protaso 302, 29017 Fiorenzuola D'Arda (PC), Italy

To whom correspondence should be addressed. E-mail: alberto.gianinetti@entecra.it

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Abstract

The hypothesis of genetic additivity states that the effects of different alleles, or different genes, add up to produce the phenotype. When considering the F_1 progeny of a cross, the hypothesis of additivity of the genetic dosages provided by the parents is tested against the mid-parent value (MPV), which is the average of parental phenotypes and represents the reference value for genetic additivity. Non-additive effects (genetic interactions) are typically measured as deviations from MPV. Recently, however, the use of MPV has been directly transposed to the study of genetic additivity in newly synthesized plant polyploids, assuming that they should as well display mid-parent expression patterns for additive traits. It is shown here that this direct transposition is incorrect. It is suggested that, in neo-polyploids, mid-parent expression has to be reconsidered in terms of reduced genetic additivity. Homeostatic mechanisms are deemed to be the obvious ones responsible for this effect. Genomes are therefore ruled by negative epistasis, and heterosis in allopolyploids is due to a decreased interaction of the parental repressive systems. It is contended that focalizing on the right perspective has relevant theoretical consequences and makes the studies of neo-polyploids very important for our understanding of how genomes work.

Key words: Genetic additivity, heterosis, homeostasis, mid-parent value, neo-polyploids, polyploidization.

Introduction

Genetic additivity occurs when the effects of different alleles, or different genes, add up in the resulting phenotype (Hayman and Mather, 1955). This effect has been widely studied in offspring derived by the crossing of parents differing for either Mendelian or quantitative traits (Allard, 1999; Gillespie, 2004). Genetic interactions cause deviations from additivity, and may occur either at the level of a single locus (intra-locus) or between different loci (inter-loci). Dominance is the classical Mendelian instance of intra-locus genetic interaction (whilst semi-dominance is the classical example of additivity), and epistasis (which occurs when one gene changes or masks the effect of another gene) includes various cases of inter-loci genetic interaction. For a given trait, non-additive effects (genetic interactions) are measured in the F_1 progeny of a cross with respect to the mid-parent value (MPV), which is the average of parental phenotypes and represents the reference value for the hypothesis of additivity of the genetic dosages provided by the parents (Hayman and Mather, 1955; Gillespie, 2004; Jackson and Chen, 2010). Recently, however, the use of MPV has also been directly transposed to the study of genetic additivity in newly synthesized allopolyploids (neo-polyploids), assuming that they too should display mid-parent expression patterns for additive traits (Albertin *et al.*, 2006; Pumphrey *et al.*, 2009; Bassene *et al.*, 2010; Qi *et al.*, 2012). It is shown here that this direct transposition, although frequently used, is incorrect, since a change in the ploidy level is involved.

Polyploidization

Polyploidy, the condition of eukaryotic organisms with multiple chromosomal sets, is an important evolutionary drive, and occurs in some animals, fungi, and many plants (Comai, 2005; Jackson and Chen, 2010; Albertin

and Marullo, 2012). Polyploid plants have been generated from evolutionary processes, crop domestication, and/or artificial synthesis (Yang *et al.*, 2011). Although the consequences of polyploidy on gene and genome have been investigated extensively, most investigations compare naturally occurring established cytotypes. This approach may confound phenotypic differences attributable to ploidy *per se* with those that result from evolution since the time of polyploid formation (Ramsey and Schemske, 2002). The production of new (synthetic) polyploids has, therefore, assumed an important role, in recent years, to study the consequences of polyploidization on phenotype and gene expression (Ramsey and Schemske, 2002; Yang *et al.*, 2011). Particular interest has been devoted to the study, at the genome level, of the additivity of genetic effects and to unravel, in allopolyploid hybrids, the mechanisms underlying heterosis, the superior performance of hybrids relative to parents (Birchler *et al.*, 2010). In fact, traditionally, polyploid plants have been studied from the perspectives of evolution, domestication, and creating crops with specific traits such as larger flowers, fruits, and yields (Yang *et al.*, 2011). These effects are chiefly relevant in stable euploids which have an integral multiple of the chromosome haploid number, in contrast to aneuploids which possess a chromosome set that is not an exact multiple of the haploid number and which usually underperform the parents and show undesirable traits (Comai, 2005). Anomalous features are also observed in odd-polyploids which have an odd multiple of the haploid number (Comai, 2005). Only polyploidization, where the full parental genomes are retained in the offspring is, therefore, considered hereafter.

Euploids can be further distinguished into auto- and allopolyploids, depending on whether the hybrid's parents belong to the same or to a different species. Actually, autopolyploidy results from a mutation in chromosome number whereas allopolyploidy results from concurrent hybridization and mutations in chromosome number (Comai, 2005). Since in the autopolyploids the number of the chromosomes is doubled but the genes are the same, once established, these polyploids are also more likely to behave as the parent diploids, apart from additive effects of increased gene number. On the other hand, in the case of allopolyploids, the merger of two divergent genomes causes a 'shock' because of obvious disequilibria into the complex system of metabolic and developmental regulations, even if, on the other side, the pairing of chromosomes is facilitated (Comai, 2005; Zielinski and Mittelsten Scheid, 2012). Eventually, a new equilibrium, representing a successful evolutionary novelty, can be obtained, or, more often, the 'shock' produces a disadvantageous phenotypic outcome.

Anyway, the division between autopolyploids and allopolyploids is not absolute. The chromosome sets of allopolyploids differ proportionally to the divergence of the parental genomes which, in turn, largely depends on their phylogenetic distance: the closer the parents, the more similar the resulting allopolyploid is to an autopolyploid (Comai, 2005). Thus, the difference between the two types of euploids as regards the effects of polyploidization on the phenotypic outcome tends to be quantitative rather than categorical. Correspondingly, the heterozygosis of the polyploid hybrid has been suggested to be important for its performance: if there is complete or nearly complete homozygosity, increasing the ploidy frequently results in a reduction of plant stature and in less biomass (Birchler, 2013).

The mid-parent value in the context of additivity: a matter of math

In simple crosses, between either diploids or polyploids, the mid-parent value is a valid criterion to assess genetic interactions (Hayman and Mather, 1955; Jackson and Chen, 2010). However, in the case of polyploidization, where the hybrid has an higher ploidy than the parents, the use of MPV to test whether genes are additive is arguable. The mid-parent value is used, for a quantitative trait, to calculate additivity (that is, a simple dosage effect) as the arithmetic average of parental effects (dosages), assuming that each parent randomly contributes half of its genome to the offspring (i.e. ½ A1A1 + ½ A2A2 = A1A2; Fig. 1). In the case of neo-polyploidy, however, the offspring receives the whole genome of each parent, hence parental values (dosages), if truly additive, should be summed up (i.e. A1A1 + A2A2 = A1A1A2A2; Fig. 2), not averaged out. Indeed, according to Hayman and Mather (1955), the phenotype is found as the algebraic sum of all parameters associated with the genotype, and, neglecting interactions, the sum of allelic dosage effects gives a first approximation to the phenotypic value. Indeed, at least according to the metabolic control theory which aims to predict phenotype from biochemistry, increased metabolic flux is expected to be monotonically related to performance (Kacser and Burns, 1981). Of course, the value of a given trait cannot increase indefinitely, but most traits do not appear to be limited by physical constraints, rather they settle at values characteristic of the parent species. Thus, the hypothesis of genetic additivity assumes that: (i) semi-dominance occurs between alleles at each locus, and (ii) the phenotype is proportional to the whole allelic dosage.

As soon as this simple point is made, we are faced with a puzzling requirement: either we admit that a doubled phenotype should be expected in the neo-polyploid with respect to its parents, or the hypothesis of genetic additivity is wrong. The apparent incongruence between what is observed (i.e. the phenotype is most often not doubled) and what the hypothesis says, is easily solved if we think that genetic additivity represents the simplest, basic hypothesis that has to be refined for building a consistent theory of genotype–phenotype relationship. Similar problems occurred for other instances of application of the hypothesis and were overcome by providing new insights into how the relationship works (e.g. classical dominance and epistatic effects, Fig. 3; de Visser *et al.*, 2011). Indeed, the hypothesis of genetic additivity has been invaluable in improving our understanding of the complexity of genotype–phenotype relationships, just by pointing out those cases in which it appeared not to work.

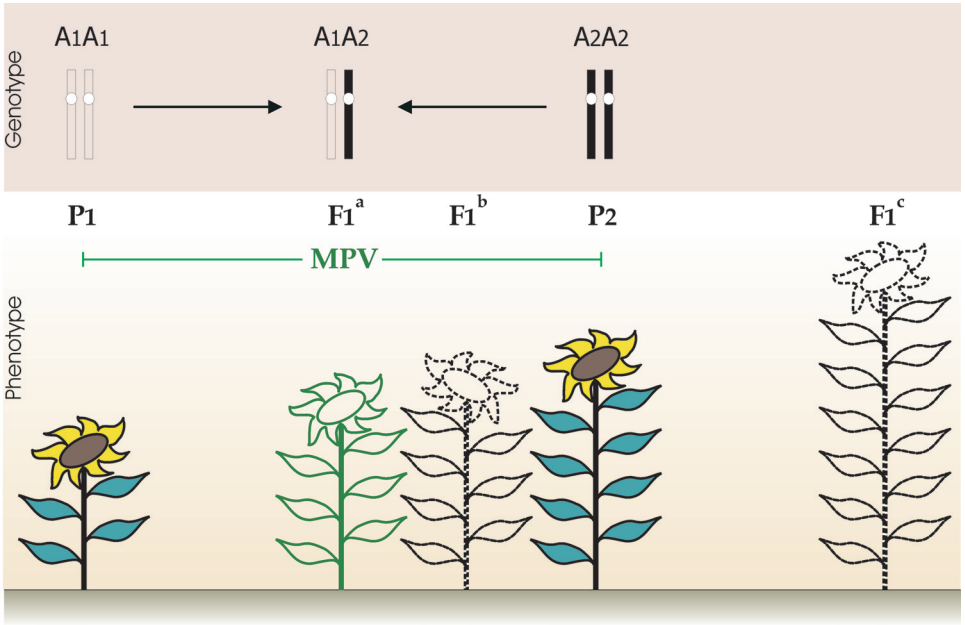

Fig. 1. Genetic additivity in a simple cross. Homozygous parents (P1 and P2) differ for the alleles (A1 and A2, respectively) at a given locus (a hypothetical gene determining the number of internodes with leaf) and can cross to generate a heterozygous F_1 genotype. Three possible offspring phenotype outcomes are shown. In F_1^a, the alleles have an additive dosage effect, and then the offspring phenotype is the average of parental values (i.e. mid-parent value, MPV). In F_1^b, the offspring phenotype deviates from MPV, i.e. the alleles show a non-additive effect (incomplete dominance). In F_1^c, the non-additive effect is so strong that the offspring phenotype is transgressive, i.e. it lies outside the range defined by parental phenotypes (in the case of favourable performances, this synergic effect is called heterosis). In classical Mendelian genetics, MPV represents semi-dominance, whereas complete dominance occurs if the F_1 offspring has the same phenotype as one or the other parent.

All the rest of this paper is aimed at showing that, even in the case of polyploidization, the discrepancy between the hypothesis of genetic additivity and the commonly observed phenotype of neo-polyploids can be explained in terms of down-modulation of the phenotypic outcome, analogously to what is done when the hypothesis is applied in similar contexts, and consistently with the findings of recent works on neo-polyploids.

From genotype to phenotype: phenotypic down-modulation is the rule in polyploidy

The main practical implication of focusing on the summed parent value (SPV; Fig. 2), here suggested as the correct yardstick for additivity in polyploidization, rather than on MPV, is that the reference value for additivity is shifted to higher levels. Therefore, phenotypic values (or gene expression levels as well) lying between MPV and SPV and previously considered to outperform the mere effect of gene dosages (i.e. to show positive interactions), should then be re-classified and included in the ranks of down-modulation. The term 'down-modulation' means a degree of phenotypic expression that is reduced with respect to what could be expected based on both the actual allelic dosage of structural genes and the hypothesis of genetic additivity (because of gene silencing, down-regulation, negative-feedback, and so on). Since SPV is the maximum expected phenotypic outcome along the scale used to measure polyploid performance with respect to their parents, and polyploid hybrids typically do not show full additivity of parent phenotypic values (case F_1^c of Fig. 2), it can be deduced that phenotypic down-modulation is the rule in polyploidy. However, analogously to what happens in the case of simple crosses, heterosis can be considered to occur when the hybrid outperforms both parents (Birchler *et al.*, 2010), that is, heterosis is defined as 'better parent heterosis'. In polyploids, therefore, the heterotic hybrid phenotype could well fall behind the theoretical value of full additivity (SPV), rather than exceeding it (as occurs with MPV in simple crosses). Hence, heterosis can coexist with phenotypic down-modulation to make polyploidy a power driver of evolution.

It can be noted that SPV is just the double of MPV. Hence, SPV can be markedly higher than the value of the better parent, and the hypothetical fully-additive hybrid phenotype would appear to show a heterotic effect that is extraordinary for neo-polyploids. SPV is, therefore, a boundary rarely crossed so that, in most cases, it does not allow further analysis of the hybrid phenotypic outcome. Nonetheless, it can be used for the analysis of gene expression. Remarkably, in many expression studies the range of deviation from MPV fell within either a 2-fold increase or a 2-fold decrease (Birchler *et al.*, 2003). This is consistent with SPV representing a threshold for full additivity of either activation or repression. Since a cut-off of twice the MPV equates to the use of SPV, results based on this threshold are correct *de facto*. Good practices are sometimes adopted before one knows why they work.

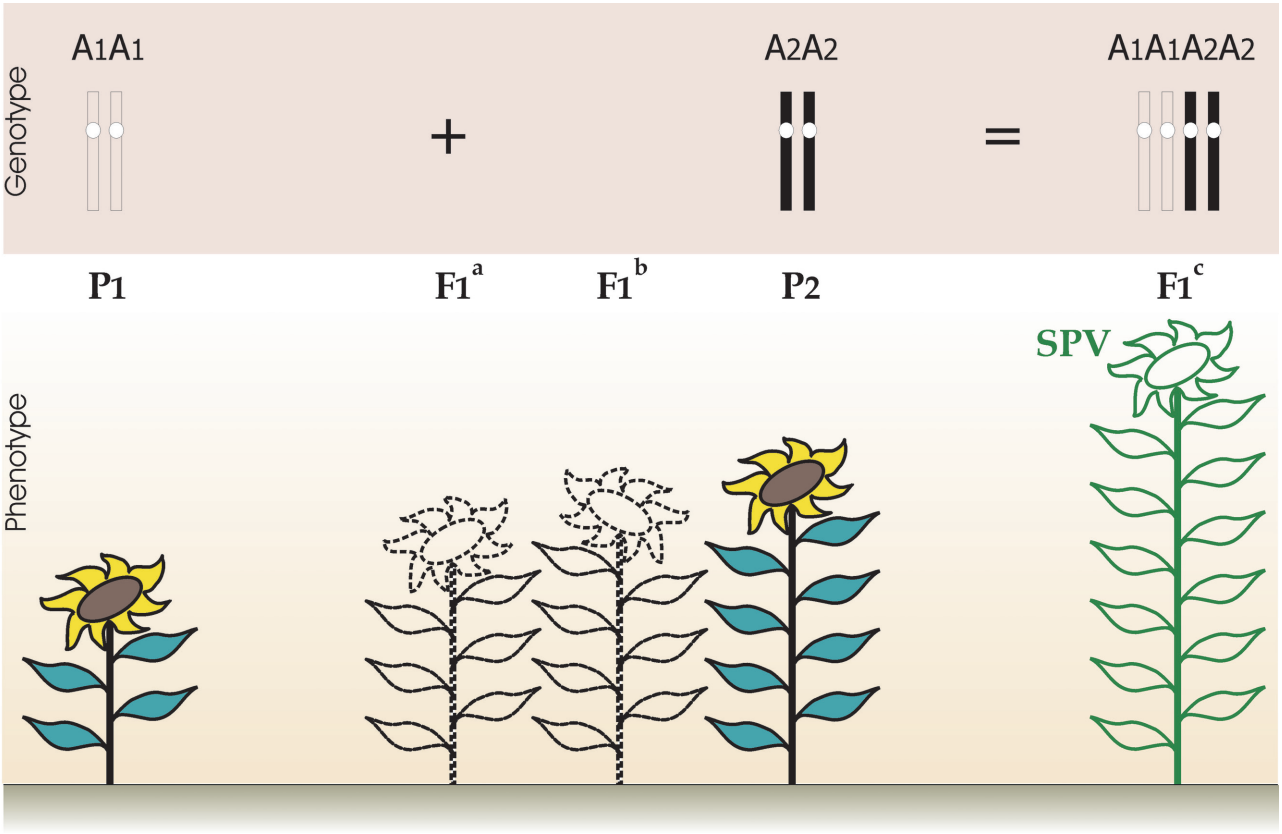

Fig. 2. In polyploidization, the diploid parental (P1 and P2) genomes do not undergo a gametic reduction and originate a tetraploid F_1. A1 and A2 designate different alleles at the same locus (or at homologous loci, in the case of an allotetraploid). If the genetic dosage is fully additive, the expected offspring phenotype is F_1^c, i.e. the sum of parent phenotypic values (SPV). However, most commonly, either F_1^a or F_1^b occur, i.e. the offspring phenotype is down-modulated with respect to full additivity. Matching of the F_1 phenotypic value with MPV is one of many possible outcomes in the case of non-additive phenotypic effects.

Homeostasis and 'protocols' as causes of phenotypic down-modulation

A first question, now, is what causes consistent down-modulation of the phenotype on the genotype–phenotype route in polyploids? The effect of polyploidization can be studied in the hybrid at different levels in addition to the final phenotype: the latter is the outcome of a sequence of dynamically controlled steps—transcription, translation, metabolism—that proceed from the genotype to the phenotype (Strohman, 2002), and each of them can be examined for changes that are consequent to polyploidization. Effects on gene expression are particularly interesting: chromosomal delections, gene silencing, and transcription down-regulation frequently occur following polyploidization (Comai, 2005; Yang *et al.*, 2011), counteracting gene redundancy and then additivity. In fact, when they do not occur, even-number ploidy has the general effect of increasing gene expression levels, on a per cell basis, in proportion to the gene dosage conferred by the ploidy level (Guo *et al.*, 1996; Yao *et al.*, 2011). In other words, gene expression, at least in maize, can be really additive. At the level of the proteome (enzymes and structural proteins) and the metabolome, most changes are, indeed, expected to be buffered by balancing of anabolic/catabolic activities and other complex regulation mechanisms (Stelling *et al.*, 2004; Jarosz *et al.*, 2010). Nevertheless, in maize, even expression for most proteins increases linearly with ploidy (Yao *et al.*, 2011). Polyploid derivatives of inbred maize lines, however, are less vigorous than the diploid progenitor (Yao *et al.*, 2011), so down-modulation must occur at the metabolic level. The resulting phenotypic changes are, therefore, largely reduced with respect to the theoretical effect of gene dosage, even if gene, or protein, expression is not. Indeed, homeostatic mechanisms are opposed to additivity and are expected to down-modulate the final phenotypic effect of increased gene number. In fact, organisms can be remarkably resistant to phenotypic change because of many cellular mechanisms that ensure the stability of the phenotype against both environmental and genetic perturbations (Jarosz *et al.*, 2010). Homeostatic settings can be seen as 'protocols' (Stelling *et al.*, 2004), that is, sets of executing rules that co-ordinate a biological system to ensure a robust performance. Inheritance of these 'packages' of rules should guarantee that the robustness of metabolism and phenotype are transferred along a phylogenetic lineage (Gjuvsland *et al.*, 2011). It should, however, be noted that these rules are built-in properties of the biological system which works as it works because it is built the way it is built; therefore

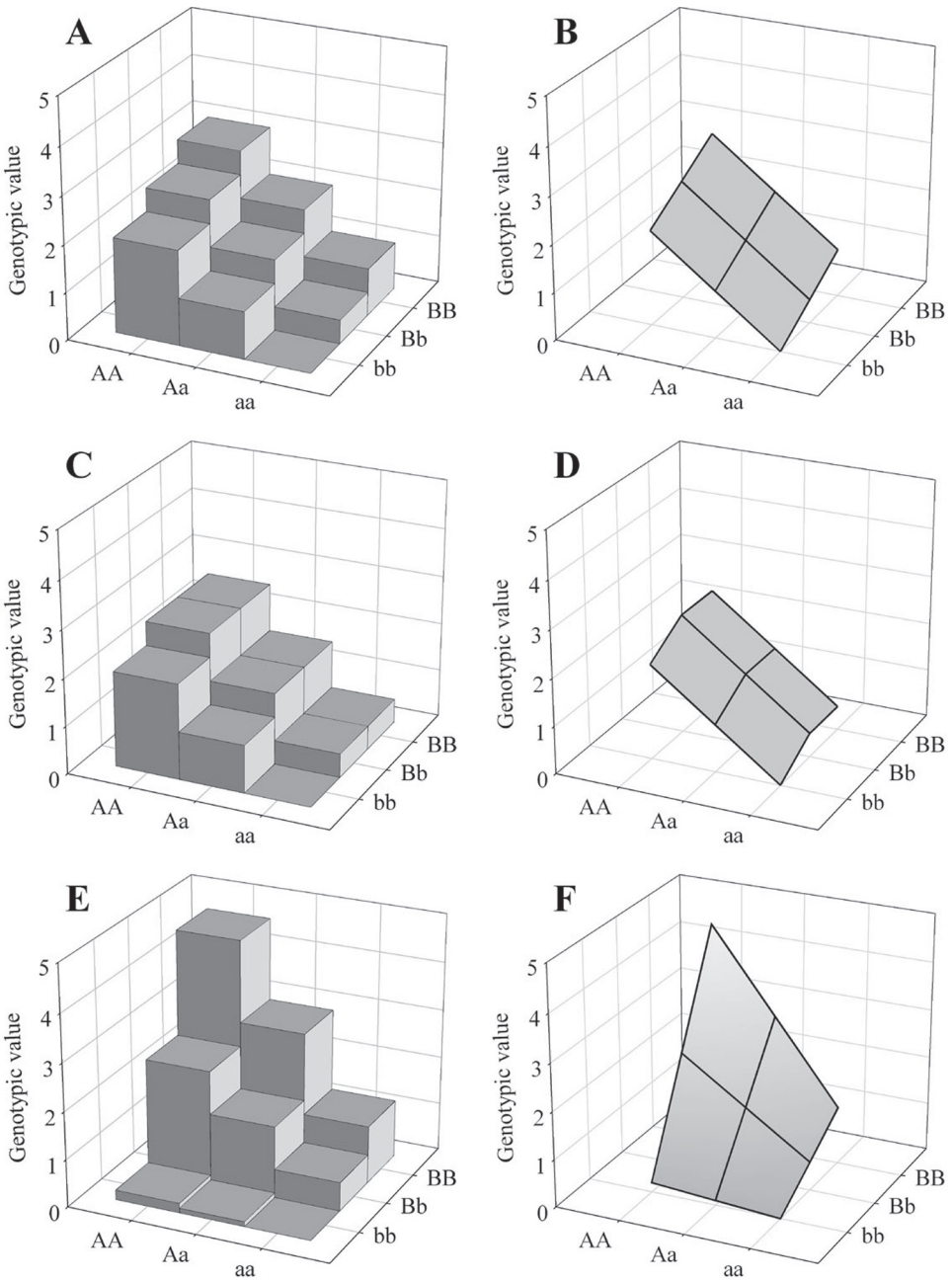

Fig. 3. Examples of deviations from additivity of genotypic values (i.e. the phenotypic outcomes that can be considered representative of genotypes once variability due to environmental and random effects is removed) in a two-loci (diploid) model. The different types of gene actions and interactions have been modelled and quantified according to Mather and Jinks (1982). On the left, genotypic values are plotted as 3D bar charts to show the contributions of different allelic doses; on the right, the corresponding 3D mesh plots highlight the overall shape of the response surface of genotypic value versus genotype to illustrate how genetic interactions represent deviations from plain additivity. (A) Full additivity: each dose of allele A gives an additive contribution (a_A) of 1 and each dose of allele B gives an additive contribution (a_B) of 0.5 to the genotypic value; all the genotypic values lie on one planar response surface (B). (C) Locus A is additive whilst locus B shows dominance (d_B), thus that $a_A = 1$, $a_B = 0.25$, $d_A = 0$, $d_B = 0.25$; the response surface is bent (D) because of saturation in the response of the genotypic value to an increasing dosage of B. (E) Both loci show intra-locus additivity, but locus B acts epistatically as an enhancer of locus A expression (additive × additive epistasis, e_{aa}), then $a_A = 0.9$, $a_B = 1.4$, $d_A = 0$, $d_B = 0$, $e_{aa} = 0.8$; because of epistasis (additive × additive), genotypic values of the four homozygotes are not coplanar, even if all intra-locus relationships are linear (F). Within each locus, the dominant allele, or the allele that confers higher genotypic value, is denoted by a capital letter.

'protocols' should be seen as inherent constraints of the biological system and not as a series of instructions that exist separately from it.

Ultimately, metabolic homeostasis at parental settings appears to play a major role in the down-modulation of neopolyploid phenotypes.

Some misunderstanding on the worthiness of MPV may have arisen just because of this consistent phenotypic down-modulation in neo-polyploids, which causes the value of most quantitative phenotypic traits to fall in the same range as that of F_1 offspring. This has probably led to the mistaken conclusion that MPV was the right theoretical reference in polyploidization as well. Indeed, biological functions are generated by hierarchical systems in which gene interaction ('epistasis' in the classical sense) represents a ubiquitous phenomenon that can greatly obscure the mapping between genotype and phenotype (Phillips, 2008; Álvarez-Castro, 2012). In the allopolyploids, the hybrid inherits from its parents two complete sets of regulative networks that are entrusted to maintain homeostasis (i.e. a stable condition of the metabolic system), but which are set at different levels of performance. The (down-modulated) hybrid performance can, then, just be the result of averaging the parental homeostatic settings. Eventually, the mid-parent-like phenotype is common in neo-polyploids (a paradigmatic example is the apparent semi-dominance observed for the flesh colour in an allotetraploid hybrid *Citrus*; Bassene *et al.*, 2009), but it is due to homeostasis and not to genetic additivity.

Back to genotype: dominance and regulatory networks

Another key question is how phenotypic down-modulation (chiefly linked to metabolic homeostasis at parental settings) should be interpreted in relation to the genetic additivity hypothesis?

A first, classical remark on the assumption of full genetic additivity is that most genes that encode metabolic functions usually show a dominant/recessive behaviour rather than being additive, in contrast to regulatory genes which, most frequently, do exhibit some type of dosage response (Birchler *et al.*, 2003). Dominance of functional alleles versus recessive null (or weak) alleles causes an increased phenotypic level in the F_1 offspring over MPV, but, on the other hand, it constrains the phenotype of the polyploid hybrid to the very same value of the dominant parent. Hence, contrary to simple crosses, dominance represents a cause of *negative* deviation from additivity in polyploids. Indeed, in diploids, dominance implies that the single dominant allele of a heterozygote provides the same function that occurs in the dominant homozygote (haplosufficiency). Actually, according to Wright's model of dominance (Wright, 1934), it is commonly assumed that a dominant allele of an enzyme-codifying gene provides a dose of enzyme sufficient to ensure the full functionality of its metabolic pathway because saturated enzyme kinetics would buffer the reduced dosage in the heterozygote (Gillespie, 2004). In its modern formulation, this theory suggests that changes in catalytic activity at one step in a pathway (due to different allelic dosages in the homo- and heterozygotes) is buffered by the response of other enzymes, even if most enzymes operate under conditions of low saturation; the whole pathway flux therefore tends to be a saturated function of the activity of each single enzyme (Kacser and Burns, 1981). Thus, metabolic control theory predicts that large increases in enzyme activity will negligibly affect fitness/flux compared with large decreases, supporting a biochemical mechanism as the basis of dominance (Kacser and Burns, 1981). However, an increased dosage of the whole battery of enzymes involved in a biochemical pathway (as takes place in neo-polyploids) should result in a proportional increment of the overall metabolic flow and of the phenotypic outcome. Since this increment usually does not occur, it can be inferred that another mechanism, outside the metabolic control theory, provides down-modulation and warrants that an increased enzyme dosage is more efficiently buffered in the polyploid than in the diploid dominant homozygotes.

A second remark, in fact, is that regulatory networks provide a more versatile and robust mechanism for homeostasis of the metabolic pathway flows as well as for proactive physiological tuning (Lenski *et al.*, 1999; Stelling *et al.*, 2004; Grüning *et al.*, 2010; Jarosz *et al.*, 2010) and they do exist as a homeostatic mechanism acting even in the case of adjusting the metabolism of a dominant/recessive heterozygote (Omholt *et al.*, 2000). Indeed, regulatory, multi-level networks typically rule the final determination of the phenotype (Grüning *et al.*, 2010; Jarosz *et al.*, 2010). For example, molecular chaperones have a central role in determining how genetic variation is translated into phenotypic diversity: they can buffer genetic variation, preventing it from having phenotypic consequence (Jarosz *et al.*, 2010). In this regard, it has recently been reported that only a small proportion of the duplicated genes have been neo-functionalized or non-functionalized in soybean (a paleo-polyploid), so that the main consequences of polyploidy in this species has to be at the regulatory level (Roulin *et al.*, 2013).

Negative epistasis is the genetic basis of phenotypic down-modulation

If homeostasis at 'protocols' settings is the physiological cause of phenotypic down-modulation, and regulatory networks are the genetic mechanisms deputed to maintain homeostasis at parental settings, then such networks act epistatically upon structural genes to tune their expression at hereditarily fixed levels, ensuring the conservation of phenotype along a phylogenetic lineage. Accordingly, a number of studies on genetic mutations support the general view that epistasis results from the buffering effects of physiological homeostasis (de Visser *et al.*, 2011). In addition, for a phenotype to be robust, its metabolism should be flexible, to cope with changing environment challenges. Flexibility implies both up- and down-modulation; thus, in ordinary conditions, the metabolism output must be below its maximum potential in order to be able to increase it when necessary. Dominance would have the same cause: haplosufficiency would be due to up-regulation; whereas, in neo-polyploids, multiallele expression should be down-regulated to the same level, the one fixed by the inherited 'protocols'. This viewpoint was actually advanced by Milborrow (1998), who proposed that, in the homozygotes, growth is genetically restricted by internal

control mechanisms to less than the maximum possible, thus that hybrid vigour would occur when the strict regulatory limitation of growth is relaxed by heterozygosity. In this view, the phenotypic function of the two alleles of the dominant homozygote is down-modulated at least to half (i.e. one allelic dose is repressed) and dominance rather than additivity would occur because of epistatic regulatory repression of redundant alleles at structural loci. However, in the case of tetraploids, four dominant alleles provide the same function that occurs in the diploid heterozygote. This means that, in the homozygous dominant tetraploid, three allelic doses of the structural gene are actually repressed. Following Milborrow's hypothesis (Milborrow, 1998), stronger repression should, therefore, occur in the hybrid polyploid than in both the diploid F_1 and in the homozygous parents to constrain the phenotype to the level of the dominant parent. Actually, with respect to the homozygous parents, activation of the dominant allele would occur in the heterozygous F_1 and repression in the neo-polyploid, suggesting that they are in a fundamentally different regulative state. Since, in many cases, the phenotype as well as gene expression are reduced with respect to the additivity hypothesis, even compared with the dominant parent, and frequently approach the levels of the recessive, low-performing parent, it can be inferred that repression predominates in most polyploids. The available evidence suggests that negative epistasis (by which a functional allele at an epistatic locus decreases the phenotypic effect of other loci) is largely responsible for the common observation of hybrid inferiority (Burke and Arnold, 2001). Epistasis is here intended with the meaning of 'compositional epistasis' as defined by Phillips (2008). Negative epistasis by repressive regulatory systems would then appear as the key violation to the additivity hypothesis and, hence, as the main genetic cause of the down-modulation of neo-polyploid phenotypes.

Indeed, even the metabolic control theory predicts that epistasis occurs between genes involved in the same pathway or biological process (Kacser and Burns, 1981; Szathmáry, 1993; MacLean, 2010). Whatever the homeostatic mechanism underlying dominance is in diploids, epistatic homeostasis represents the key opponent to the linear additivity of the allelic effects in establishing the value of a quantitative character in neo-polyploids. Studies of gene expression as well as enzyme and metabolite concentrations at different ploidy levels will help to clarify the roles of the kinetic and of the regulative mechanisms, which, however, are not necessarily mutually exclusive. Thus, neo-polyploids represent interesting genetic materials for confronting the different theories of dominance, just like, on the other side, the haploid organisms (Orr, 1991).

Theoretical consequences for heterosis

A valuable feature of neo-polyploids is that, by helping to clarify the basic mechanisms of genetic additivity, they also provide a complementary perspective to study the causes of heterosis. In the diploid F_1 much of the evidence points to the importance of additive factors as the genetic basis of heterosis (Ramsey and Schemske, 2002). Indeed, quantitative traits would be genetically controlled in large part by multiple dose-dependent regulatory hierarchies (Birchler *et al.*, 2010), and the existence of order-preserving design principles (in other words, homeostatic protocols) in the regulatory machinery causes a dramatic increase in the proportion of the additive variance explaining the phenotype of a diploid offspring (Gjuvsland *et al.*, 2011). Along this line of thought, an additive model, involving subunit dosages at multimeric regulatory complexes, has been proposed to explain heterosis, including progressive heterosis in polyploids (Veitia and Vaiman, 2011). That model assumes that phenotype linearly relates to the concentration and functionality of these complexes (that is, they are activators/enhancers). However, rebuttal of the MPV concept in polyploidization implies that, in neo-polyploids, heterosis is instead based on reduced additivity of the parental repressive regulatory hierarchies, that is, on relieved repression. Indeed, heterosis in parental diploid *Brassica* species appears to be unrelated to heterosis at the alloploid level (Bansal *et al.*, 2012); therefore, different mechanisms seem to operate in the two conditions. In addition, progressive heterosis would be quite difficult to explain in terms of complementation of detrimental recessives (Birchler *et al.*, 2005), and then by the metabolic control theory. Actually, none of the current genetic models completely explain heterosis in polyploid plants, as allelic and genomic dosage may have a greater role than the allelic complementation or interactions (Bansal *et al.*, 2012).

From a genetic point of view, polyploid heterosis (below SPV) can then be interpreted in terms of reduced negative epistasis. Reduced epistatic constraints could derive from higher genetic diversity between the parents, explaining why polyploids with increasing genetic diversity exhibit progressively greater heterosis (Veitia and Vaiman, 2011; Bansal *et al.*, 2012). Merging two divergent genomes in a nucleus may lead to trans-activation and repression due to the divergence in parental regulatory machineries that become reunited in the hybrid, resulting in both novel patterns of homoeologue activation and repression (Yoo *et al.*, 2013). The net effects of these regulatory interactions is commonly reflected as expression level dominance (i.e. the overall pattern of gene expression in the hybrid corresponds to that of one parent), but it can also produce transgressive expression. This suggests a form of trans-activation or repression, entailing the joint up- or down-regulation of homoeologues due to the novel regulatory environment of the polyploid nucleus (Yoo *et al.*, 2013). Thus, in the hybrid, reduced interaction between the inherited parental repressive systems might well be consequent on the fact that the integration of two slightly different regulatory systems can result in a new regulatory protocol with fewer restraints to metabolic fluxes than occurs in both parents and then cause heterosis (Milborrow, 1998). However, polyploid hybrids tend to perform poorly on average, and only some hybrid genotypes outperform their parental counterparts (Burke and Arnold, 2001; Zielinski and Mittelsten Scheid, 2012; Birchler, 2013). It appears that, in analogy to what occurs in bureaucracy, increasing the number of protocols most often results in a stronger repression of productive

activity, and better performance is an exception rather than the rule.

Applications: a common theme and a conceptual framework

Some exemplifications of the concepts presented here are provided below, on the basis of studies already published. The common theme is that the genomes are composed of structural and regulatory genes and MPV is used as a reference for the additivity hypothesis with the aim of measuring dominance effects, and the epistatic effects of some genes (e.g. regulatory ones) on others (e.g. structural genes), as well as the reciprocal effects of complementary genes (Phillips, 2008; Álvarez-Castro, 2012). However, in neo-polyploids, the additive effects of structural genes should lead to a summed-parent phenotype rather than to a mid-parent one, and it is therefore on the SPV that dominant/epistatic effects should be measured. By using the MPV, what is measured is not the overall effect of dominance/epistasis, but the deviations from additivity of 'protocols'. This can still be valuable information, but it is not what it is assumed to be. In fact, the protocols are informational entities hierarchically higher than genes and even than multigene complexes encoding regulated biochemical pathways (Stelling *et al.*, 2004; Gjuvsland *et al.*, 2011).

In the following examples the additive model of Veitia and Vaiman (2011) has been adopted as a convenient conceptual framework, even if it has not yet been demonstrated, to show how repressive regulatory systems could be working in neo-polyploids (both auto- and allopolyploids).

The flesh fruit colour in Citrus

As a first instance, consider that, in the aforementioned case of *Citrus* (Bassene *et al.*, 2009), the hybrid possesses the whole battery of functional genes that are able to give a deep orange colour in the fruit flesh of the Willowleaf mandarin parent. Therefore, even if the Eureka lemon parent (having a pale yellow fruit flesh) carried null alleles at these loci, the hybrid should have the same deep colour as the mandarin parent. It doesn't have it, so the functional gene battery must be repressed. According to Birchler *et al.* (2003) the regulatory genes appear to work additively, so, in the hybrid, repression is stronger possibly because of a doubled concentration of multimeric regulatory complexes or because the subunits of the regulatory complex governing carotenoid expression in the lemon parent are more strongly repressive than those of the mandarin parent, thus that the hybrid regulatory system consists of a balanced mix of strong and weak-acting subunits and, then, the regulatory complexes have a repressive action on the hypostatic biochemical pathway that is intermediate between those of its parents (according to the 'repressive' version of the model of Veitia and Vaiman, 2011, proposed here). The apparent mid-parent phenotype is therefore determined by the additive, semi-dominant, behaviour of the parental repressive systems. Actually, at the metabolic level, the allotetraploid hybrid produced the same pigments as mandarin but at very low concentrations, largely below MPV. Thus, Bassene *et al.* (2009) hypothesized that a new gene regulation profile, unfavourable to carotenoid accumulation, could occur in the hybrid, which is entirely consistent with the view presented here. In fact, the 'protocol' could essentially be based on the additive model of Veitia and Vaiman (2011), in which a balanced mix of strong and weak-acting subunits and a doubled concentration of multimeric regulatory complexes, combine to repress carotenoid concentrations below MPV. In genetic terms, the genes for potential pigmentation inherited by the mandarin parent are epistatically constrained by the hybrid regulatory system to a MPV, even if the structural genes would be available to give a SPV phenotype.

The case of size

In many newly synthesized polyploids the size of the plant increases (Ramsey and Schemske, 2002). This is evident, for example, for the size of the spike in triticale (Jung and Lelley 1985), of the fruit in *Actinidia* (Wu *et al.*, 2012), and of the leaves in *Lolium* (Sugiyama, 2005) and alfalfa (Stanford *et al.*, 1972) neo-polyploids. In fact, a larger size of the cells and thicker and broader organs (like leaves, bracts, and petals), are common in polyploids (Stebbins, 1971; Mizukami, 2001; Balao *et al.*, 2011). Interestingly, a larger size of the cells implies that, due to geometrical scaling (Símová and Herben, 2011), the biomass of the plant organs should get close to SPV better than other traits; although this does not necessarily reflect on the biomass of the whole plant (Birchler, 2013). With regard to the additivity, these instances would be consistent both with a relieved repression mechanism and with the original version of the additive model for multimeric regulatory complexes acting as enhancers/activators (Veitia and Vaiman, 2011). In the latter case, additivity should be expected for both the concentrations of regulatory complexes, as well as for the allelic dosage of structural genes involved in the growth process (like those for GA synthesis and response). If so, then a doubled SPV could be expected as the phenotypic outcome as a result of the simultaneous increment in the number of copies of structural genes and of their activators. However, the increased cell size counteracts the effect of higher levels of activators and metabolites by 'buffering' their concentration and so dampening the phenotypic outcome until a new equilibrium is reached. It is worth noting that if activators can increase the output of structural genes up to a SPV phenotype, it means that activators themselves were previously set (in the diploids) for a lower output. As even this process has to be regulated, it turns out that repression has only shifted to a higher hierarchical level of regulation. Reduced activation is still a form of down-modulation. The heterotic effect observed for these traits, and not for most others, might occur because plant growth is frequently curbed by environmental constraints and then a looser 'protocol' has evolved for this feature. A regulatory network allowing for the additivity of structural genes and of activators/enhancers, as well as for the supervening effect of repressors, and also more open to environmental cues, must be involved.

Yield

It is highly possible that the higher grain yield offered by the hexaploid common wheat (Dubcovsky and Dvorak, 2007) is due to the disruption of the yield-limiting protocols occurring in tetraploid durum wheat, rather than to some miraculous highly-effective gene, or allele, carried by a very poor-yielding *Aegilops* species. In fact, it has been suggested (Feldman *et al.*, 2012) that, when loss or silencing of genes do not take place, the process of cytological diploidization leading to exclusive intra-genomic meiotic pairing and, consequently, to complete avoidance of inter-genomic recombination, can provide a means for the fixation of positive heterotic inter-genomic interactions. As noted above, such heterotic interactions could well occur because of additivity. So, this may be regarded as a case of reduced negative epistasis, intermediate between the previous examples.

Gene expression and translation

Concerning gene transcription, the aforementioned case of maize (Guo *et al.*, 1996; Yao *et al.*, 2011) seems to be uncommon, even if it is of great relevance since it demonstrates that gene expression can indeed be additive. Not surprisingly, the substantial majority of genes show mid-parent expression in the synthetic allohexaploid wheat nucleus (Pumphrey *et al.*, 2009). Although this is not what is expected based on the additivity hypothesis, it confirms that inheritance of protocols guarantees that robustness of metabolism and phenotype are transferred to the offspring (Gjuvsland *et al.*, 2011). Interestingly, in the allopolyploid cotton hybrid, as well as in other species, merged parental genomes frequently show expression level dominance, that is, the total gene expression level resembles that of one of the two parents (Yoo *et al.*, 2013). Lack of parental equivalence with respect to the expression level (i.e. lack of a mid-parent expression level) seems to be caused by bias in trans-acting of homoeologue regulation. In fact, for most genes both homoeologues are equally expressed despite differential gene expression between the diploid parents (Yoo *et al.*, 2013). Thus, up- or down-regulation from the dominant parent is the most common cause of expression level dominance and is particularly evident on the expression level of the homoeologue from the non-dominant parent (Yoo *et al.*, 2013). In other words, as previously mentioned, the hybrid inherits from its parents two complete sets of regulative networks that are entrusted to maintain homeostasis, but which are set at different levels of performance. However, in this case, it appears that one is dominant over the other.

A mid-parent, or a parent-like, phenotype at the expression, translation, and metabolic levels makes sense, since otherwise the cytoplasm would become too crowded, if cell volume does not increase as well. Correspondingly, in the *Brassica napus* neo-polyploids, only some proteins show an expression higher than that of the higher parent (Albertin *et al.*, 2006; Kong *et al.*, 2011), but this actually means that most proteins exhibit a non-additive expression repatterning. It is expected that a quite stable mid-parent, or a parent-like, protocol governs overall protein expression and concentration, avoiding molecular over-crowding.

In this regard, the widely observed increase in cell size that occurs in polyploids suggests that a larger cell volume is a primary homeostatic response to polyploidization, aimed to maintain the proper concentrations of gene products. It would then be compelling, in every study of new-polyploids, to report about differences in cell size between the hybrid and its parents, since this appears to be a key aspect for a meaningful understanding of any rearrangement of metabolic processes.

Conclusion

In all the above cases, the expected phenotype should be SPV, since genomes add up rather than average out; but, more often than not, the phenotypic outcome is subdued, because regulative mechanisms control genotype translation to phenotype and their settings (i.e. the protocols) indeed average out. This is the reason why, usually, the polyploids are not the sum of their constituent genomes (Otto, 2003). Failing to acknowledge this fact leads to a loss of the main significance of the experimental results obtained with neo-polyploids: on one side, for a few traits (specifically, those related to cell size), a SPV phenotype confirms that neo-polyploids can indeed attain full additivity; on the other side, most commonly, a mid-parent phenotype provides straightforward evidence of Milborrow's 'repressive theory', that is to say, metabolome, proteome, and transcriptome are down-modulated, and, then, the genomes are ruled by negative epistasis. Homeostatic mechanisms aimed to preserve parental metabolic settings (protocols) are here suggested to be the rationale of this phenomenon.

There are, of course, physiological constraints to the possible increase in the value of each trait, but it appears that, in neo-polyploids, most characters studied are genetically restrained well below such constraints and polyploidy is now recognized as a characteristic feature of all angiosperm genomes (Jiao *et al.*, 2011).

Indubitably, studies on neo-polyploids will have a relevant impact in unravelling the physiological and genetic bases of additivity and heterosis. In such a context, the use of the MPV as a reference for phenotype needs to be revised.

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