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RESEARCH ARTICLE

Biased Polyphenism in Polydactylous Cats Carrying a Single Point Mutation: The Hemingway Model for Digit Novelty

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Abstract Point mutations in a *cis*-regulatory element of Sonic Hedgehog are frequently associated with preaxial polydactyly in humans, mice, and cats. The Hemingway mutant in the Maine Coon cat exhibits polyphenic effects of polydactyly that are not equally distributed. A statistical analysis of a comprehensive data base of Hemingway mutants reveals a biased and discontinuous distribution of extra digits. Further biases exist in the difference of effects in fore- versus hind-limbs and in left–right asymmetry. These non-equally distributed phenotypic effects cannot be explained by the point mutation alone. We propose a double mapping model, termed the Hemingway Model, to account for the biased distribution of supernumerary digits. The model is based on the random bistability of individual cells in the limb area affected by the mutation and on the application of the Central Limit Theorem. It proposes two kinds of mapping events that (a) transform a mutational effect of single additive changes into a continuous distribution, and (b) transform the continuous distribution into discrete character states via developmental threshold effects. The threshold widths for the occurrence of discrete extra digits are specified as units of standard deviations of the continuous variable. This makes it possible to specify the generation of empirical developmental variables (the liability of quantitative genetics) as a result of developmental parameters that give rise to biased morphological patterns and phenotypic novelty.

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Introduction

Polydactyly is a widespread phenomenon in vertebrates, including numerous cases in all tetrapod classes. Even though certain regularities of digit addition to the species specific ground state can be discerned, there is significant intra- and inter-individual variability in both the number and appearance of the supernumerary digits. Since this is true even for populations that share the same genetic background, an interesting problem arises for the explanation and modeling of polydactyly: What are the factors that determine the polydactylous phenotype? Below we address this issue in a systematic study of digit variation in polydactylous cats, and, based on the results, we formulate a new model for supernumerary digit formation. We begin with an overview of the current models of polydactyly.

Two major trends can be identified in the approaches and interpretations of past investigations of polydactyly: One is primarily genetic, associating the appearance of extra digits with specific mutations, and the second is based on the developmental mechanisms of limb patterning. In the first approach, several mutations are shown to be associated with the appearance of polydactyly in vertebrates. Most of these occur in highly conserved *cis*-regulatory elements that directly or indirectly affect the Sonic Hedgehog (SHH) signal transduction pathway (Albuisson et al. 2011; Bimonte et al. 2011; Cameron et al. 2009; Chen et al. 2004; Driess 2005; Dunn et al. 2011; Farooq et al. 2010; Haycraft et al. 2007; Klopocki et al. 2008; Krawchuk et al. 2010; Lettice et al. 2008, 2012; McFadden et al.

2002; Park et al. 2008; Patterson et al. 2009; Semerci et al. 2009; Wieczorek et al. 2010; Yada et al. 2002; Zhang et al. 2010; Zhao et al. 2009). In other cases the Indian Hedgehog pathway is affected, such as in the Doublefoot mutant (Babbs et al. 2008) or in the Cilia mutant, where SHH (Haycraft et al. 2007) or BMP (Dahte 2009) signaling are also affected. It is argued that specific point mutations (Lettice et al. 2008), duplications (Lettice et al. 2003; Dahte 2009) or deletions (Babbs et al. 2008) disrupt conserved non-coding elements (CNEs) such as the ZPA regulator sequence (ZRS), a *cis*-regulatory element that controls the expression of *shh* in the limb. Indeed, preaxial polydactyly can be associated with ectopic *shh* expression in developing limbs, forming an additional ZPA like region in the anterior area of the limb bud (McFadden et al. 2002; Yada et al. 2002; Lettice et al. 2003, 2008, 2012). Besides the SHH related effects, polydactyly has been associated with mutations in the *Hoxa* and *Hoxd* clusters (Fromental-Ramain et al. 1996). In another case, extra synpolydactylous toes are induced via mutated *Hoxd13*, as well as by the reduction of retinoic acid (Kuss et al. 2009). Interaction of *Hoxd13* with *Gli3* can result in severe cases of synpolydactyly, generating up to twice the number of digits in a limb. Here SHH regulation is explicitly mentioned to be irrelevant (Sheth et al. 2007). Other important signaling pathways in the context of polydactyly are FGF, WNT and NOTCH signaling. For a more complete summary of molecular alterations that underlie polydactyly in mice see Talamillo et al. (2005) and Zuniga et al. (2012).

The second class of polydactyly explanations is based on the cell and tissue processes involved in limb patterning. Typically, generative systems are capable of a number of dynamical responses to perturbations, regardless of whether these have genetic or non-genetic triggers (Newman and Müller 2005). Here the phenotypic specificity of the modified form is explained by the developmental properties of digit formation, not by its molecular proxies. A particular case of developmental patterning, often applied to the limb system, is based on reaction–diffusion processes (Turing 1952). Today, Turing models are no longer based on chemical reaction–diffusion alone but include spatial transport mechanisms as well as "local autoactivation—lateral inhibition" processes (Meinhardt and Gierer 2000) and other self-organizing properties that have led to two- and three-dimensional simulations of limb patterning (Hentschel et al. 2004; Cickowski et al. 2005; Chaturvedi et al. 2005; Newman and Müller 2005; Alber et al. 2008; Christley 2008; Newman et al. 2008; Zhu et al. 2010). Reaction–diffusion dynamics also apply to activator–activator systems as well as to "short range inhibition–long range activation" systems (Madzvamuse et al. 2010), creating diffusion based instabilities characteristic of Turing systems. A recent model by Sheth et al. (2012) suggests that *Hox* genes are involved in the regulation of digit patterning by controlling the wavelength of a Turing-type mechanism.

The pattern forming consequences of these properties often become apparent only after growth of the entire region in which these dynamics play out is included (Crampin et al. 2002a, b; Miura et al. 2006), resulting in simulations with the capacity to generate concrete forms of polydactyly. Such models indicate how bifurcation type digit duplications can result from simple elongations of the chondrogenic domain. Miura et al. (2006) generate Doublefoot phenotypes, and Zhu et al. (2010) show how entirely new digits may arise in modeled limb buds. In these kinds of simulations additional digits or toes arise from a modification of the overall geometry of the limb forming system, but without the requirement of specific signals providing positional coordinates, an information theoretical view that had dominated early notions of pattern formation (Wolpert 1999).

Lettice et al. (2008) describe a highly polydactylous cat population living in Hemingway's house in Key West, Florida, with a mutation in a distant sonic hedgehog *cis*-regulator which they term the "*Hw*" mutant, as well as "*Uk1*" and "*Uk2*" mutations found in British mutant cats with PPD. Hamelin (2011) describes another mutation in the Maine Coon cat which could not be localized in the ZRS and neighboring DNA regions. In the cats analyzed by Lettice et al. (2008) and Hamelin (2011) PPD is inherited as an autosomal dominant trait with high penetrance. Cats with the *Hw* mutation (Fig. 1) exhibit the following phenotypes in the forelimb: (A) Elongation of digit one; (B) in addition to the elongation, a proximal bifurcation of digit

Fig. 1 Preaxially polydactylous Maine Coon with the CLP 7-7-5-5. Forelimbs: elongated first toe, one bifurcated PPD toe, one extra toe. (Photo published with kind permission of P. Shevtsova)

one (Fig. 2); (C) both of these effects, plus a completely new anterior digit (Danforth 1947a; Hamelin 2011) (Figs. 2, 3). Conditions B and C are known among breeders as "mitten paws." In some cases there is a dewclaw, a soft tissue appendage with a claw but without internal skeletal elements. In rare cases an elongated digit one is accompanied by a "free floating" skeletal element not connected to any other skeletal structure of the autopod (Fig. 3). Hamelin also describes size variations and fusions in the carpal bones. In most cases the *Hw* forelimb can be clearly distinguished from other PPD mutants, such as the Canadian mutant (Hamelin 2011), which only rarely shows the forelimb mitten paw. Instead, in most cases, it has a complete extra digit. In the hindlimb, cats with the *Hw* mutation exhibit the following phenotypes: One or two complete additional digits can occur (Figs. 2 and 3). No bifurcations are seen in hindlimbs.

In the *Hw* mutant strains, as well as in other cases, variations of the PPD effects are observed within the same individual and among individuals, i.e., the phenotypes of the initiating point mutation are polyphenic. One or several extra digits can appear. The effects may occur in one limb alone, symmetrically in either forelimbs or hindlimbs, in the limbs of one body side, or in all limbs of an individual. Similarly, extra digit variations can be observed among individuals of the same litter or between litters. So far, such kinds of digit polyphenisms received little attention and were assumed to represent chance events. Exceptions are the classical paper of Sewall Wright on guinea pigs (Wright 1934a) and the study of Danforth (1947a, b) on cats. Wright analyzed 23 strains of inbred guinea pigs for the presence of atavistic extra toes. He concluded that the formation of the extra toe depends on a combination of genetic and non genetic threshold factors, without specifying the nature of these factors. Danforth (1947a) studied the variable forms and numbers of extra toes in polydactylous cats bred in a controlled laboratory setting. No statistical analyses making use of the extensive extant data bases on feline polydactyly have been performed, which could determine whether the frequencies of the different forms of PPD exhibit any kind of bias. Nor were the evolutionary aspects of such polyphenic consequences of point mutations considered. These issues are the primary focus of the present investigation.

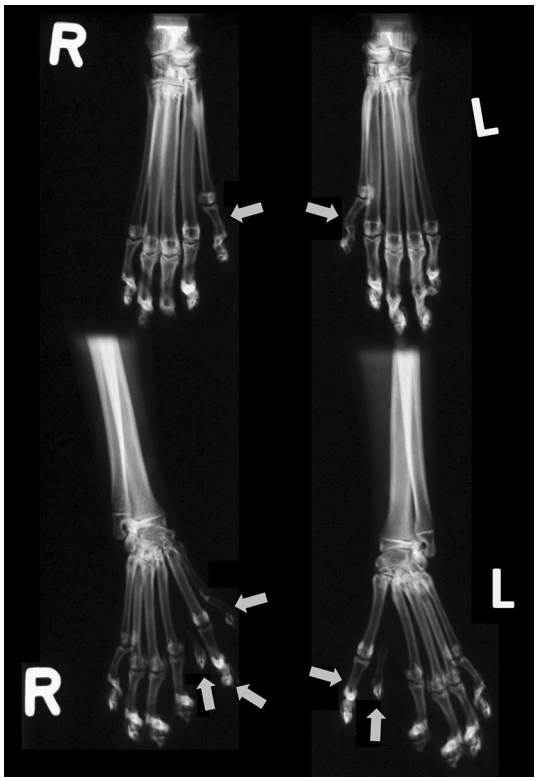

Fig. 3 Rare *Hw* mutant with CLP 6-7-5-5. *Top* hindlimbs: One extra anterior digit in both limbs (*arrows*). *Bottom* forelimbs: *Left limb* (L) elongated first digit; free floating element posterior to digit one. *Right limb* (R) elongated first digit; free floating element posterior to digit one; one thin extra anerior digit. Photo published with kind permission of S. Otten-Boult

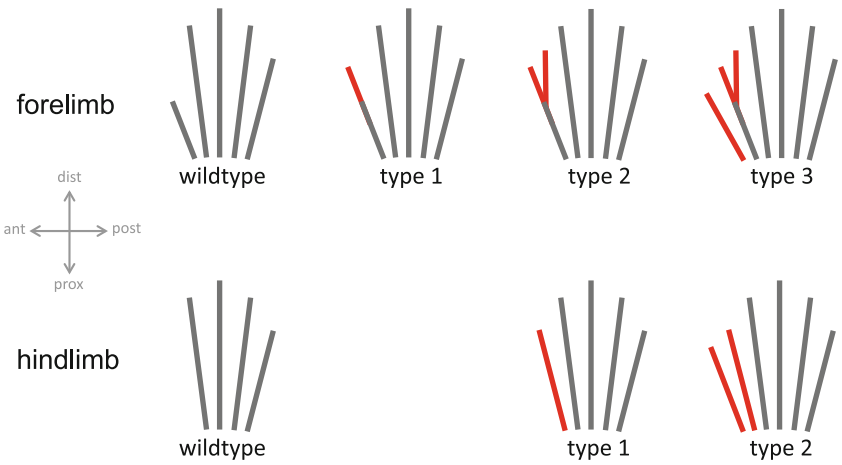

Fig. 2 *Hw* mutant limb patterns. Right limbs dorsal views: *Above* Forelimb: (Type 1) Elongated digit one; (Type 2) same as (1) plus posterior bifurcation of digit one; (Type 3) same as (2) plus complete extra anterior digit. *Below* Hindlimb: (Type 1) one additional anterior digit, (Type 2) two additional anterior digits

In the present study we examine the occurrence of phenotypic variants in the limbs of Maine Coon cats carrying the *Hw* point mutation described by Lettice et al. (2008). We show that the polyphenic PPD effects do not represent an equal distribution as one might expect of a single point mutation. Rather, those mutants exhibit various kinds of discrete phenotypes. They vary in their patterns concerning (1) individual limbs, (2) their forelimb versus hindlimb distributions, and (3) their left–right distributions. We analyze the frequencies of these distributions and propose a model that explains how a continuous distribution of developmental parameters can give rise to the discontinuous distribution of extra digits. We interpret our results in the context of patterning models of limb development and the potential of such generative systems for discontinuous evolutionary variation.

Materials and Methods

Polydactyly data were obtained with consent from the PolyTrak database. PolyTrack (www.polytrak.net) has been registering PPD specimens of *Felis catus*, the Maine Coon, since 2006. As of January 2011, 975 entries of individual cats have been included, listing 526 individuals (54 %) with PPD. The database does not provide genetic information for the polydactylous cats. Based on PolyTrak and on the pedigree database PawPeds (www.pawpeds.com) we were able to distinguish the *Hw* lineage (485 individuals) and the Canadian mutant lineage (41 individuals). Since in 2011 only a single British breeder was listed, who had imported her polydactylous breeder cat from Colorado USA (thus a *Hw* mutant), there was no potential presence of *Uk1* and *Uk2* mutants in the database at the time we used it. The analysis of the fore- to hind-limb differences used the PolyTrak data present in June 2011. For comparative purposes, we used the empirical data provided by Hamelin (2011) for a polydactylous cat lineage with a Canadian point mutation.

Sampling

We distinguish between individual limb patterns (ILPs) and combinatorial limb patterns (CLPs) in the analysis of the *Hw* sample (Tables 1, 2). All 485 cat specimens were used for the analysis of the ILPs and for the estimates of left–right asymmetries of CLPs. The notation for CLP patterns follows the sequence: left forelimb–right forelimb–left hindlimb–right hindlimb. The wild type pattern is 5-5-4-4. Extra digits are added to the wild type numbers, such as 6-6-4-4 in the case of a symmetrical addition of 1 digit on each forelimb. "P" next to a digit number refers to thumb elongation. The CLP 5P-5P-4-4 (18 toes, 0 additional ones) corresponds numerically to the wild type, because it does not involve any additional digits, but it does show a variation in form of an elongated first digit. An elongated thumb is always characterized by one additional interphalangeal joint. (For information on the classification of preaxial PPD forms, including elongated thumbs, we refer to Online Mendelian Inheritance in Man (OMIM: ncbi.nlm.nih.gov/omim/603596). A bifurcated digit is counted as a complete extra digit (Tables 1, 2). The terms "toe" and "digit" are used synonymously in the present paper.

Since the majority of observed CLPs are symmetrical, we focused on bilaterally symmetrical patterns. For these analyses we chose a stratified sampling (Table 1). We proceeded in three steps: First, the symmetrical CLPs were extracted. Second, from all symmetrical CLPs with identical toe numbers the CLPs with the highest frequency were used, i.e., for 20 toes the pattern 6-6-4-4 (142 individuals), for 22 toes the pattern 6-6-5-5 (109 individuals) and for 24 toes the pattern 7-7-5-5 (38 individuals) (Table 1, column D; Table 2, column E). Third, in order to exclude maternal effects, each kitten born by the same mother is represented

Table 1 Stratified random sampling of *Hw* mutants

A Combinatorial limb patterns (CLP) Total number	B Total HW mutant specimens (n = 485)	C Symmetrical CLPs in % of B (n = 485)	D Sample in % (n = 485)	E Sample absolute (n = 317)	F Sample weighted (n = 100)
0 PPD toes (5P-5P-4-4)		4.8 %	3.7 %	18	5
2 PPD toes (6-6-4-4)		37.9 %	29.3 %	142	59
4 PPD toes (6-6-5-5)		29.1 %	22.5 %	109	24
6 PPD toes (7-7-5-5)		10.1 %	7.8 %	38	10
8 PPD toes (7-7-6-6)		2.7 %	2.1 %	10	2
Total	100 %	85 % of 485	65.4 % of 485	317	100 %

A—types of combinatorial limb patterns (CLP). B—total sample size of *Hw* mutants. C—symmetrical CLPs. D—most frequent symmetrical CLPs. E—the most frequent symmetric CLPs when the total number of toes occurs in multiple CLPs. F—weighted frequency excluding effects due to the same mothers of individuals

Table 2 Total number of toes per *Hw* mutant specimen with multiple CLPs

A Phenotype	B Number of symmetrical toes	C Combinatorial limb patterns (CLP)	D Symmetrical CLPs	E CLPs with highest frequency	F E with maternal effects excluded
1	18	5P-5P-4-4	18	18	5
2	20	5P-5P-5-5	14		
3	20	5R-5R-5-5	5		
4	20	6-6-4-4	142	142	59
5	22	6-6-5-5	109	109	24
6	24	6-6-6-6	26		
7	22	7-7-4-4	13		
8	24	7-7-5-5	38	38	10
9	26	7-7-6-6	10	10	2
Totals			375	317	100

A—phenotype classes. B—symmetrical toe numbers. C—combinatorial limb patterns (CLP). D—symmetrical CLPs. E—the most frequent symmetrical CLPs. 317 individuals or 65.4 % of 485 cats are used in our sampling. F—when weighted (to exclude effects due to the same mothers of individuals) 100 individuals remain

as a weighted input, e.g., three PPD-kittens born by the same mother were counted as 0.3333 each (Table 1 column F, Table 2 column F).

Statistical Procedures

For the analysis of ILP structures we determined the order and form in which each PPD toe at fore- and hind-limbs appears. For calculating the theoretical maximum number of CLPs we used a combinatorial model (Table 3). From a combinatorial perspective a set of 5R (regular), 5P (polydactylous), 6, and 7 toes the possible number of forelimb patterns is nf = 4. Whereas from a set of 4 (regular), 5, and 6 toes the possible number of hindlimb patterns nh = 3. The combinatorial calculations show variations with repetition: $Vf = 4^2 = 16$ in forelimbs and $Vh = 3^2 = 9$ in hindlimbs. The maximum of mutant CLPs result in Nm = Vf * Vh = 144 as the theoretical limit. Excluding the regular pattern (wild type) (5-5-4-4) the maximal number reduces to 143.

For the analysis of the CLP distribution of the symmetrical cat specimens we listed the stratified sample in ascending order of the total toe number per cat specimen. For the analysis of fore- and hind-limb differences we built the differences between both forelimbs and both hindlimbs of the stratified sample, in order to detect how different PPD frequencies are represented in the fore- and hind-limbs. A goodness of fit test was used to detect left–right asymmetry.

Projection From Continuous to Discontinuous Distributions

In the manner of quantitative genetics (Wright 1934b; Falconer 1989) we set the observed frequency distributions of discrete PPD patterns as a starting point for reciprocal projections between discontinuous and continuous distributions. As a continuous and limiting distribution we assumed the standard normal. The projection was calculated via the Gaussian integral, starting from the left by a cumulative summarizing of the observed PPD pattern distribution. This permitted the construction of the probability density function of the standard normal distribution. Starting from the standard normal distribution the thresholds were determined.

Table 3 Maximum number of combinatorial PPD limb patterns

Possible individual limb patterns	Variation with repetition	Maximum number of CLPs
nf = 4	Vf = 16	Nm = 144
nh = 3	Vh = 9	

16 possible forelimb patterns times 9 possible hindlimb patterns = 144 maximal CLPs. Since one CLP is the wild type, 143 is the theoretical maximum number of mutant CLPs

Results

The polydactyly data from PolyTrak show several kinds of unexpected phenotypic biases, which we term the Hemingway Effect. Only a limited set of PPDs is realized out of the theoretically possible number of patterns. Furthermore, biases of PPD realizations can be observed concerning (1) the individual pattern in a single limb (2) the combined limb pattern in a complete animal, (3) the forelimb versus hindlimb distributions, and (4) the left–right distributions. As noted above, an individual limb pattern (ILP) is a specific configuration of a single polydactylous limb, for

example 6 toes on a forelimb. A combined limb pattern (CLP) is a specific configuration of toe numbers that includes all four limbs. For example: left forelimb 6, right forelimb 6, left hindlimb 5, right hindlimb 5, representing the CLP 6-6-5-5. The wild type CLP is 5-5-4-4. Below we describe the observed patterns of the *Hw* mutants in detail.

ILP Patterns

In the forelimbs, the ILPs of the *Hw* mutants appear in a strict order and form. Type 1 shows an enlarged thumb. Type 2 only appears when Type 1 is present already: a bifurcation is added on the posterior side of the thumb. Type 3 has the condition of Type 2 plus a full extra digit. In the hindlimbs only full digits appear, never bifurcations. All observed fore- and hind-limb ILPs are schematically represented in Fig. 2.

CLP Patterns

In our sample, out of the 143 possible CLPs (excluding the wild type pattern), only a total of 60 different CLPs are observed, corresponding to a percentage of 42 %. Many of the theoretically possible patterns are not realized even once. 42 out of the 60 CLPs are bilaterally symmetrical, 18 are asymmetrical. Figure 4 shows the ten most frequent CLPs, representing 82.5 % of the data set. The symmetrical CLPs dominate the picture. More than 90 % of the most frequent patterns are symmetrical. The two asymmetrical exceptions show the patterns 6-7-4-4 and 7-6-5-5.

Figure 5 shows how the PPD toe numbers are distributed in the 100 most frequent symmetrical *Hw* cats. The most frequent pattern (mode) is reached at 20 toes (2 PPD toes) with the CLP of 6-6-4-4. Above this number the frequencies diminish steadily. The mean of PPD toes is reached at 20.9 (rounded 3 PPD toes), the median is at 22 (4 PPD toes). Thus the distribution is characterized by the relation of mode (2.0) <mean (2.9) <median (4.0).

Fore- and Hind-limb Differences

In addition to the described CLPs, further regularities can be observed in the fore- and hind-limb patterns. Thus, for

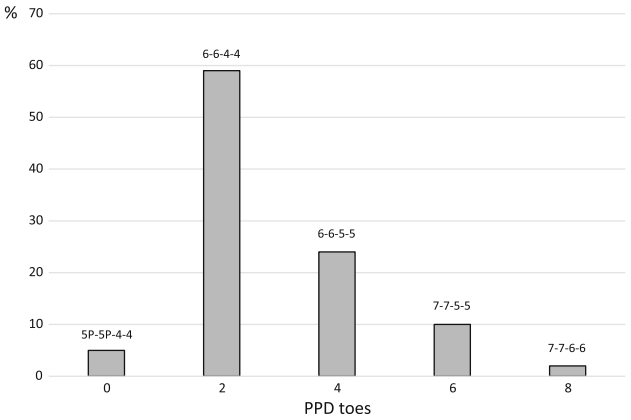

Fig. 5 Distribution of bilaterally symmetrical CLPs in 317 *Hw* mutant individuals

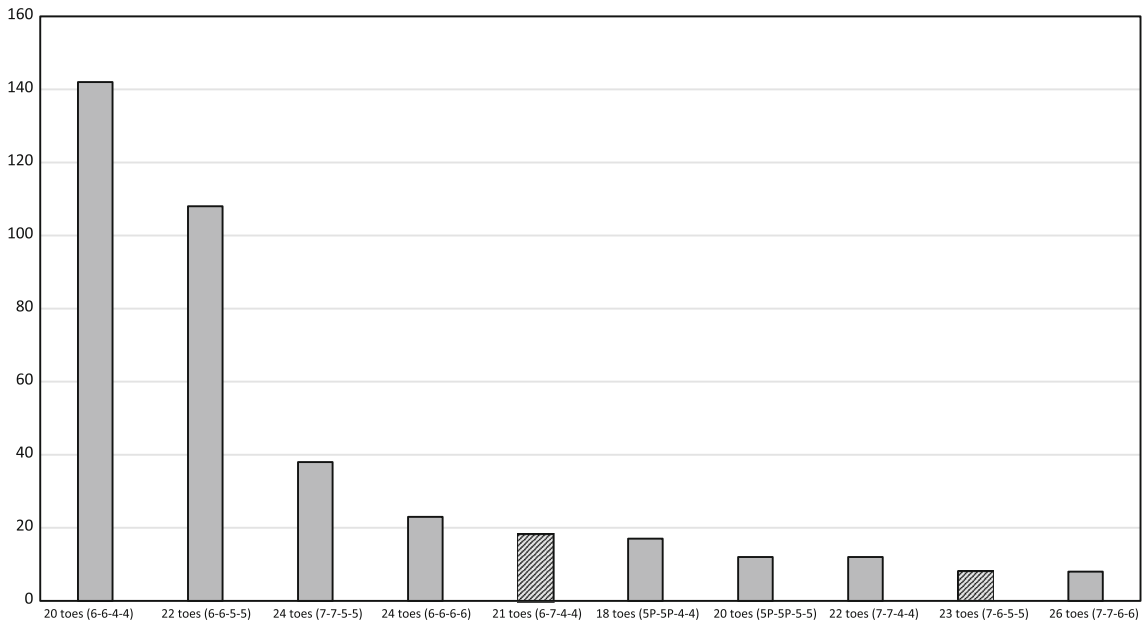

Fig. 4 The ten most frequently realized CLPs in *Hw* mutants from 143 theoretically possible combinations (485 *Hw* individuals). *Gray* bilaterally symmetrical CLPs. *Crosshatched* asymmetrical CLPs

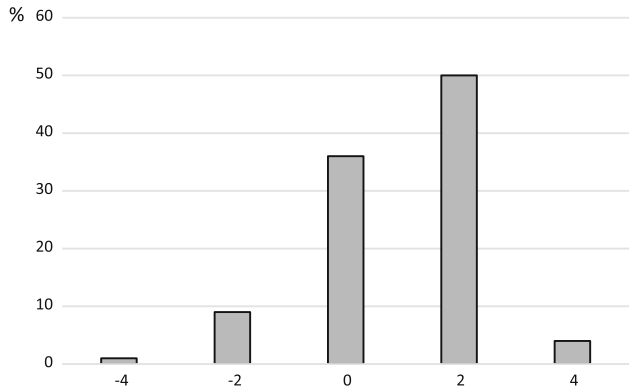

Fig. 6 Differences in PPD number between forelimbs and hindlimbs. *x* axis: −4 = Hindlimbs with two more PPD toes on each foot as compared with the forelimbs. −2 = Hindlimbs with one more PPD toe on each foot as compared with the forelimbs. 0 = same number of PPD toes in forelimbs and hindlimbs. +2 = one more PPD toe on each forelimb as compared with the hindlimbs. +4 = two more PPD toes in each forelimb as compared with the hindlimbs

example, there exist differences in the number of PPD toes realized in the forelimbs and hindlimbs respectively. Figure 6 shows the distribution of the differences in our sample of 375 symmetrical animals. There are cases in which the forelimbs contain a total of 4 more toes than are present in the hindlimbs. Alternatively, up to 4 more PPD toes can be present in the rear as compared to the front. A steady increase of the frequency of PPD toe distribution between fore- and hind-limbs can be seen in Fig. 6, beginning with two more PPD toes in each hindlimb (1 %), followed by one more PPD toe in each hindlimb (9 %), going towards the same number of PPD toes in both fore- and hind-limbs (37 %), and reaching the maximum frequency at a difference of one more PPD toe in each forelimb (50 %). Two more PPD toes in each forelimb again has a low frequency (3 %). This distribution is characterized by the relation of mean (0.9) <mode (2) = median (2).

Left–Right Asymmetry

Only a small number of individuals exhibit PPD in the forelimbs or the hindlimbs alone, or only on one side of the body. In 35 out of 485 individuals an asymmetry is present, with the PPD appearing more frequently in limbs on the left side of the body. Out of the 35 individuals, 6 cats exhibit PPD only in the left forelimb and 3 only in the right forelimb (Table 4). In the hindlimbs 17 individuals show PPD exclusively on the left side, as compared with 9 individuals with PPD only on the right side. Although the number of 35 cats is relatively small compared with the total number of 485 individuals, a goodness of fit test of equal left–right-distribution provides a statistical hint that a true left–right-asymmetry exists ($p = 0.061$).

Table 4 Left right asymmetry

Left forelimb	Right forelimb
6	0
Left hindlimb	Right hindlimb
17	0
Left forelimb	Right forelimb
0	3
Left hindlimb	Right hindlimb
0	9

35 out of 485 mutants exhibit either only *left* sided (23) or only *right* sided (12) PPDs. Left sided PPD is slightly more frequent

Canadian PPD Mutants

The analysis we described for the *Hw* mutant was performed in the same way for the "Canadian" mutant (Hamelin 2011). Similar regularities of digit variation appear, but there is a slight divergence in the distribution of the phenotypes. For a total of 32 cats the data of Hamelin show 26 symmetrical individuals (81.25 %). The numbers of toes of the symmetrical individuals are distributed in the following way: 1 × 20 (6-6-4-4), 4 × 22 (6-6-5-5), 18 × 24 (6-6-6-6), 3 × 26 (7-7-6-6), corresponding to a relation of mean (5.8 PPD toes) <median (6 PPD toes) = mode (6 PPD toes). The Canadian mutants mostly exhibit complete PPD toes and only rarely contain bifurcations.

Distribution Thresholds

Since the observed PPD patterns in the *Hw* mutants are discrete, but are all based on a single point mutation affecting a single gene product, the existence of developmental threshold parameters must be assumed. By applying a set of procedures that establish a projection between an assumed continuous distribution of an additive gene product and the phenotypic polydactyly patterns we demonstrate such threshold conditions.

Using the Gaussian integral, the phenotype patterns can be inscribed under a curve (Fig. 7b). Each area under the curve delimited by red lines represents a probability density for a developmental threshold, realized by the different PPD toe numbers. The threshold widths (0, 2, 4, 6 and 8 PPD-toes) are specified as units of standard deviations of the continuous variable (threshold units). For example, the appropriate value for the threshold between 0 and 2 polydactylous digits is -1.64 standard deviations; the value for the threshold between 2 and 4 extra digits is 0.36 standard deviations. Thus the threshold unit between the two thresholds is equivalent to the difference of these two, which represents 2.0 units of standard deviations.

Discussion

Polydactyly is a frequent occurrence in vertebrates, but very few systematic analyses of the phenomenon exist. Sewall Wright (1934a) analyzed Guinea pigs with one additional toe in the hindlimbs, but other specimens that exhibited multiple extra toes were not included. The Norwegian Lundehund is another example of variable toe numbers (Galis et al. 2001; Park et al. 2008). Although the majority of the Lundehund specimens show six toes on each limb, cases with eight toes have been reported. The genetic background for polydactyly was not known in any of these cases. Cats provide a unique opportunity to study the genetic and epigenetic conditions for polydactyly formation and the ensuing genotype-phenotype relation. We discuss the results of our study in their genetic and developmental contexts and suggest a developmental threshold model for polydactyly formation.

Biased Plasticity

The present analysis of a large data set of polydactylous Maine Coon cats with a single point mutation shows high degrees of phenotypic plasticity but significantly fewer realization patterns than are theoretically possible. Five kinds of biases characterize the polydactyly phenotype in *Hw* mutant cats: First, extra digits are added to the ground state in a given sequence and position. Second, the bilaterally symmetrical patterns are not equally distributed but form at different frequencies. Third, fore- and hind-limbs differ in the numbers of extra toes, favoring the appearance of extra digits in the forelimbs. Fourth, a left–right asymmetry slightly favors the occurrence of extra digits on the left side of the body. Fifth, the addition of a new digit is not a gradual process of adding more elements to a new digit rudiment (e.g., by progressing subdivision) but is a discontinuous all-or-nothing phenomenon.

While our results confirm a slight forelimb emphasis for polydactyly in *Hw* mutants, earlier works have suggested a more significant forelimb preference. Lettice et al. (2008) describe exclusive forelimb effects amounting to as much as 80 % of the cases, whereas in our study, out of 375 symmetrically polydactylous *Hw* mutants, only 46.9 % of the polydactyly phenotypes are restricted to the forelimbs. Actually, in the majority of our cases both fore- and hindlimbs are affected, even though the sums of extra toes are higher for the forelimbs. Since forelimb bud development in mammals usually slightly precedes hindlimb development, this effect could be based on the timing of onset or duration of the mutant gene expression. Alternatively, because the usual pattern is 5 digits in the forelimb and 4 digits in the hindlimb, the regulatory elements involved could activate at different magnitudes.

Left–right asymmetry is also infrequent, symmetrical polydactyly effects clearly prevail. Nearly all of the possible bilaterally symmetrical CLP patterns have been realized. Merely two out of 11 possible patterns are absent: 5P-5P-6-6 and 5R-5R-6-6 never occur in our sample, although several other CLPs have 6 toes in one or both hindlimbs. By contrast, asymmetrical patterns, such as 5P-6-5-5 (7 cases) or 5P-6-4-4 (6 cases) are rare. Many asymmetrical CLPs, such as 5P-6-5P-6 or other forms of asymmetry in both fore- and hind-limbs, don't occur even once, the absence of the former further supporting the left side preference. Interestingly, a number of ILPs that are realized in one limb never occur bilaterally. Such indications of directional asymmetry in the limb coincide with findings of Pitx1-mediated asymmetry of pelvic reduction (Shapiro et al. 2006), a gene that is also important in hindlimb development.

The frequencies of the polydactyly patterns determined in the present study are stable. In general the following rule applies: the more toes, the lower the frequency. In the *Hw* mutants, the elongated thumb is a precondition for true extra digits to appear, i.e., extra digits never develop without a long thumb. Only very few individuals develop an elongated thumb without a separate, full extra digit. The optimum pattern realized in the *Hw* mutants is 6-6-4-4 (Fig. 5). Beyond this CLP with a total of 20 toes the frequencies diminish steadily. It seems to become more and more difficult to form a limb bud large enough for developing 7 or even 8 toes.

The Canadian mutant lineage shows a similar CLP distribution for the bilaterally symmetrical CLPs as the *Hw* mutant, both following mean $<$median $\leq$mode. However, in the Canadian lineage the 6-6-6-6 pattern is prevalent, a fact that is well known and preferred by breeders. Hence, the effect could have been stabilized by artificial selection. But the phenotype with 24 toes dominates the picture here whereas 20 toes is the most frequent CLP in the *Hw* mutants. Furthermore, in the Canadian mutant only one CLP exists at a time for a specific symmetrical toe number. This result provides even higher significance to our finding of discretely distributed frequencies of the bilaterally symmetrical CLPs in the *Hw* mutants. Thus, polyphenic variability that results in response to a single point mutation outside the coding region is significantly higher than previously suggested (Chakravarti and Kapoor 2012), but the biased forms of variation require additional explanation beyond the genetic mutation itself.

Genetic and Developmental Conditions for Polydactyly Formation

Current mutational and developmental models of polydactyly explanation were discussed in the introduction, but

the relationship between them is little explored. Lettice et al. (2003, 2008) relate three point mutations in the highly-conserved, non-coding element ZRS with preaxial polydactyly (PPD) in humans, mice, and cats. Using transgenic mice, these authors describe the ectopic expression of SHH in the anterior regions of limb buds, opposite the zone of polarizing activity (ZPA) in which SHH is naturally expressed. Nissim et al. (2007) show that the natural production of SHH in the anterior region is usually suppressed by TBX2 signaling from the ectoderm. Spatial SHH expression is controlled by Ets transcription factors, acting directly on the ZRS, defining the boundaries of the ZPA (Lettice et al. 2012). It has been suggested that the Etv4/5 transcription factor implicated in SHH restriction is disrupted in the polydactylous mutants, allowing its ectopic anterior expression (Zhang et al. 2010). Whereas all these factors seem to be causally involved in polydactyly formation, the question remains, why does this result in discrete phenotypic effects?

Several authors (Li 2011; Gu 2004) regard fluctuating changes in the expression of a single gene as a stochastic process, essentially representing a Gaussian distribution of a continuous variable. Fluctuating Shh transcription may represent such a stochastic process. Raj and van Oudenaarden (2008) also emphasize the rates of transcription. When the transcription rate is high, variability in protein level is low, but when the transcription rate is lowered and the translation rate is raised, gene expression is far noisier. SHH dosage and timing of expression are potential additive variables. A relation between SHH dosage and digit number has been described (Yang et al. 1997; Lettice et al. 2008). Lettice et al. (2012) measured the relative ectopic SHH expression boundaries in transgenic mice. Unfortunately transgenic mice don′t express the polyphenism in the same way the cats do.

Gene products other than SHH, namely GLI3, GLI3R, FGFs, WNT, TBX2, BMP, HAND2 and others involved in limb patterning also need to be taken into account (Litingtung et al. 2002; Niswander 2003; McGlinn and Tabin 2006; Nissim et al. 2007; Bénazet and Zeller 2009). In particular, Lai et al. (2004) argue that the *Shh* network has evolved and exploited positive *Gli3* feedback, creating a switch in *Gli3* expression, as well as negative Patched (*Ptc*) feedback maintaining the robust properties of the switch. In the wild type *Gli* and *Ptc*, both are not expressed anteriorly in the limb (Platt et al. 1997). This network is maintained in the polydactylous cat since ectopic SHH expression induces anterior *Gli* and *Ptc* expression (Dillon et al. 2003). SHH flips cells between alternate functional states at key threshold concentrations' generating a 'bistable genetic switch'. 'Stochastic effects' can cause the spontaneous switching between two distinct states of different *Gli* transcription factor concentrations (Lai et al. 2004). A high concentration of GLI corresponds with enhanced mitotic activity and cell proliferation. Also the influence of *Hox* genes on digit number is established, such as *Hoxd8*, *Hoxd4*, *Hoxa10*, and *Hoxa7* (Tabin 1992) as well as *Hoxa13* and *Hoxd11* to *Hoxd13* (Sheth et al. 2012).

SHH or other gene products do not build limbs, and the mutation of a *cis*-regulatory element is not by itself explanatory of a discrete phenotypic pattern. Hence cell based processes that account for three-dimensional cell arrangements and patterning, which can be modulated by the regulatory gene products, must be considered. The activator-inhibitor model for vertebrate limb bud patterning (Newman and Bhat, 2007) describes a mechanism that mobilizes the autoregulatory skeletal condensation formation circuit, based on the interaction between cells expressing TGF-β, which is positively autoregulatory and enhances the production of fibronectin as well as its own inhibitor. The enrichment of glycoproteins, such as fibronectin in the extracellular matrix, favors the forming of cell condensation that will form cartilage later. The simultaneous expression of an inhibitor of TGF-β restricts the condensations; therefore they are limited to the vicinity of the TGF-β maxima. Because the lateral inhibition system (possibly involving the *delta/notch* pathway) leads to equally spaced pre-chondrogenic digit elements, the number of elements that can be realized depends on the size of the active zone in the limb bud. Experimental studies support the relation between the size and geometry of a limb bud and the number of digits it can form (Saunders and Gasseling 1968; Tickle et al. 1975; Tickle 1981, 2006; Smith and Wolpert 1981; Honig and Summerbell 1985; Wilson and Hinchliffe 1987; Zhu et al. 2010).

Autoregulatory processes, such as the one described above, necessarily include non-linear properties. Developmental threshold effects are a precondition for translating continuous variation in one (or several) parameters into discrete units of phenotypic structure (Newman and Müller 2005; Müller 2010). Since supernumerary digit formation is discrete, exhibiting an all-or-nothing principle, developmental threshold effects are likely to be implicated. Threshold mechanisms can stabilize random fluctuation of gene expression, which eventually becomes homogeneous in all cells within the same threshold width (Nijhout 2004). Here, the same mechanism controls the threshold and the degree of stochasticity of the final phenotypic pattern. Computer simulations of an autoregulatory, reaction–diffusion based model of limb skeletal formation also indicate the existence of threshold effects in the formation of discrete limb patterns. Zhu et al. (2010) describe the proximo-distal length and the anteroposterior widths of the limb as the dominant factor accounting for the number of toes that can be formed in a growing limb bud. Based on these considerations we

suggest a tentative model for discrete polydactyly formation in the tetrapod limb.

The Hemingway Model—A Dual Mapping Dynamics of Polydactyly Formation

We suggest a two step mapping process to account for the discontinuous realization of extra digits, based on the continuous (gradient like) distribution of a key variable in the limb patterning system (Fig. 7). In the present case, SHH—affected by the Hw mutation—is taken to represent the key variable, but the model is equally suited to account for other pattern influencing variables in the limb system. Our formulation of the model is based on the "Central Limit Theorem" (CLT; Adams 2009; Fischer 2011) which states that the sum of the effects of a sufficiently large number of independent random variables (each with a well-defined expected value and well-defined variance) will be approximately normally distributed.

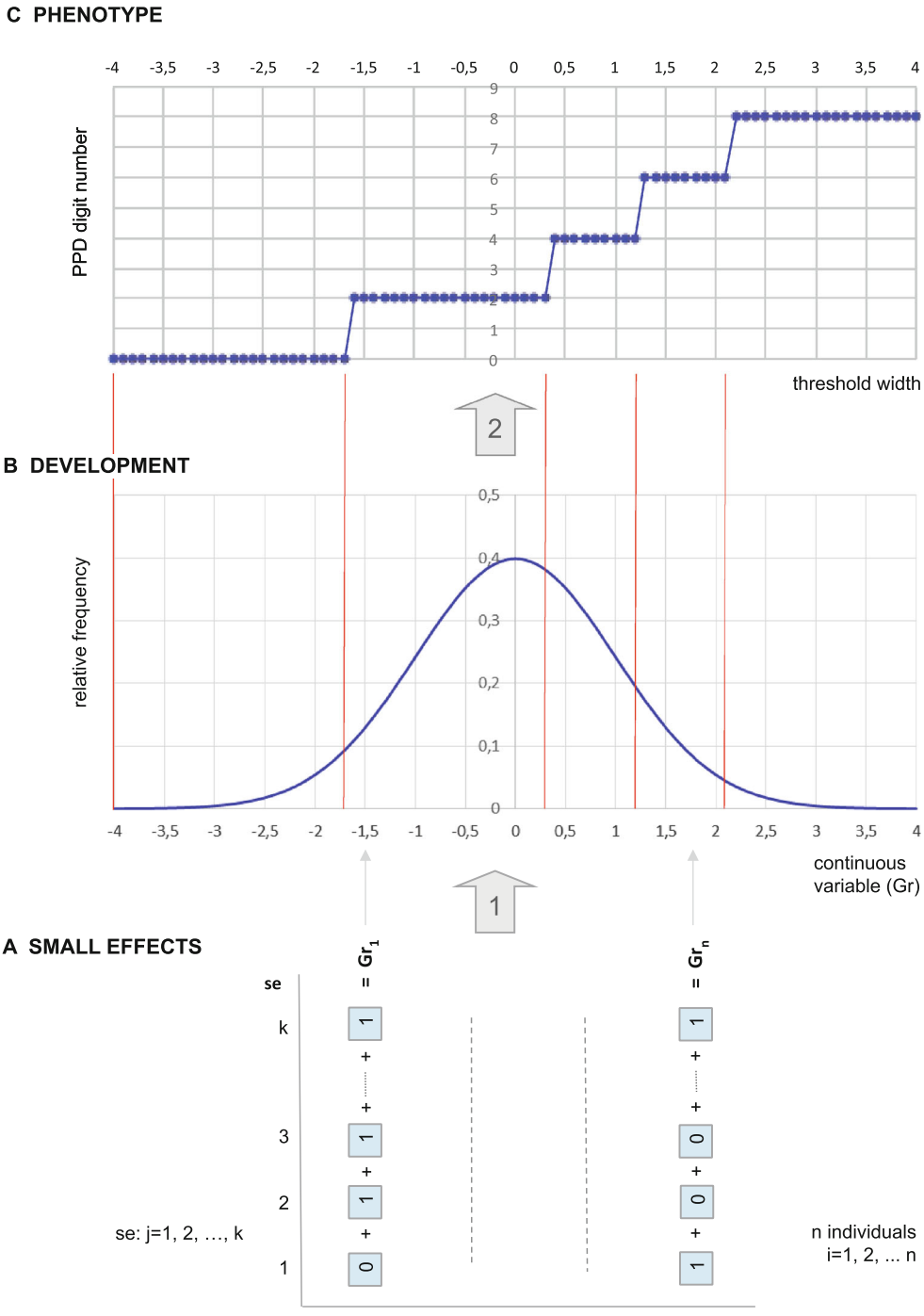

Fig. 7 The Hemingway Model. The *Hw* mutation affects a developmental factor (e.g., SHH dose expressed in the limb buds) causing a stochastic distribution of bistable states of the affected cells. **a** The summation of small effects (se: j = 1, 2,…, k) per individual cell (n individuals, i = 1, 2,…, n) generates a continuous variable (the combined cell states), termed developmental gradient (Gr). **b** The continuous variable (Gr) is modeled by an approximated standard normal distribution (probability density function). **c** Threshold widths of polydactyly phenotypes (present study), expressed in units of standard deviation. The *diagram* shows two steps of development-to-phenotype mapping. Step 1 (*arrow 1*) mapping of small summational effects (in our case the result of bistable switches, see text) onto a continuous developmental gradient. Step 2 (*arrow 2*) mapping of the standard normal distribution of the continuous variable onto the discrete phenotypic realisations of polydactyly. **b** and **c** use the same abscissa in units of standard deviation. The threshold limits are displayed by *vertical red lines* that relate the developmental thresholds to discrete phenotype classes

A set of preconditions underlies the formulation of the model: (1) The limb system consists of more than 30 additively acting independent factors (e.g., cell types, cell number, regulatory molecules, gene products, etc.). (2) A pattern-affecting variable is expressed in a posterior-to-anterior graded continuum in early limb buds, functioning as a proliferation enhancer and polarizing agent (e.g., SHH). (3) Ectopic expression of the key variable in the anterior region of limb buds (e.g., by a point mutation in a distant *cis*-regulator of *Shh*) flips individual cells between alternate functional states at threshold concentrations, generating a 'bistable genetic switch' [e.g., ectopic SHH levels causing a spontaneous switching between two states of GLI concentration (Lai et al. 2004)].

The specific model we propose for the polyphenic and biased polydactyly formation consists of the combination of two mapping events:

Step one consists of the mutation acting in a developmental system that consists of more than 30 additive factors. The ectopic expression of the point mutation affects an area of at least 400 cells (Tickle 1981), and each one of these cells represents a random bistable switch (Lai et al. 2004). The outcome of each and every affected cell (either "off" or "on") additively combines to form a continuous effect variable. By the cluster of these responsive cells and their bistable productions alone, well over 400 independent variables are present in the system, thus justifying the application of the CLT. The consequence of the CLT is a graded mapping of the switching effects in the sense of a continuous variable exhibiting an approximately normal distribution across the limb system (Fig. 7a, b).

Step two is the mapping of the graded distribution to discrete character states (digit number) which we propose to happen via the transgression of threshold levels of active cells, causing the autonomous pattern generating limb system to produce different numbers of discrete extra digits, depending on the number of cells available to form separate precartilage condensation (Fig. 7b, c). The differences in the threshold widths are responsible for the biased digit numbers.

The Hemingway Model is a threshold model (sensu Wright 1934b; Falconer 1989) based on a mechanism for summing up a large number of small additive effects in the limb system, corresponding with the requirements of the Central Limit Theorem. The number of extra digits resulting from these two mapping events depends on the threshold widths within a given limb system which can be determined by the frequency at which extra digits are found. It highlights the fact that the relation between genotype and phenotype is not deterministic. The limb system of the *Hw* mutant is decanalized but biased. The model enriches existing simulations, such as by Zhu et al. (2010), by adding two mapping components to the genotype-phenotype transformation in digit formation. A particular property of our model is its potential to compare different distributions of threshold widths across a variety of polydactylous conditions in vertebrates, regardless of the specific mutation initiating the change in the affected limbs. The model is independent from the details of empirical molecular findings, but it is in agreement with autoregulatory and Turing based models of limb development (Zhu et al. 2010; Sheth et al. 2012).

Evolutionary Consequences

The existence of discontinuous and biased phenotypic reactions to mutational change has major implications for evolutionary theory. Unlike in cases of gradual and continuous variation on which the standard theory rests, here a set of discrete morphological responses results from a single point mutation. The polydactyly example shows that if the mutationally, selectionally, or environmentally induced disturbance of the limb developmental system is too large to be buffered by its plastic response capacities, the system is decanalized and its self-organizing properties are mobilized, resulting in new digits. Whereas threshold based models for the transition from continuous to discrete character states represent a standard explanation in quantitative genetics since the 1930s (Wright 1934a, b), the biological origins of the continuous variable (i.e., the liability) remain unaccounted for (Falconer 1989). The present model adds a preceding step to the standard account, explaining the generation of continuous variables in development that have inherent threshold characteristics, which are based on empirical developmental parameters—such as SHH in the present case. However, the model is equally suited for other kinds of polydactyly formation as well as for the generation of wild type patterns.

Furthermore, the cellular core processes in the limb bud and the responsiveness of the pattern forming processes define the setting for the generation of new phenotypes, or morphological novelties. Such new elements do not have a homologous precursor. The evolution of the vertebrate limb is a paradigmatic case for the appearance of novel elements that lack a homologous precursor structure (Wagner and Chiu 2001). The scenario for polydactyly formation described above corresponds with the predictions from epigenetic innovation theory (Newman and Müller 2005; Müller 2010). Any change in the initiating conditions that affect one parameter of the limb system, such as the timing, size, or geometric shape of the embryonic limb bud, will automatically give rise to different numbers and spatial arrangements of skeletal condensations. Thus developmental plasticity can be seen as a capacitator for evolutionary change (Kirschner and Gerhart 2010), and the specific quality of the phenotypic

result will be defined by the biases that exist in a given developmental system. In our case, the modification of a continuous variable leads to discontinuous and discrete morphological states that cannot be predicted from genetic variation alone. By contrast, knowledge of the dynamics of the limb forming system and its underlying threshold qualities allows to make such predictions, which is why the standard variational theory needs to be supplemented by a theory of innovation, as well as other concepts that are able to describe the origin of non-adaptive variation and novelty (Pigliucci and Müller 2010; Peterson and Müller 2013), in order to account for the origin of discrete phenotypic states.

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