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Cryptic genetic variation uncovers evolution of environmentally sensitive parameters in *Caenorhabditis* vulval development

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SUMMARY Understanding the robustness of developmental systems requires insights into the sensitivity of underlying molecular and cellular parameters to perturbations, and how such sensitivity evolves. We address these issues using vulval cell fate determination—a reproducible and robust patterning process regulated by a cross-talk of EGF-Ras-MAPK and Delta-Notch pathways. Although the final vulval cell fate pattern is identical in all *Caenorhabditis* species, the patterning process underlies extensive cryptic genetic variation between and within species. Here, we tested whether this cryptic genetic variation translates into variation in developmental sensitivity to environmental perturbations. We disrupted vulval patterning using thermal perturbations to quantify and compare environmental sensitivity of different system parameters between distinct genotypes of *C. elegans* and *C. briggsae*. Thermal perturbations globally debuffered vulval development, triggering diverse pattering variants, whose frequency and spectra were strongly species- and genotype-dependent. This condition-dependent variation indicates that environmental sensitivity of different system properties, such as vulval competence or vulval induction, is subject to evolutionary change. High temperature induced a genotype-specific decrease of secondary fate induction and corresponding Notch pathway activity in the *C. elegans* N2 strain; in contrast, hypoinduction of the primary cell fate was never observed. Vulval precursor cells therefore differ in temperature sensitivity and such cell-specific sensitivity shows evolutionary variation. We further compared spectra of temperature-induced vulval variants to the ones induced by mutation accumulation in the same genotypes. In response to either perturbation, we observed similar genotype-dependence of variant production, allowing identification of distinct system features most sensitive to both mutation and environment. Taken together, we show how sensitivity of system parameters regulating *Caenorhabditis* vulval development depends on subtle interactions between perturbations and genetic background. Our results imply that cryptic genetic variation may reflect evolutionary variation in developmental robustness, therefore potentially contributing to the maintenance of phenotypic precision when facing perturbations.

INTRODUCTION

Organismal development is inherently sensitive to variation in the external environment, but such environmental sensitivity may or may not translate into variation of corresponding phenotypic outcomes. Relative insensitivity of a phenotype to environmental variation is termed environmental robustness and contrasts with phenotypic plasticity where developmental sensitivity to the environment results in different phenotypic outcomes (Flatt 2005; Wagner 2005; Masel and Siegal 2009). Environmental robustness of any developmental system has limits and may break down in response to specific environmental conditions. Such developmental debuffering may lead to the production of "novel," usually deleterious phenotypic variants, which are indicative of environmentally sensitive aspects of the underlying developmental processes. Developmental errors induced by the environment may therefore be informative by revealing the type and spectrum of environmentally sensitive aspects of a given developmental system (Braendle and Félix 2008, 2009; Braendle et al. 2010). In classical developmental biology, the application of heat shock or other environmental perturbations to induce phenocopies or to determine the timing of a developmental process of interest provides an example of how environmental debuffering has been used to characterize developmental mechanisms and their environmental sensitivity (Goldschmidt 1935; Peterson 1990; Welte et al. 1995). Upon developmental debuffering by a given environmental perturbation, the frequency, type and spectrum of phenotypic variants induced may be highly variable, often showing genotype-dependence (Braendle and Félix 2008, 2009; Braendle et al. 2010). Experimental demonstration that environmental sensitivity of development shows genetic variation is provided by the classical *Drosophila* experiments performed by Waddington (1953, 1956). These experiments revealed the presence of cryptic genetic variation, that is, standing genetic variation that is usually phenotypically silent

but may become expressed in response to environmental and genetic perturbations (Gibson and Dworkin 2004; Paaby and Rockman 2014). The phenotypic manifestation of cryptic genetic variation is therefore condition-dependent variation arising through genotype-by-environment interactions or genetic interactions (epistasis). Empirical evidence reporting the presence of cryptic genetic variation is thus old, and recent research shows that such "hidden" genetic variation is very common (Gibson and Dworkin 2004; Wagner 2005; Chandler et al. 2013; Chari and Dworkin 2013; Paaby and Rockman 2014). This research emphasizes the need for a better understanding of condition-dependent phenotypic variation, both from a mechanistic and an evolutionary perspective. While there are many examples of biological systems in diverse organisms harboring cryptic genetic variation (Gibson and Dworkin 2004; Wagner 2005; Chandler et al. 2013; Chari and Dworkin 2013; Paaby and Rockman 2014), the molecular genetic identity of such variation has only rarely been elucidated (Gibson and Hogness 1996; Dworkin et al. 2003; Duveau and Félix 2012), and despite important claims (Rutherford and Lindquist 1998; Queitsch et al. 2002), the evolutionary significance of cryptic genetic variation in adaptive phenotypic evolution is still unclear (Meiklejohn and Hartl 2002; Mitchell-Olds and Knight 2002; Braendle and Flatt 2006).

The phenomenon of cryptic genetic variation is tightly linked to the notion of developmental robustness (Gibson and Dworkin 2004; Wagner 2005; Félix and Wagner 2006; Masel 2006; Masel and Trotter 2010). Robustness that causes phenotypic insensitivity to genetic perturbations (e.g., the accumulation of novel mutations) is the supposed key property generating cryptic genetic variation. Thus, developmental robustness may lead to evolutionary stability at the phenotypic level in the presence of evolutionary change in the underlying genetic architecture (Gibson and Dworkin 2004; Wagner 2005; Félix and Wagner 2006; Paaby and Rockman 2014). Although difficult to evaluate, both theoretical and empirical evidence suggest that environmental robustness causes genetic robustness, and vice versa (Meiklejohn and Hartl 2002). Consistent with this scenario, different sources of perturbations may disrupt the same features of a given developmental process, that is, genetic and environmental perturbations may act interchangeably in the production of specific developmental defects (cf. phenocopies and genocopies) (Goldschmidt 1935; Welte et al. 1995).

Debuffering of a developmental system leading to an usually invariant phenotypic outcome allows exploration of possible phenotypic defects and underlying developmental deregulation, thus allowing identification of the system's most sensitive parameters to the applied perturbation. However, very little research has systematically quantified the spectrum and frequency of inducible developmental variants or errors, which would permit such inferences about developmental system sensitivity. Moreover, whether type and spectrum of such condition-dependent developmental variants show evolutionary variation (i.e., cryptic genetic variation) has rarely been quantitatively examined (Rutherford and Lindquist 1998; Braendle and Félix 2008; Braendle et al. 2010). Here, we therefore aimed to explore the environmental sensitivity of a robust developmental system, which generates an invariant phenotype across different genotypes and species. We used the well-characterized process of vulval cell fate patterning in *Caenorhabditis* nematodes to characterize which specific parameters of this process are most sensitive to extreme environmental perturbations, and to quantify how this developmental sensitivity to the environment varies among different genotypes.

The *C. elegans* hermaphrodite vulva differentiates from a subset of six ventral hypodermal cells, called Pn.p cells, through a molecularly well-understood signaling network regulating the induction of specific vulval cell (Sternberg 2005) (Fig. 1). P3.p to P8.p acquire competence to adopt vulval cell fates through expression of the HOX gene *lin-39*, regulated by the canonical Wnt pathway (Fig. 1A). The key steps of vulval induction and fate differentiation are regulated by an interplay of EGF-Ras-MAPK and Delta-Notch pathways (Fig. 1B) (Sternberg 2005; Félix and Barkoulas 2012). During the late L2/early L3 stage, the gonadal anchor cell (AC) releases the EGF-like ligand LIN-3, which induces the primary (1°) vulval cell fate in the closest cell, P6.p, through activation of the EGF-Ras-MAPK pathway (Hill and Sternberg 1992). In turn, activation of this pathway in P6.p triggers a lateral intercellular signaling event, mediated by the Delta-Notch pathway, promoting the adoption of a secondary (2°) cell fate by the neighboring cells, P5.p and P7.p. Notch activity in these cells further inhibits the 1° cell fate by activating negative regulators of the EGF-Ras-MAPK pathway (Greenwald et al. 1983; Sternberg and Horvitz 1986; Sternberg 1988; Berset et al. 2001; Yoo et al. 2004) (Fig. 1B). Moreover, a switch from the canonical Ras-Raf pathway to a Ras-RGL-1-RAL-1 signaling pathway promotes the 2° cell fate in P5.p and P7.p (Zand et al. 2011), and the Wnt pathway, primarily involved in vulval competence, may contribute to vulval induction (Gleason et al. 2002). The remaining vulval precursor cells (VPCs) adopt non-vulval cell fates (3° and 4°) as they do not receive sufficient doses of either signal: in *C. elegans*, P4.p and P8.p adopt the 3° cell fate while P3.p shows stochastic variation, either adopting a 3° or 4° cell fate (Sulston and Horvitz 1977; Pénigault and Félix 2011a, 2011b). Cell ablation experiments indicate, however, that P3.p, P4.p and P8.p are competent to adopt 1° or 2° vulval cell fates and capable of replacing missing cells of the P5.p-P7.p group (Katz et al. 1995; Sternberg 2005; Braendle and Félix 2008). The different cell fate patterns (1° to 4°) correspond to distinct, invariant cell division patterns of the Pn.p cells, which occur during the mid to late L3 stage (Fig. 1C). The canonical cell fate sequence of P3.p to P8.p is therefore 3°/4°-3°-2°-1°-2°-3–a pattern that is largely conserved within the *Caenorhabditis* genus comprising 26 described species

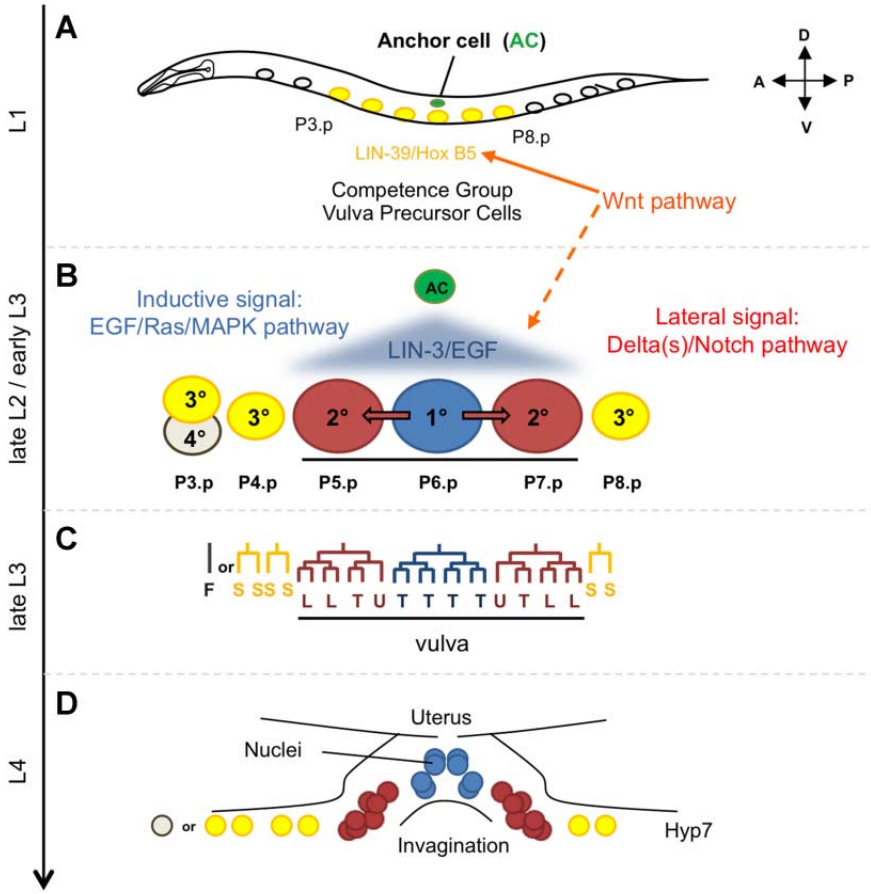

Fig. 1. *Caenorhabditis* vulval cell fate patterning. The *Caenorhabditis* vulva develops from a set of six ventral hypodermal cells, P3.p to P8.p. (A) L1 stage: P3-8.p cells express the Hox gene *lin-39* and acquire competence to adopt vulval cell fates. (B) Late L2 /early L3 stage: the anchor cell (AC) releases the morphogen-like LIN-3/EGF inductive signal. P6.p receives the highest level of LIN-3/EGF inducing a 1° cell fate (blue) via EGFR-Ras-MAPK activation, which in turn activates lateral signaling through the expression of the Delta ligands targeting the Delta-Notch pathway in its neighbors, P5.p and P7.p. This lateral signaling induces the 2° vulval cell fate (red) via Delta-Notch activity and further represses the 1° cell fate in these cells. The competent cells, P4.p and P8.p, adopt a non-vulval 3° fate (yellow), while the fate of P3.p varies among individuals, either adopting a 3° fate or a 4° fate (gray, also referred to as F(used) fate). Therefore, of a total of six potential vulval precursor cells, only P5.p, P6.p and P7.p adopt actual vulval cell fates in a 2°-1°-2° sequence, which is conserved among *Caenorhabditis* species. (C) Mid to late L3 stage: Vulval cell divisions. The fate assignments correspond to stereotypical cell division patterns that are invariant (with exception of P3.p). The three cells adopting the 2°-1°-2° vulval fates generate a total of 22 vulval cells by the end of the L3 stage. T: transverse (left-right) division, L: longitudinal (antero-posterior) division, U: undivided, SS: fusion to the epidermal syncytium (hyp7) after a single division (3° fate); F: fusion to the syncytium in the L2/L3 stage with no prior division (4° fate); 3° and 4° fates are non-vulval fates. (D) L4 stage: vulval morphogenesis.

(Félix 2007; Kiontke et al. 2007; Kiontke et al. 2011; Pénigault and Félix 2011a). P3.p, the only cell with variable vulval cell fate in *C. elegans*, may show different ratios of 3°/4° cell fates depending on species and isolates, and has lost vulval competence completely in some species; in addition, the competence of P4.p and P8.p to adopt vulval cell fates shows variation between species (Félix 2007; Pénigault and Félix 2011a, 2012).

The *Caenorhabditis* vulval signaling network possesses a wide range of properties that contribute to a precise patterning output, including partial redundancy and crosstalk among signaling pathways, manifold regulatory inputs and feedback loops within the EGF-Ras-MAPK (Sternberg 2005; Félix and Wagner 2006; Braendle and Félix 2008; Braendle et al. 2008; Braendle and Félix 2009; Félix 2012a; Félix and Barkoulas 2012). Key among these properties is the regulatory cross-talk between EGF-Ras-MAPK and Delta-Notch pathways, which ensures a reproducible establishment of the 2°-1°-2° vulval cell fate pattern of P5.p to P7.p despite extensive variation in parameter space (Hoyos et al. 2011; Félix and Barkoulas 2012; Barkoulas et al. 2013).

Caenorhabditis vulval cell fate patterning—a relatively simple cell fate determination process involving a molecularly very well-characterized signaling network—has emerged as an

important model system for quantitative developmental studies, system robustness and evolution as well as cryptic genetic variation (Félix and Barkoulas 2012). Comparative developmental studies of intra- and interspecific variation in *Caenorhabditis* nematodes have revealed extensive cryptic variation in genetic and developmental parameters underlying the evolutionarily conserved vulval pattern (Félix and Barkoulas 2012). Cryptic variation has been revealed through application of genetic (e.g., mutation accumulation, introgression of vulval mutations into different wild isolates, insertion of transgenes into different species, accumulation of spontaneous random mutation) and environmental perturbations (Delattre and Félix 2001; Félix 2007; Braendle and Félix 2008; Milloz et al. 2008; Braendle et al. 2010; Hoyos et al. 2011; Pénigault and Félix 2011a; Duveau and Félix 2012; Barkoulas et al. 2013). These results indicate that diverse system properties (e.g., cell competence, pathway activities, and their interactions) may evolve without leading to changes in the final vulval cell fate pattern.

Quantitative analysis of vulval development in different environments (intermittent starvation, passage through the dauer stage, different temperatures, liquid culture) indicates that vulval pattern establishment is robust despite environmental sensitivity of underlying signaling cascades (Braendle and Félix 2008). Of 6000 animals assessed in these different environments only 0.25% showed apparent vulval defects and an additional 2.10% showed non-canonical vulval patterning variants with an intact 2°-1°-2° sequence. Moreover, certain environmental conditions induced specific variants whose frequencies were further strongly genotype-dependent. For example, starvation during the L2 stage consistently induced vulval centering shifts on P5.p in the *C. elegans* N2 strain but very rarely in other strains of *C. elegans* or *C. briggsae* (Braendle and Félix 2008). This and other examples suggest that certain system properties (e.g., a specific pathway or a specific Pn.p cell) are more environmentally sensitive than others, and that this sensitivity is further subject to evolutionary change. However, these conclusions are based on limited data because previously examined environments induced variant and defective patterns only at very low frequencies, rendering quantitative analysis of environmental sensitivity and its evolution difficult (Braendle and Félix 2008).

In this study, we took advantage of the *Caenorhabditis* vulval developmental system to characterize its response to extreme environmental perturbations, low and high temperature extremes, and how such responses evolve. In contrast to previous studies (Braendle and Félix 2008; Braendle et al. 2010), we aimed to maximally disrupt the precision of the patterning process to be able to better understand (a) which underlying developmental and cellular aspects are most sensitive, (b) whether such specific system sensitivity evolves within and between species, and (c) whether genotype-specific patterns of such environmentally induced phenotypic variation correlate with mutationally induced phenotypic variation.

MATERIAL AND METHODS

Strains

We examined the same *C. elegans* and *C. briggsae* strains (wild isolates) as used in Braendle et al. (2010): the reference lab strain, *C. elegans* N2 (Bristol, UK) ("N2 ancestral"), *C. elegans* PB306 (CT, USA), *C. briggsae* HK104 (Okayama, Japan), *C. briggsae* PB800 (OH, USA). These strains had been used to derive mutation accumulation lines over 250 generations (Baer et al. 2005) and we used the same stocks from Charlie Baer's laboratory (University of Florida, Gainesville, FL, USA) that were originally obtained from the *Caenorhabditis* Genetics Center.

Temperature assays

To quantify and characterize temperature effects on the production of vulval pattern variants and defects, we exposed the four strains to three temperatures, 6°C, 20°C (control), and 30°C, during the time window of vulval cell fate patterning (mid L2 to mid L3). We arbitrarily chose two sublethal temperature extremes at which development and reproduction of *C. elegans* and *C. briggsae* strains are completely inhibited or strongly compromised. At 6°C, all strains arrest development whereas at 30°C development may progress although at a slower rate than at 25°C. Note that *C. elegans* ceases reproduction at temperature higher than 27°C, while certain (e.g., tropical) *C. briggsae* isolates maintain reproduction up to approximately 30°C (N. Poullet & C. Braendle, unpublished data) (Harvey and Viney 2007; Anderson et al. 2011; Prasad et al. 2011). Strains were derived from frozen stocks and maintained on NGM agar plates (1.7% agar) seeded with *E. coli* OP50 (Wood 1988) at 20°C. For each experimental repeat, mid-L2 individuals from each of the four strains were randomly allocated to the three temperature treatments. In an initial assay, we determined the time required to reach the mid-L2 stage as inferred from gonad characteristics (germ cell numbers, somatic gonad cells, size) using Nomarski microscopy. The four strains had very similar developmental times, reaching this stage at approximately 33–36 h after egg-laying. In the cold treatment, animals were exposed to 6°C for 20 h, then transferred back to 20°C for 24 h prior to scoring of the vulval phenotype (note that developmental progression was strongly slowed down, if not arrested, at 6°C). In the heat treatment, animals were exposed to 30°C for 16 h, immediately after which the vulva phenotype was scored. Control animals at 20°C reached the mid L4 stage at the same time as animals of the heat treatment and were scored in parallel.

Scoring of vulval cell fates and variant patterns

We scored the vulval phenotype of 300 individuals for each strain in each of the three temperature treatments ($N_{total} = 3600$ scored individuals), derived from 15 experimental

repeats (blocks) in each of which the four strains were scored in parallel as outlined above. Cell fate patterns of P3.p to P8.p were inferred through observation by Nomarski optics in early to mid L4 individuals as previously described (Sternberg and Horvitz 1986; Braendle and Félix 2008; Braendle et al. 2010).

Measurement of temperature effects on *lip-1::gfp* activity

We inferred Delta-Notch pathway activity in P5.p to P7.p using a transgenic strain containing an integrated transcriptional reporter construct, *lip-1::gfp* (strain AH142, derived from the reference strain N2) (Berset et al. 2001). Experimental populations were age-synchronized by hypochlorite treatment and liquid arrest at 20°C (Wood 1988). Young L1 larvae were transferred to NGM plates until they reached the mid L2 stage at which point they were randomly allocated to 20°C (control) or 30°C treatments. *lip-1::gfp* quantification was performed when individuals had reached the lethargus L2/L3 or early L3 stages. The Pn.p cells of live, anesthetized individuals were first identified using Nomarski optics, followed by measurement of signal (pixel) intensity in P5.p, P6.p, and P7.p for each individual as previously described in Braendle and Félix (2008) using an Olympus BX61 epi-fluorescence microscope equipped with a Coolsnap HQ2 camera (at 40× magnification). This experiment was repeated once, and a total of 33 and 34 individuals were scored at 20 and 30°C, respectively.

Statistical analysis and data presentation

Effects of environment, species and strain on frequency of vulval variants

Data representation in Figures 3 and 4 represent the mean percentage of classes and their variants averaged across experimental repeats ($N = 15$). We performed an REML (JMP 9.0, SPSS statistics) testing for the random effect of block, fixed effects of species, strain (nested in species), temperature and the interactions of species × temperature and strain (species) × temperature using Class A–C variant frequencies obtained per experimental repeat as a response variable ($N = 15$).

Quantification of lip-1::gfp activity

We performed an ANOVA (JMP 9.0, SPSS statistics) testing for the effects of *block* (i.e., experimental repeat, $N = 2$), *temperature* (20°C vs. 30°C), *cell* (P5.p, P6.p, P7.p), *individual* and all possible interactions using mean signal intensity as a response variable. Including individual as an effect allowed controlling for the non-independence between measures of P5.p, P6.p, and P7.p taken from the same individual. Data were log-transformed prior to analysis (Sokal and Rohlf 1981). Post-hoc tests (Tukey's HSD) were then performed to determine differences in signal expression between temperature, treatments and cells (P5.p. P6.p, P7.p). Data represented in Figure 5C show Least-Square Means of the *cell* × *temperature* interaction.

RESULTS

Characterization of vulval cell fate pattern variants

The characteristics of different vulval cell fates and corresponding cell lineages are described in Figure 1. Phenotypic characterization of vulval cell fate patterns variants deviating from the canonical *Caenorhabditis* 3°/4°-3°-2°-1°-2°-3 pattern follows the variant classes established by Braendle et al. (2010) (Fig. 2). We refer to any deviation from the canonical pattern as *variant* while *defect* only refers to variant patterns of class A, that is, patterns where the 2°-1°-2° adoption of vulval cells is disrupted. We defined 15 distinct non-canonical cell fate patterns, which are grouped into Class A, B, C and D variant categories as described in detail in legend of Figure 2. Note that we established an additional class C variant other than listed in Braendle et al. (2010): #14, that is, individuals that showed a 4° fate for both P4.p and P8.p.

Extreme temperatures debuffer vulval development inducing diverse variants and defects

The two temperature extremes caused a significant increase in vulval pattern variants deviating from the canonical pattern. Such increases in developmental variance thus indicate debuffering and reduced precision of the vulval developmental system (Braendle et al. 2010). Types of temperature-induced vulval variants varied greatly, covering the whole range of previously observed variants (Braendle et al. 2010) yet at much higher frequencies (Fig. 4, Table S1). At the standard temperature of 20°C, variants deviating from the canonical vulval cell fate patterns were rare for all strains, with defects (Class A) occurring at a frequency of $< 1\%$ (Fig. 3A). At 20°C, as previously found (Delattre and Félix 2001; Braendle and Félix 2008; Braendle et al. 2010; Pénigault and Félix 2011a), *C. briggsae* strains, compared to *C. elegans*, showed an increased tendency of P3.p, P4.p, and P8.p to adopt 4° fates (Class C and D) (Table 1C: main effect *Species*). Exposure to temperature extremes caused a significant increase in all variant classes for all strains (Table 1A–C: main effect *Temperature*) with an overall highest increase at 30°C, leading to defect frequencies (Class A) of up to 12% (Fig. 3A). For all strains, variants of class C, that is, P4.p and/or P8.p adopting a 4° fate, were consistently highest at 30°C (Figs. 3 and 4). Strains and species differed in the frequency of P3.p adopting the 4° fate, but the ratio of 4°:3° fates of P3.p was not sensitive to temperature variation (Fig. 4E); this contrasts with previous results where the ratio of 4°:3° cell fate for P3.p was found to be highly sensitive to environmental variation (Braendle and Félix 2008).

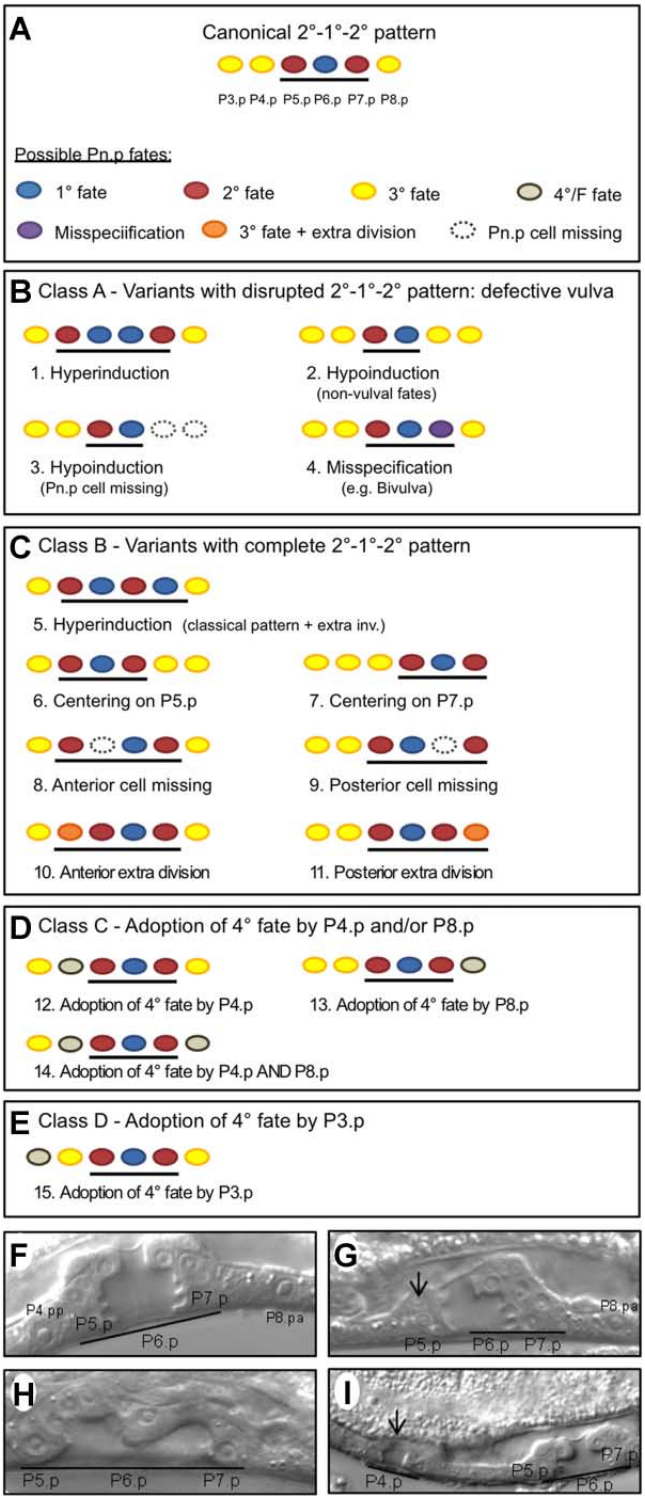

Fig. 2. Variant patterns of *Caenorhabditis* vulval precursor cells. (A) The canonical cell fate pattern of P3.p to P8.p is represented with P3.p adopting a 3° fate, and cells adopting a vulval cell fate are underlined. We defined 14 non-canonical subcategories of variants relative to P(4-8).p fates, grouped into three different classes. As P3.p fate is a highly variable trait, it is presented in its own class. (B) Class A: Disrupted 2°-1°-2° pattern leading to a defective vulva. (C) Class B: Complete 2°-1°-2° pattern but altered fates for the VPCs. (D) Class C: Complete 2°-1°-2° pattern and variable adoption of 3° versus 4° fate by P4.p and/or P8.p. (E) Class D: Complete 2°-1°-2° pattern and variable adoption of 3° versus 4° fate by P3.p. Detailed description of vulval pattern variants: (B) Class A (1–4): This class groups vulval variants that cause a strongly disrupted vulval pattern, likely leading to a defective vulval organ. (1) Hyperinduction: more than three induced VPCs (1° or 2° fate) that prevent the formation of a functional vulva (e.g., example adjacent 1° fate cells) (2) Hypoinduction due to adoption of a 3° or a 4° fate, resulting in fewer than three induced VPCs (1° or 2° fate). For example: P7.p adopts a 3° cell fate. (3) Hypoinduction due to missing cells: fewer than three induced cells because of the absence of one or several Pn.p cells. For example: missing P7.p and P8.p, leading to only two induced cells (P5.p and P6.p). (4) Misspecification of vulval fates (other than hyper- or hypoinduction): three VPCs are induced but their lineages deviate from the canonical pattern. For example, P7.p misspecification: the canonical UTLL lineage is replaced by LLTU, referred to as Bivulva phenotype (Inoue et al. 2004). (C) Class B: This class groups variants with complete 2°-1°-2° vulval patterns, yet deviating from the overall canonical pattern of VPCs. Vulva formation is not obviously disrupted, however, the impact of such variants on egg-laying or other functions remains unclear (Braendle and Félix 2008). (5) Hyperinduction: more than three induced VPCs. For example: P4.p adopts a vulval fate (2°) and creates a second, non-functional invagination. (6–7) Centering shifts: the three correctly induced VPCs are shifted to the anterior (centering on P5.p) or posterior (centering on P7.p). For example: anterior centering, P5.p adopts a 1° cell fate and its neighbors, P4.p and P6.p, adopt a 2° fate; the anchor cell is centered on P5.p. (8–9) Missing cells: One or more VPCs are missing. For example: P8.p adopts a 2° fate because P7.p is missing; in this case, it is not possible to distinguish whether this variant was due to a missing P7.p or P8.p cell. We can only distinguish whether the missing cell(s) is anterior (P3.p to P5.p) or posterior (P7.p and P8.p). (10–11) Supernumerary cell divisions: P3.p, P4.p, or P8.p divides more than once, generating three to four daughter cells that fuse with the hypodermis. For example: P4.p divides twice (ssss lineage instead of SS). (D) Class C: P4.p or P8.p adopts a 4° instead of a 3° fate. (12) P4.p adopts a 4° fate, fusing with the hypodermis without prior division. (13) P8.p adopts a 4° fate. (14) P4.p and P8.p adopt 4° fates. (E) Class D: Adoption of 4° fate instead of 3° fate by P3.p. It corresponds to variant (15). (F–I) Nomarski images of vulval cells in the mid L4 stage: (F) canonical vulval pattern, (G) hypoinduction (variant #2), (H) misspecification (variant #4), (I) hyperinduction (variant #5).

Genotype-dependence of temperature-induced vulval pattern variants

Frequency and type of temperature-induced vulval variants differed between species as well as between strains within each of the species (Figs. 3 and 4; Table 1). The two species showed converse responses to the two temperature extremes as indicated by the significant *species* × *temperature* interaction (Table 1A). *C. elegans* strains showed an increased frequency of variants, including vulval defects, at 30°C yet no increase at 6°C, while *C. briggsae* strains were more sensitive to 6°C (Fig. 3). The

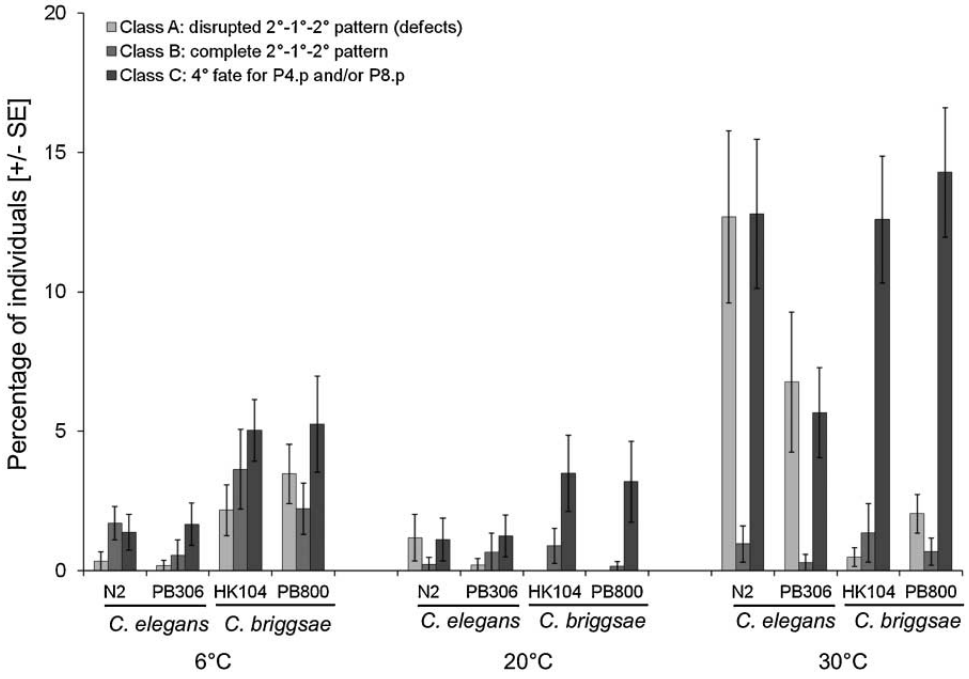

Fig. 3. Effects of temperature and genotype on variant class frequencies. Bars indicate the mean percentage of individuals showing vulval variants in three classes A–C ($N = 15$ experimental repeats, $N = 300$ individuals per strain/temperature, $N = 900$ per strain). Error bars indicate ± 1 SE. For detailed variant descriptions, see Table S1.

increased frequency of vulval variants was mainly due to an increase of class C variants (P4.p and/or P8.p adopting a 4° fate) in *C. briggsae* whereas in *C. elegans* we observed an increase of class C and class A (i.e., defect with disrupted 2°-1°-2° pattern) variants.

Table 1. Effects of environment, species and strain on frequency of vulval variant classes A–C

Source	DF	DFDen	*F* ratio	*P*-value
(A) Class A				
Species	1	154	3.61	0.0593
Strain (species)	2	154	2.72	0.0692
Temperature	2	154	17.58	<0.0001
Species × temperature	2	154	18.07	<0.0001
Strain (species) × temperature	4	154	1.06	0.3787
(B) Class B				
Species	1	154	2.45	0.1194
Strain (species)	2	154	1.45	0.2369
Temperature	2	154	5.94	0.0033
Species × temperature	2	154	1.02	0.3623
Strain (species) × temperature	4	154	0.56	0.6892
(C) Class C				
Species	1	154	18.34	<0.0001
Strain (species)	2	154	1.07	0.3442
Temperature	2	154	37.86	<0.0001
Species × temperature	2	154	0.36	0.6966
Strain (species) × temperature	4	154	1.47	0.2131

Mixed-model (REML) testing for the fixed effects of *species*, *strain* (nested in *species*), *temperature* and the interactions of *species* × *temperature*, *strain* (*species*) × *temperature*, and controlling for the random effect of *block*.

C. briggsae strains showed similar variant frequencies across temperatures for most variant types in all three classes (Fig. 4, C and D). In contrast, variant types varied qualitatively and quantitatively between the *C. elegans* strains at 30°C; although both strains exhibited a high frequency of vulval defects (Class A), the nature of these defects was different: in N2, the majority of defects (7.7%) were due to hypoinduction (variant #2) whereas this variant was very rare (0.2%) in PB306, which mainly showed defects due to other misspecification events of the VPCs (6.0%) (Fig. 4, A and B). In addition, also at 30°C, N2 showed an higher increase of P4.p and P8.p fusion (variants #12–14) relative to PB306.

Environmental sensitivity of specific system features

Vulval hypoinduction (variant #2) at high frequency (7.7%) was specifically displayed by *C. elegans* N2 individuals exposed to 30°C, and we found that this increase was consistently observed in N2 strains kept in other laboratories (Fig. S1). N2 hypoinduction at 30°C was primarily due to adoption of 3° fates by P5.p and P7.p (23 out of 25 individuals) rather than 4° fates (2 of out of 25 individuals), and never due to missing cells (Fig. 5A). In contrast, the central 1° fate cell, P6.p, was never hypoinduced. Induction of 2° but not 1° fate vulval cells, P5.p and P7.p, is thus sensitive to high temperature.

We therefore next asked how high temperature affects the activity of the key signaling pathway involved in 2° vulval fate determination, the Delta-Notch pathway (Sternberg 2005). Using the highly sensitive transcriptional reporter gene *lip-1::*

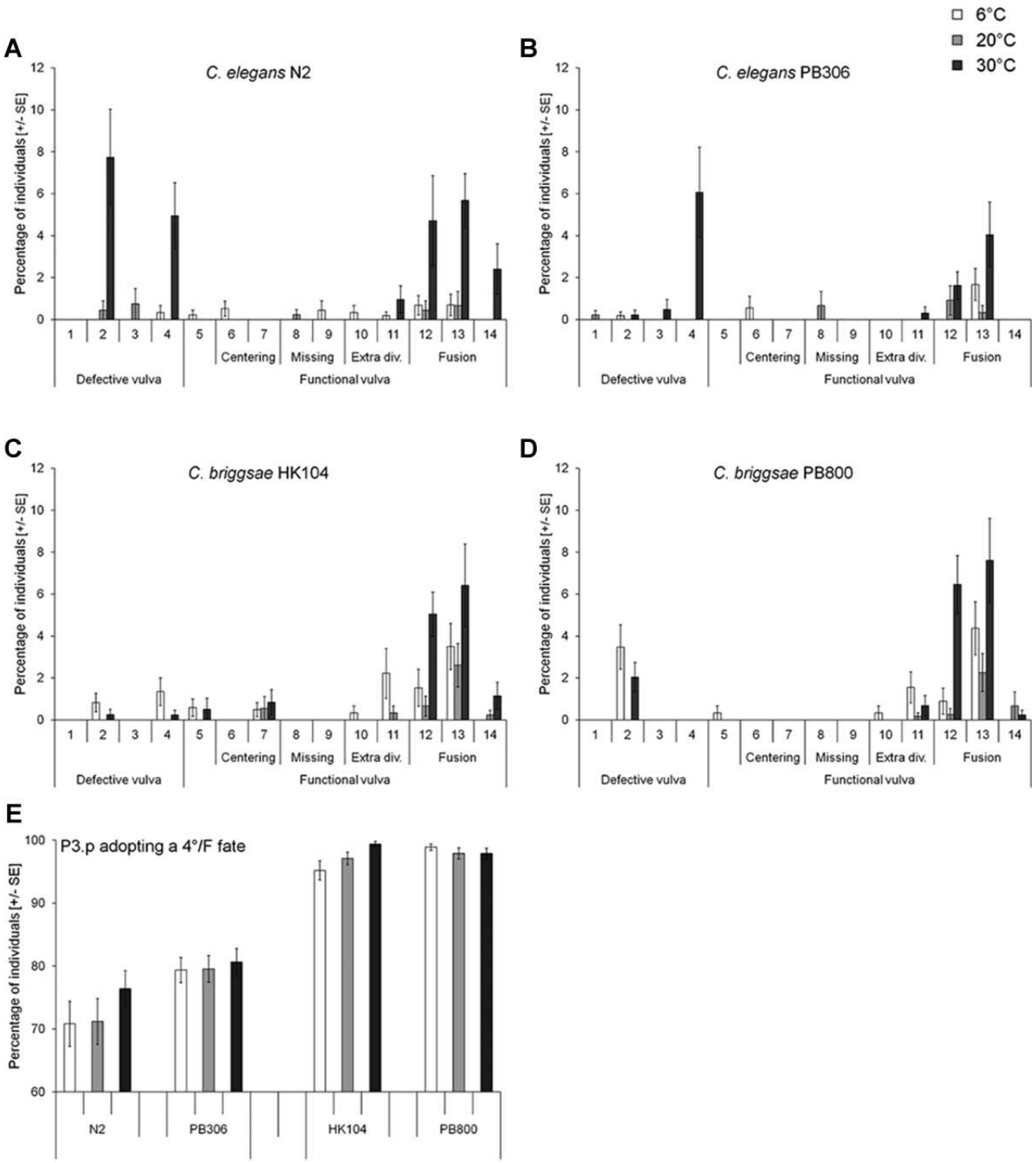

Fig. 4. Effects of temperature and genotype on frequencies of specific vulval variants. Frequency of specific vulval variants at different temperatures for the *C. elegans* strains N2 (A) and PB306 (B), and the *C. briggsae* strains HK104 (C) and PB800 (D). (E) Frequency of individuals with P3.p adopting a 4° fate. Bars indicate the mean percentage of individuals with a given vulval variant ($N = 15$ experimental repeats, $N = 300$ individuals per strain/temperature, $N = 900$ per strain). A detailed description of different types of vulval variants is given in Figure 2. Error bars indicate ± 1 SE. For detailed variant descriptions, see Table S1.

gfp in the N2 background (Berset et al. 2001), we quantified pathway activities at 20°C and 30°C during vulval fate patterning in P5.p to P7.p (lethargus L2/L3 and early L3 stages) (Fig. 5B). Temperature affected Delta-Notch activity in a cell-specific manner: at 30°C *lip-1::*GFP expression, was significantly decreased in P5.p while it was increased in P6.p (Fig. 5C). These observations are consistent with a reduced stability of 1°-2°-1° pattern establishment at high temperature, which seem to primarily disrupt the induction of 2° cells as reported above (Fig. 5A). The detection of reduced mean Delta-Notch activity specifically in P5.p may account for its higher proportion of hypoinduction compared to P7.p (Fig. 5A). In contrast, despite an apparent mean increase of its Delta-Notch activity, P6.p was never found to be hypoinduced at 30°C; however, multiple other misspecification defects (variant #4), observed for N2 at 30°C, also affected P6.p (Fig. 4A).

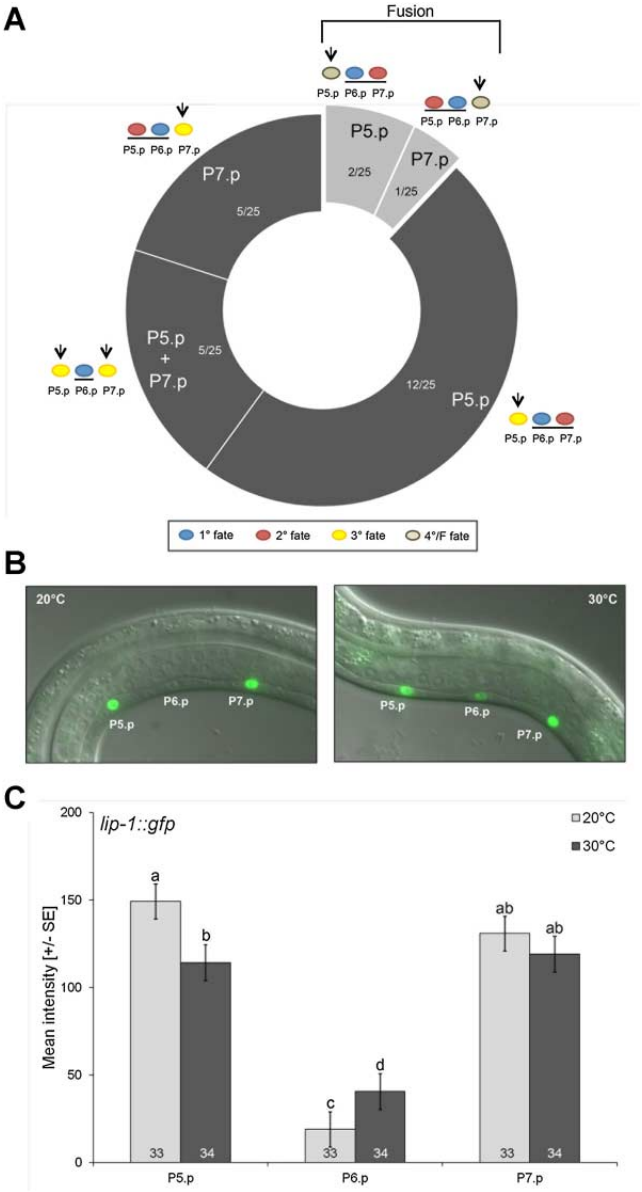

Fig. 5. Effects of high temperature on *C. elegans* N2 vulval induction and Delta-Notch pathway activity. (A) Vulval hypoinduction (variant #2) in *C. elegans* N2 at 30°C: 25 of 300 individuals showed variant patterns of this type. The figure shows the different fates (3° or 4°/Fusion fate) adopted by the different vulval precursor cells in the 25 affected individuals. Considering the total of 25 hypoinduced variants, P5.p was more frequently affected than P7.p (Fisher's exact test, $P = 0.043$) and the majority of variants were due to adoption of the 3° cell fate ($N = 22/25$). Note that Pn.p hypoinduction due to 3° cell fate may reflect partial hypoinduction of the corresponding cell, that is, either Pn.pa or Pn.pp was hypoinduced (for detailed description of hypoinduced variants, see Table S1 "N2 hypoinduction variants"). (B) Merge of Nomarski and fluorescence images of *lip-1::*GFP individuals (early L3 stage) exposed to 20°C and 30°C. (C) Measurement of Notch pathway activity through *lip-1::*GFP analysis at 20°C and 30°C. Bars indicate mean signal (pixel) intensity, that is, Least Square Means for the interaction *cell* × *temperature* ($F_{2,72} = 8.03$, $P = 0.0007$), controlled for block and individual effects. Values labeled with different letters are significantly different (Tukey's HSD). Number of individuals assayed are shown in bars. Error bars indicate ± 1 SE.

Environmental versus mutational perturbation of vulval cell fate patterning

A previous study (Braendle et al. 2010) had quantified vulval variants of mutation accumulation (MA) lines derived from the same *C. briggsae* and *C. elegans* strains used here, allowing comparison of mutationally versus environmentally induced vulval variants. MA lines were generated under strongly relaxed selection, that is, single larval transfer across generations, and contain numerous spontaneous random mutations, leading to reduced fitness (Baer et al. 2005), increased developmental variance (Baer 2008), and a decay of vulval patterning precision characterized by the presence of diverse patterning variants (Braendle et al. 2010). Here, we found that overall the frequency of vulval variants after extreme temperature stress exposure was much higher than after 250 generations of MA (Fig. 6). Across species and strains, both MA and exposure to temperature extremes triggered a similarly wide spectrum of vulval developmental variants, yet variant spectra for most strains were overall narrower after temperature perturbations. For example, *C. briggsae* PB800 had a 50% reduced variant spectrum in response to thermal perturbations relative to the MA assay. In addition, we also found cases where variant types in certain strains were only generated in response to temperature but not MA. For example, variant #13 (4° of P8.p) in the N2 strain was never observed after MA but occurred at relatively high frequency in this strain at 30°C (Fig. 4A). Similarly, hypoinduction variants (#2) frequently observed at high temperature for N2 (Fig. 4A) were very rare after MA for the same strain (Braendle et al. 2010). While quantitative

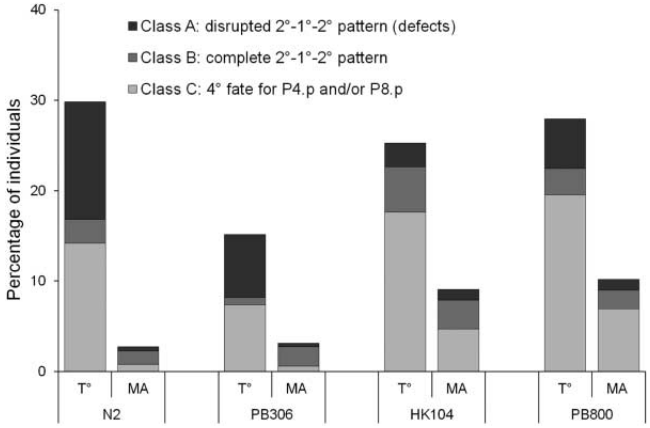

Fig. 6. Comparison of vulval variants induced by temperature versus mutation accumulation. Proportion of individuals with vulval variants after temperature exposure to 6 and 30°C (T°) or after mutation accumulation (MA) (Braendle et al. 2010). Bars indicate the total percentage of individuals with a variant pattern of the three classes after the two treatments (T° or MA). For details of the MA experiment, see Braendle et al. (2010).

comparison between mutationally and environmentally induced vulval variants is difficult given the low frequency of most variants after MA, several examples illustrate that genotype-dependence of variant production can be observed in response to either source of perturbation. For example, the 3° VPCs, P4.p and P8.p, are most sensitive, frequently adopting a fused 4° fate (Class C variants) upon either mutational or environmental perturbation in both *C. elegans* and *C. briggsae*, yet the frequency of 4° fate adoption is strongly species- and isolate-dependent (Figs. 3 and 4). In another example, centering variants on P7.p (variant #7) were never observed for *C. elegans* N2 after either type of perturbation, while they occurred at low frequency in all other strains. Hence, mutational and environmental perturbation of vulval cell fate patterning may disrupt the same specific developmental system features (e.g., specific precursor cells), yet for both types of perturbations the degree and type of disruption was usually dependent on the genetic background of individuals examined.

DISCUSSION

We used extreme temperatures to perturb the *Caenorhabditis* vulval cell fate patterning process, allowing us to quantitatively assess debuffering of this developmental system through analysis of non-canonical vulval patterning variants. Applying such strong environmental perturbations enabled us to detect environmental sensitivity of different system parameters as well as their evolutionary variation. Figure 7 summarizes observed evolutionary variation in temperature sensitivity of each VPC, indicating that the environmental sensitivity of VPCs varies both within and among genotypes.

Environmental sensitivity of the vulval cell fate patterning process shows evolutionary variation

Extreme temperatures consistently impaired the precision of vulval cell fate patterning in all tested strains. We found that extreme temperatures induce vulval patterning variants in $>10\%$ of individuals while such variants are absent or very rare in control conditions. Type and frequency of variant vulval patterns were temperature-, species- and genotype-dependent, showing significant differences among tested species and strains within species (Table 1). Although exposure to 30°C induced the highest proportion of variant patterns for all strains, only *C. elegans* strains showed an increased frequency of defects (Class A variants) at this temperature. In contrast, such defects were increased at 6°C for *C. briggsae* strains, suggesting that the two species differ in their thermal tolerance to hot versus cold temperatures, respectively. Thermal preference analysis indicates that *C. briggsae* is generally more tolerant to high temperature compared to *C. elegans* (Harvey and Viney 2007; Anderson et al. 2011; Prasad et al. 2011) and our data suggest that *C. briggsae* may also show reduced cold tolerance. Therefore, thermal tolerance of reproductive traits may correlate with thermal tolerance of other traits, such as the developmental patterning process examined here.

Species differences were most marked for pattern variants affecting P3.p, P4.p and P8.p, that is, VPCs adopting non-vulval cell fates in the canonical situation. As found in previous studies (Delattre and Félix 2001; Braendle and Félix 2008; Braendle et al. 2010; Pénigault and Félix 2011a), *C. briggsae* strains showed a significantly higher proportion of individuals with P3.p adopting the 4° fate compared to *C. elegans* strains (at all temperatures). Additionally, even at the standard temperature of 20°C, P4.p and P8.p showed a low, yet consistently elevated propensity to adopt the 4° fate instead of the canonical 3° cell fate (Class C, variants #12 to 14), which further increased at both temperature extremes. Similarly, increased Class C variants for *C. briggsae* strains have been observed after exposure to starvation conditions (Braendle and Félix 2008). In *C. elegans*, the proportion of the same variants was increased only at 30°C, yet more so in N2 than in PB306. Although their frequency was modulated by both temperature and genotype, Class C variants were overall most frequent, indicating that fate specification of P4.p and P8.p is sensitive to environmental perturbations, particularly in *C. briggsae*.

Different features of the vulval patterning process vary in their environmental sensitivity in a genotype-dependent manner

C. elegans strains generated more defects than *C. briggsae* strains in response to 30°C, a temperature which specifically induced vulval hypoinduction in the N2 strain. The triggering

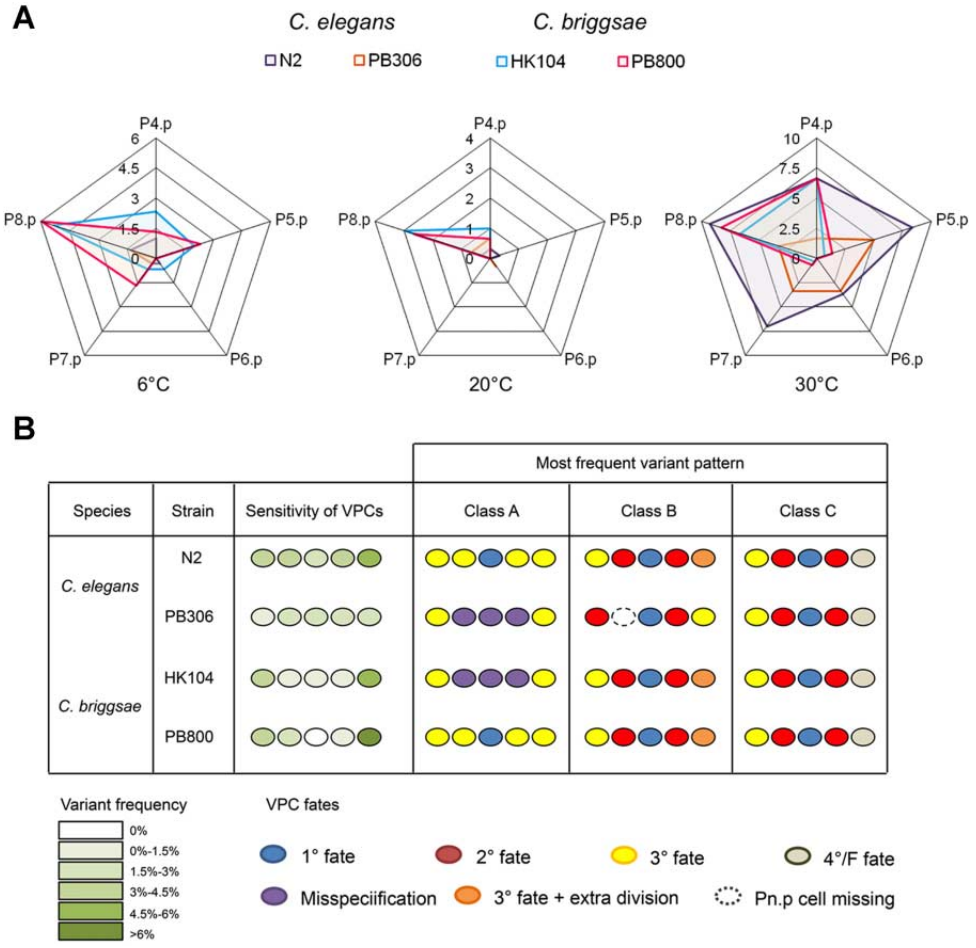

Fig. 7. Evolutionary variation in cell-specific temperature sensitivity. (A) Radar charts plotting the percentage of each VPC affected (i.e., adoption of a non-canonical cell fate of the Pn.p cell) in different strains across the three temperatures (all variants pooled, except for centering variants that were excluded from this representation for simplicity's sake). (B) Illustration of evolutionary variation in sensitivity of different VPCs to extreme temperatures (6°C and 30°). Different degrees of thermal sensitivity, measured as the percentage of non-canonical variants, are indicated for each VPC, labeled from low sensitivity (white) to high sensitivity (dark green). The right panel indicates the most frequently observed variant pattern for each class across species and genotypes.

of this developmental defect was primarily due to adoption of non-vulval cell fates (3° fate) by P5.p and P7.p while the 1° fate of P6.p remained unperturbed. Temperature perturbations therefore preferentially disrupted induction of 2° rather than 1° vulval cell fates, and their corresponding Pn.p cells. Consistent with these observations we found that high temperature modulates the Delta-Notch pathway – the central pathway for 2° fate specification – through reduction of its activity in 2° cells and an increased activity in the 1° cell. This temperature effect on Delta-Notch activity may result through increased activity in P6.p alone (e.g. through down-regulation of the EGF-Ras-MAPK pathway), which would then weaken lateral activation of the Delta-Notch pathway in P5.p and P7.p. Alternatively, Delta-Notch and/or EGF-Ras-MAPK are directly affected in all three cells, P5.p to P7.p. In either case, the observed effects indicate a weakened cross-talk between EGF-Ras-MAPK and Delta-Notch pathways, resulting in a reduced reinforcement of the 2°-1°-2° vulval fate pattern. The lower activity of Delta-Notch in 2° fate cells suggests lower levels of vulval inductive signal, consistent with the frequent hypo-induction defects of these cells in *C. elegans* N2. However, decreased Delta-Notch activity at 30°C in 2° fate cells was asymmetric, more frequently affecting P5.p than P7.p. It is unclear why P5.p is more sensitive to high temperature than P7.p, but several studies indicate that *C. elegans* VPCs, including P5.p to P7.p, differ in expression of Wnt-regulated Hox genes involved in vulval competence and sensitivity to inductive signals (Clandinin et al. 1997; Pénigault and Félix 2011b, 2011a). Therefore, VPCs are not equivalent in their potential to adopt vulval cell fates, and our results suggest that they further show distinct sensitivities to the same environmental perturbation.

Environmental and mutational perturbations reveal genotypic biases in the production of vulval developmental variants

Comparison of vulval developmental variants induced by temperature extremes versus variants induced after 250 generations of mutation accumulation (MA) (Braendle et al. 2010) indicates that environmental and genetic perturbations may affect the same features of this developmental system. For example, the frequency of 4° fate adoption by P4.p and/or P8.p (Class C variants), became overall most easily augmented by either type of perturbation in both *C. elegans* and *C. briggsae*. In contrast, induction of the 1° fate, usually by P6.p, seemed generally most robust, that is, least affected by different perturbations as shown in this study and before (Braendle and Félix 2008; Braendle et al. 2010). Certain Pn.p cells and their specific properties (e.g., competence, cell fate) are therefore more sensitive to both environmental and genetic perturbations. Moreover, genotype-dependence of the frequency and type of vulval patterning variants may occur, so that a given genotype produces a biased spectrum of variants irrespective of the type of perturbation. Examples include the near-absence of certain variant patterns (centering on P7.p, hyperinduction) in *C. elegans* N2, which are regularly observed in other strains, or the increased frequency of Class C variants in *C. briggsae* relative to *C. elegans* strains (Braendle and Félix 2008; Braendle et al. 2010). Furthermore, in this study we found that *C. elegans* N2 showed an increased tendency for hypoinduction errors (yet no hyperinduction errors) in response to high temperature. In contrast, hypoinduction errors were rare (0.2%) in *C. elegans* PB306. One potential explanation for this difference is that basal levels of the vulval inductive signal are higher in PB306 than in N2, consistent with previous measurements of EGF-Ras-MAPK pathway activity in these two strains (Braendle et al. 2010). Thus, if high temperature similarly reduced vulval induction in both strains, this reduction would not be sufficient to elicit hypoinduction errors in PB306. A similar difference in the production of hypo- versus hyperinduced variants between N2 and PB306 has also been observed after MA (Braendle et al. 2010): hypoinduction was rare for PB306 but common in N2, while hyperinduction was frequent in PB306 but virtually absent in N2. Both MA and high temperature therefore induced a consistent, genotype-biased pattern of hypo- versus hyper-induced variants, which are congruent with the difference of basal EGF-Ras-MAPK activity detected in these two *C. elegans* strains (Braendle et al. 2010). These observations suggest that specific properties of the vulval developmental system, such as the inductive signal level, are more sensitive to both environmental and genetic perturbations; and this sensitivity may consistently vary among different genotypes. Understanding such developmental and genotypic biases in the production of developmental variants is relevant as they are indicative of the mutationally accessible phenotypic spectrum, which modulate potential evolutionary trajectories (Yampolsky and Stoltzfus 2001; Arthur 2004; Dichtel-Danjoy and Félix 2004; Braendle et al. 2010; Félix 2012b).

Overall, however, the frequency and type of induced vulval pattern variants observed for a given strain in response to temperature treatments did not recapitulate the patterns observed after MA. For example, in *C. elegans* N2 we repeatedly found P8.p fusion (variant #13) in response to temperature perturbations, yet this variant was never found after MA (Braendle et al. 2010). Such comparison between mutationally and temperature-induced developmental variants is obviously limited given that MA induces a wide spectrum of random mutations while temperature represents only one specific condition out of an infinite range of environmental conditions to assess. Moreover, vulval pattern variants induced by MA occurred at very low frequencies, making the detection of such correlations between the two treatments difficult. Taken together, the currently available data suggest that there is environmental specificity in the induction of variants, which is further genotype-dependent. For example, Braendle and Félix (2008) found that starvation exposure in the L2 stage consistently induced shifts of vulval patterns centered on P5.p (variant #6) in *C. elegans* (N2 but not in other *C. elegans* or *C. briggsae* strains). These examples indicate that environmental sensitivity of the vulval developmental system depends on subtle interactions between specific environments and genetic background.

Characterizing cryptic genetic variation to study developmental evolution of environmental sensitivity

The observed genotype-dependence of vulval variant production in response to temperature reflects cryptic genetic variation uncovered by environmental perturbations, and thus corresponds to genotype-by-environment interactions. Because many of the temperature-induced variant patterns represent defects or deviants decreasing precision of the pattern establishment, this variation reflects evolutionary variation in environmental sensitivity of vulval development. Such evolutionary variation in the environmental sensitivity of the vulval patterning output can—as explained above—likely be traced back to evolutionary variation in underlying system parameters, including differential activity of vulval signaling pathways (Braendle and Félix 2008; Milloz et al. 2008; Braendle et al. 2010; Duveau and Félix 2012; Félix and Barkoulas 2012) or different levels of competence in VPCs (Félix 2007; Braendle and Félix 2008; Braendle et al. 2010; Pénigault and Félix 2011a). However, open questions remaining are to what extent such cryptic genetic variation arises because of the vulval system's robustness to genetic and environmental variation and whether this variation is largely selectively neutral. As shown here, cryptic genetic variation corresponds to genetic variation in environmental sensitivity and therefore may also include adaptive variation contributing to

developmental robustness, for example, resulting from evolution in divergent environmental conditions.

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Author Contribution

C.B. and S.G. conceived and designed the experiments; S.G. performed the experiments; S.G. and C.B. analyzed the data; C.B. and S.G. wrote the paper.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Fig. S1. Frequency of vulval variant patterns of different *C. elegans* strains at 30°C.

Table S1. Data table: Vulval variant patterns of *C. briggsae* and *C. elegans* strains at different temperatures