

**ROBUSTNESS AND PLASTICITY IN *DROSOPHILA* EMBRYOGENESIS: A
COMPARATIVE ANALYSIS OF NEARLY ISOGENIC AND NON-ISOGENIC
MASS POPULATIONS**

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By

Tunahan alıkođlu

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We certify that we have read this thesis and that in our opinion it is fully
adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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ABSTRACT

Robustness And Plasticity in *Drosophila* Embryogenesis: A Comparative Analysis of Nearly Isogenic and Non-Isogenic Mass Populations

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Robustness and plasticity are fundamental aspects of early development, but how they are shaped by genetic background and environment remains unclear. To address this, we examined the effect of temperature perturbation on the early embryonic markers in *Drosophila melanogaster* across widely used isogenic laboratory lines with potential genetic differences, natural populations from Saimbeyli/Adana, and weak *bcd* mutants. Early embryonic markers included the Bcd gradient and *Hunchback* (*hb*) and *even-skipped* (*eve*) stripe positions.

At 25°C, most wild-type isogenic strains had comparable *hb* pbps on average. On the other hand, Canton S had the most variable (5.2) *hb* pbp %EL, whereas the Saimbeyli Mass population had the most stable, least variable (1.5) pbps at the same temperature. At 29°C, Oregon R *hb* pbps shifted posteriorly, while the Saimbeyli Mass line remained unchanged compared to 25°C. Canton S had the highest variation (5.05) Oregon R had the lowest variation (1.4). *Eve* second stripe was generally more robust against temperature change than *hb*, though Berlin K, Oregon R, and Saimbeyli Mass showed significant shifts towards posterior. For *eve* second stripe at 29°C, Saimbeyli 2 had the highest variation (2.8) and Berlin K had the lowest (0.8). In weak *bcd* mutant lines, steepness of Bcd gradient increased at 22°C, which correlated with the increase in embryo-to-embryo variations. They also showed lower survival rates which correlated with increased mutant cuticle phenotypes. In contrast, wild-type isogenic and Saimbeyli lines maintained high viability under all conditions. Notably, the Saimbeyli mass line showed more robust (robust averages across temperatures) but more variable (increased embryo-to-embryo variation) *hb* pbps, but greater variability and plasticity in *eve* expression.

These findings indicate that robustness and plasticity are not uniform outcomes of development, but depend on the segmentation network, the developmental marker, and

maternal inputs. Development can buffer some traits while allowing flexibility in others, balancing stability in response to the environmental perturbations.

Keywords: *Drosophila melanogaster*, Phenotypic variation, Isogenic lines, Temperature perturbation



ÖZET

***Drosophila* Embriyogenezinde Robustluk ve Plastisite: Neredeyse İzogenik ve İzogenik Olmayan Kitle Popülasyonlarının Karşılaştırmalı Analizi**

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Sağlamlık (robustluk) ve plastisite, erken gelişimin temel özellikleridir; ancak genetik arka plan ve çevre tarafından nasıl şekillendirildikleri belirsizliğini korumaktadır. Bunu ele almak için, *Drosophila melanogaster*'da sıcaklık pertürbasyonunun erken embriyonik belirteçler üzerindeki etkisini; olası genetik farklılıklar taşıyan, yaygın kullanılan isojenik laboratuvar hatları, Saimbeyli/Adana'dan doğal popülasyonlar ve zayıf bcd mutantları boyunca inceledik. Erken embriyonik belirteçler arasında Bcd gradyanı ile *Hunchback* (*hb*) ve *even-skipped* (*eve*) şerit konumları yer aldı.

25°C'de, çoğu yabani tip isojenik soy ortalama olarak karşılaştırılabilir *hb* arka sınır konumlarına (pbp'lere) sahipti. Öte yandan Canton S, *hb* pbp'de en değişken değeri (5,2) gösterirken, Saimbeyli Mass popülasyonu aynı sıcaklıkta en kararlı, en az değişken (1,5) pbp'lere sahipti. 29°C'de, Oregon R'ın *hb* pbp'leri posteriora kayarken, Saimbeyli Mass hattı 25°C ile karşılaştırıldığında değişmeden kaldı. Canton S en yüksek varyasyona (5,05) sahipti, Oregon R ise en düşük varyasyona (1,4). *eve* ikinci şeridi genel olarak *hb*'ye kıyasla sıcaklık değişimine karşı daha sağlamdı; ancak Berlin K, Oregon R ve Saimbeyli Mass posteriora doğru anlamlı kaymalar gösterdi. 29°C'de *eve* ikinci şeridi için Saimbeyli 2 en yüksek varyasyona (2,8), Berlin K ise en düşük varyasyona (0,8) sahipti. Zayıf bcd mutant hatlarında, Bcd gradyanının dikliği 22°C'de arttı ve bu artış embriyodan embriyoya değişimdeki artışla ilişkiliydi. Bu hatlar ayrıca artmış mutant kutikula fenotipleriyle ilişkili olarak daha düşük yaşam oranları gösterdi. Buna karşılık, yabani tip isojenik ve Saimbeyli hatları tüm koşullarda yüksek yaşama oranlarını korudu. Dikkat çekici olarak, Saimbeyli Mass hattı *hb* pbp'lerde daha sağlam (sıcaklıklar arasında sağlam ortalamalar)

ancak daha deęişken (embriyodan embriyoya artmış deęişkenlik) bir yapı sergilerken, *eve* ifadesinde daha büyük deęişkenlik ve plastisite gösterdi.

Bu bulgular, saęlamlık ve plastisitenin gelişimin tekdüze çıktıları olmadığını; segmentasyon ağına, incelenen gelişimsel belirtece ve maternal girdilere baęlı olduğunu göstermektedir. Gelişim, bazı özellikleri tamponlarken dięerlerinde esnekliğe izin verebilir ve böylece çevreye duyarlılık ile kararlılık arasında bir denge kurabilir.

Anahtar Kelimeler: *Drosophila melanogaster*, Fenotipik varyasyon, İzojenik hatlar, Sıcaklık pertürbasyonu



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DEDICATION



To my beloved Mother..

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1. INTRODUCTION

1.1 Background and Significance

1.1.1 Phenotypic Variation

Phenotype is the set of observable and measurable traits that are molecular, cellular, anatomical, physiological, and behavioral and that stems from how a genotype is expressed, with additional factors such as the environment and random events during development. Examples include simple physical features like height or eye color. Within a population, organisms differ genetically through common variants such as single-nucleotide polymorphisms and small insertions/deletions. Together with environmental factors and developmental randomness, these differences generate phenotypic diversity.

The roles of genotype and environment in shaping phenotypic diversity have been studied extensively. The environmental context can change both the magnitude of the variation and its consequences. For example, temperature shifts biochemical rates and can change the stability of intermediate states, altering how much variability shows up in the phenotype without changing the genotype (Begasse et al., 2015).

Overall, phenotypic variation can be grouped into three interacting sources: (i) genetic differences among individuals, (ii) environmental influences that alter reaction kinetics or state stability, and (iii) intrinsic stochastic (random) processes during development.

Studies at single-cell and developing-tissue scales show that even genetically identical (clonal) cells raised in the same macro-environment exhibit measurable molecular and cellular variability (Elowitz et al., 2002; Raj & van Oudenaarden, 2008; Geiler-Samerotte et al., 2013). We refer to these genotype- and environment-matched differences as random variation, arising from intrinsic noise and stochastic events in development or gene expression. How much this random component contributes to phenotypic differences—even among clonal or isogenic individuals—remains poorly understood, as do its evolutionary and developmental consequences. (Elowitz et al., 2002; Raj & van Oudenaarden, 2008; Geiler-Samerotte et al., 2013). The contribution of random variation—small differences among genetically identical individuals raised

under the same conditions—is still not well explored, and its evolutionary and developmental consequences remain poorly understood. Here, “random variations” refer to intrinsic noise and stochastic events during development or gene expression that may account for differences even among clonal or isogenic individuals.

Early embryogenesis is a sensitive window where small upstream perturbations can either be amplified or filtered by gene-regulatory networks. Therefore, early spatial readouts are very informative for probing how genotype, environment, and chance come together to shape phenotypic variation (Gregor et al., 2007; Petkova et al., 2019).

1.1.2 Sources of Non-Genetic Phenotypic Variation - Random Variations

Random variation can arise even when genotype and the macro-environment are held constantly. It reflects variability from intrinsic molecular processes and from small, hard-to-control microenvironmental differences among cells or embryos (Félix & Barkoulas, 2015; Elowitz et al., 2002; Raj & van Oudenaarden, 2008). In early embryogenesis, the spatial and temporal precision of gene expression reflects the combined action of these intrinsic and extrinsic factors. Intrinsic noise comes from the stochastic nature of transcription, translation, and molecular interactions, and from uneven partitioning of low-copy components during cell divisions. These processes cause cell-to-cell or embryo-to-embryo differences even under controlled conditions (Félix & Barkoulas, 2015; Elowitz et al., 2002; Raj & van Oudenaarden, 2008). Extrinsic influences include subtle differences in local physiology and microenvironment that set reaction rates and stability. Local temperature at the micro-level for example, shifts its reaction kinetics, affects protein stability and alters behavior of regulatory networks, and thus changes gene-expression dynamics (Rohner et al., 2013; Begasse et al., 2015).

The impact of intrinsic variation depends on stage. Perturbations early in development are more likely to propagate and affect downstream outcomes, whereas late-stage noise may be buffered and filtered by the genetic network or have limited effect (Moreno-Ayala et al., 2021; Waymack et al., 2020). Studying early embryonic patterning is therefore a sensitive way to ask how random variation shapes developmental trajectories (Gregor et al., 2007; Petkova et al., 2019). In the syncytial blastoderm, several factors introduce variance in early readouts: transcriptional

bursting; how often enhancers and promoters meet; limited sampling windows set by nuclear cycles (nc10–nc14); and geometric constraints on nuclei (shape, position, size) that affect how inputs are integrated (Bothma et al., 2014; Fukaya et al., 2016, Foe & Alberts, 1983). Together, these factors determine where boundaries and stripes form unless the network filters them (Little et al., 2013).

Cooperative enhancer logic, overlapping transcriptional inputs, and redundant cis-regulatory elements such as shadow enhancers reduce the phenotypic impact of molecular randomness, though they do not eliminate it, helping preserve positional precision despite intrinsic and extrinsic fluctuations (Perry et al., 2012; Félix & Barkoulas, 2015). Maternal factors (e.g., maternal age) can also shift initial conditions such as mRNA/protein loading and stability and may influence how embryos read morphogen inputs. We do not analyze these explicitly in this thesis but acknowledge them as plausible non-genetic contributors to variation in otherwise standardized experiments.

Taken together, intrinsic noise and small, practically uncontrollable extrinsic microenvironmental differences form a third axis of phenotypic variation alongside genotype and environment. By quantifying their effects at early spatial readouts such as the *hb* pbp of the anterior domain and *eve* stripe-2 position we can directly test buffering and sensitivity in the embryonic network (Gregor et al., 2007; Petkova et al., 2019) and relate these measurements to developmental robustness and plasticity.

1.1.3 Fluctuating Asymmetry as a Readout of Developmental Noise

Fluctuating asymmetry (FA) means small, random left–right differences around perfect bilateral symmetry. Because these differences average out to nearly zero at the population level, their variance is used as a readout of developmental instability caused by random variation rather than directional genetic or environmental effects (Palmer & Strobeck, 1986; Van Valen, 1962; Van Dongen, 2006). In Waddington’s canalization framework, robust development buffers perturbations so that bilateral traits match on both sides. When canalization weakens, buffering drops and intrinsic noise shows through; a common morphological result is increased FA (Palmer & Strobeck, 1986; Van Dongen, 2006; Félix & Barkoulas, 2015).

FA is most often measured in paired, bilaterally symmetric traits—classically wing size and vein patterning in *Drosophila*, and limb or digit measures (including digit ratios) in vertebrates—because left and right sides share genotype and the same macro-environment. Any leftover left–right difference is then attributed to random developmental variation that accumulated while the trait was forming (Klingenberg, 2003; Pelabon et al., 2006; Carter et al., 2009; Leamy & Klingenberg, 2005).

First, test for DA; if present, it biases FA upward, so center (L–R) or drop the trait. Second, estimate Measurement Error (ME) with repeats and remove it from FA; otherwise FA is inflated. Standard protocols emphasize these steps and recommend reporting standardized FA indices so studies can be compared (Palmer & Strobeck, 1986; Van Dongen, 2006). Because FA values are small, adequate sample sizes and replication are essential to separate true FA from error. FA is widely used to study robustness under stress, and higher FA after temperature shifts, toxins, pathogens, or poor nutrition has been reported in many taxa—consistent with weaker buffering in challenging conditions (Lens et al., 2002; Møller & Swaddle, 1997).

Molecular buffering contributes to canalization and can shape FA. Hsp90, a heat-shock chaperone with high baseline expression and further induction under stress, stabilizes and folds client proteins—including mutated or unstable forms—thereby buffering phenotypic effects of genetic variants and supporting stress responses and genome stability. Lowering Hsp90 function reveals cryptic genetic variation and can raise phenotypic variance and FA in several systems, including *Drosophila* and zebrafish (Rutherford & Lindquist, 1998; Queitsch et al., 2002; Debat et al., 2006; Yeyati et al., 2007; Specchia et al., 2010). Epigenetic regulators also help keep development symmetric. When they are impaired, buffering decreases and developmental instability increases, linking chromatin-level regulation to canalization (Ringrose & Paro, 2004; Frankel et al., 2010).

Conceptually, FA is a non-invasive readout of the noise experienced during the developmental window of a given bilateral structure. It folds together intrinsic noise (e.g., stochastic gene expression and signaling) and local microenvironmental variation into a single variance measure of left–right differences. This makes FA useful for comparing robustness across traits or conditions once bilateral morphology is set and measurement protocols are standardized (Klingenberg, 2003; Van Dongen, 2006).

FA is particularly informative at later morphological stages, but our study looks earlier, at embryonic gene-expression patterns, before bilateral morphology forms. We therefore use positional readouts (*hb* posterior boundary of the anterior domain; *eve* stripe-2 position) to capture how noise and buffering appear during early patterning. In parallel, we score larval cuticle morphology only in sensitized weak *bcd* mutants to connect early variation to terminal phenotypes; this complements our method of studying the effect of random variation, isogenic populations and the early readouts we obtain from them without relying on FA metrics.

1.1.4 Isogenic Populations as Tools to Study Random Variation

Using isogenic populations groups of individuals that are completely or nearly genetically identical is a practical way to distinguish non-genetic sources of phenotypic variability. In such populations, any differences we observe can be traced to developmental noise or environmental inputs rather than to standing genetic differences (Raj & van Oudenaarden, 2008; Félix & Barkoulas, 2015). This setup is especially powerful for analyzing stochastic gene expression and the effects of controlled environmental perturbations.

Isogenic systems are widely used across developmental biology, genetics, and systems biology. Model organisms such as *Drosophila melanogaster*, *Caenorhabditis elegans*, *Mus musculus*, and inbred plant lines, as well as clonally derived cell lines, keep the genetic background constant so researchers can prove how noise and microenvironment shape phenotype (Raj & van Oudenaarden, 2008). Likewise, clonal mammalian cell lines derived from a single cell are used *in vitro* to dissect molecular pathways under a fixed genetic background (Snijder et al., 2009). Inbreeding first exposes harmful recessive alleles (inbreeding load) and reduces fitness. Over many generations, selection can remove (‘purge’) the most deleterious alleles, so the surviving lines become healthy and stable. In *Drosophila*, extended inbreeding often purges much of this load, yielding robust isogenic stocks (Pérez-Pereira et al., 2021)

Because genotypes are designed to be constant in isogenic lines, variation in morphology, gene expression, timing, or stress responses can be attributed to intrinsic sources (e.g., transcriptional noise, partitioning errors) and extrinsic sources (e.g., small microenvironmental differences, temperature). Using these lines helps separate

intrinsic noise, environmental sensitivity, and genetic contributions with high precision, and is useful for quantifying developmental robustness, plasticity, and canalization (David et al., 2004; Via & Lande, 1985; Félix & Barkoulas, 2015; Fraser & Schadt, 2010; Lehner, 2010). For plasticity studies, isogenic designs also increase statistical power because line-to-line genetic variability is removed, which is why they are used in many systems (Sommer, 2020).

In this thesis, isogenic *Drosophila* lines let us attribute embryo-to-embryo differences in early positional readouts: *Bcd* gradient steepness *hb* posterior boundary of the anterior domain and *eve* stripe-2 position—to random variation and controlled temperature regimes rather than to genetic differences. Similar approaches in *Drosophila*, from early patterning to wing morphology, have quantified non-genetic noise in spatial gene-expression patterns and fluctuating asymmetry (Chalancon et al., 2012). Perturbing isogenic populations with defined environmental variables (e.g., temperature shifts, nutrient stress) then provides direct tests of robustness and plasticity under changing conditions, linking isogenic design to clear tests of canalization in early patterning.

One limitation of isogenic lines is that homozygosity can reduce allelic buffering (for example, recessive deleterious alleles are no longer masked) and may weaken canalization if the fixed allele set is not optimal for buffering. In contrast, heterozygosity in diverse mass populations can increase buffering and flexibility. Thus, both robustness and plasticity can be altered in homozygous backgrounds.

1.1.5 Developmental Plasticity and Robustness

Development is influenced by intrinsic fluctuations (such as stochastic gene expression) and by extrinsic factors (like temperature or nutrient availability), yet organisms still build complex body plans with high reproducibility (Raj & van Oudenaarden, 2008; Félix & Barkoulas, 2015). Two properties help explain this: robustness—the tendency to keep phenotypes stable despite genetic, environmental, or random perturbations and plasticity the capacity of a single genotype to adjust its phenotype across different environments (Waddington, 1942; West-Eberhard, 2003).

1.1.5.1 Developmental Robustness

Developmental robustness in the context of canalization is the ability to produce consistent phenotypes despite genetic, environmental, and random (stochastic) perturbations (Waddington, 1942; Félix & Barkoulas, 2015). It can be described as the tendency of the developmental pathways to focus inputs resulting from both genetic background and environmental cues towards a stable reproducible phenotypic outcome (Tronick, E., & Hunter, 2016). Several mechanisms support this: genetic redundancy (for example, duplicated genes or shadow enhancers), feedback and cross-regulation within networks, modular organization of gene-regulatory circuits, and molecular chaperones such as Hsp90 that stabilize protein function (Félix & Barkoulas, 2015; Manu et al., 2009; Perry et al., 2010; Rutherford & Lindquist, 1998).

Examples across systems illustrate the point. In *Caenorhabditis elegans*, invariant cell lineages lead to nearly identical cell fates across individuals, showing robustness in both space and time (Sulston et al., 1983). In vertebrates such as zebrafish and frogs, feedback in the BMP pathway buffers dorsal–ventral specification so tissues still differentiate properly even when signaling gradients vary (De Robertis, 2008). In *Drosophila melanogaster*, the maternal Bicoid (Bcd) gradient provides precise anterior–posterior positional information even when Bcd levels differ between embryos; likewise, the posterior boundary of the anterior *hunchback* (*hb*) domain—a direct Bcd target—shows high positional accuracy, pointing to tight regulatory control and effective noise filtering (Gregor et al., 2007; Perry et al., 2012). Together, these findings support the idea that early patterning is a robust process run by networks tuned for precision, even though transcription is globally stochastic (Little et al., 2013). Beyond positional precision, we also quantify the robustness of upstream encoding by estimating the Bicoid gradient length scale, λ , from single-component exponential fits along the A–P axis, $I(L) = A e^{-(L/\lambda)} + B$, and ask whether λ stays stable across genotypes and temperatures.

Classic precision studies usually examine one laboratory background rather than multiple strains, so they capture embryo-to-embryo precision but not cross-strain dispersion. We will extend this by comparing our positional analyses with (i) published measurements of *hb* mRNA concentration variability, where available, and (ii) reports on natural variation in early patterning (for example, line-specific differences in

segmentation gene patterns). This lets us relate concentration-level fluctuations to positional metrics such as the *hb* pbp and discuss how cross-strain dispersion compares to embryo-to-embryo precision (Jiang et al., 2015).

1.1.5.2 Developmental Plasticity

Developmental plasticity is the capacity of a genotype to adjust development when the environment changes, producing different phenotypes across conditions (West-Eberhard, 2003). It includes polyphenism, temperature-dependent sex determination, and phenotypic accommodation, where growth or patterning is rerouted to keep the organism viable under non-optimal conditions (West-Eberhard, 2003; Scheiner, 1993).

In *Drosophila*, temperature changes the pace and timing of early gene-expression waves during segmentation without wiping out the overall pattern. Embryogenesis still progresses in the same order across a broad thermal range (Kuntz & Eisen, 2014). These timing shifts are plastic adjustments that keep the body plan on track (Kuntz & Eisen, 2014). In *Caenorhabditis elegans*, nutritional stress during larval stages can trigger entry into dauer, an alternative trajectory controlled by insulin/IGF-1 and TGF- β pathways—an example of environment-cued switching between life-history strategies (Fielenbach & Antebi, 2008). Among vertebrates, amphibians such as *Rana temporaria* show plastic growth rates and metamorphosis timing in response to temperature and pond-desiccation risk; endocrine control (notably thyroid hormone, with context-dependent roles for corticosterone) mediates these shifts (Denver, 1997; Laurila et al., 2001).

In this study, we measure plasticity at the embryonic level as systematic, environment-dependent shifts in early readouts: the *hb* posterior boundary of the anterior domain and *eve* stripe-2 position. We also quantify the Bicoid gradient's length scale (λ) at nc14 using single-component exponential fits, $I(L)=A e^{(-L/\lambda)}+B$, across constant-temperature regimes. As a late endpoint, we score larval cuticle morphology only in sensitized weak *bcd* mutants to connect early patterning shifts to final outcomes.

1.1.5.3 Evolutionary Implications of Plasticity and Robustness

The balance between robustness and plasticity is central to evolvability the ability of populations to generate heritable, selectable variation. Robust developmental systems can hide or “store” cryptic genetic variation that only becomes visible under stress or mutation (Rutherford & Lindquist, 1998; Masel & Siegal, 2009). By contrast, plastic responses let the same genotype adjust its phenotype when the environment changes, creating new phenotype–environment combinations that selection can act on (Masel & Siegal, 2009).

From an evolutionary perspective, organisms need both robustness and plasticity. Robustness keeps core developmental processes on track under fluctuating conditions, whereas plasticity allows traits to shift when environments change. (Félix & Barkoulas, 2015). *Drosophila* offers a clear test case: by examining how segmentation gene expression changes with temperature, we can ask how much gene regulation is robust to perturbations and how much can adjust plastically (Kuntz & Eisen, 2014; Little et al., 2013; Petkova et al., 2019).

Mechanistically, several features of the network help reconcile precision with the possibility of change. Shadow enhancers and overlapping inputs provide redundancy; cross-regulation among gap genes stabilizes patterns yet allows parameters to drift over evolutionary time (Perry et al., 2010; Manu et al., 2009; Frankel et al., 2010). Hsp90 illustrates the same idea from a molecular angle: when its buffering capacity drops, previously hidden allele effects appear, supplying raw material for selection in new environments (Rutherford & Lindquist, 1998; Queitsch et al., 2002; Rohner et al., 2013).

Data from natural populations support this view. Lines can differ in early *eve* stripe timing or spacing, yet adults still form normal segments because later steps compensate—evidence that robustness can mask selectable diversity (Jiang et al., 2015). In our study, we make this concrete by measuring how two positional readouts *hb* posterior boundary of the anterior domain and *eve* stripe-2 position change across constant temperatures and in sensitized *bicoid* backgrounds. If we weaken a maternal input and then see either more embryo-to-embryo scatter or a shift in the average position, that means the network had been buffering noise; removing that buffer

exposes “cryptic” variation, showing that part of its apparent precision comes from buffering and that hidden variation can become available to selection

1.1.6 *Drosophila melanogaster* as a Model for Studying Embryonic Patterning and Gene Expression Variation and Robustness

Drosophila melanogaster is a well-established model for studying how intrinsic noise and random variation affect development. It has a short generation time, produces many offspring, is easy to handle, and comes with mature genetic and imaging toolkits that now allow very precise measurements. Phenotypic and molecular differences are easy to observe across life stages from classic mutant traits (wings, eyes, color, segmentation) to spatial gene-expression patterns that change over time, including during early embryogenesis (Lawrence, 1992; Nüsslein-Volhard & Wieschaus, 1980; Tolwinski, 2017).

In the embryo, the syncytial blastoderm lets us read out morphogen decoding and segmentation-gene regulation at the level of individual nuclei, which makes early patterning unusually accessible to quantitative analysis. Maternal gradients and the gap/pair-rule networks have been mapped and perturbed in detail, giving a clear framework to measure positional accuracy and variability (Driever & Nüsslein-Volhard, 1988; Struhl et al., 1989; Small et al., 1992; Surkova et al., 2008; Gregor et al., 2007; Dubuis et al., 2013). The system also supports designs that isolate non-genetic contributions: isogenic laboratory lines reduce background genetic noise, and controlled environments such as constant-temperature incubation across a defined range let us test robustness and plasticity in early patterning (Chalancon et al., 2012; Kuntz & Eisen, 2014).

Taken together, tractable genetics, standardized quantitative imaging, and well-characterized spatial and temporal patterning networks make *D. melanogaster* a practical, information-rich system. It lets us measure how intrinsic fluctuations and small environmental differences translate into spatial gene-expression phenotypes and tests how developmental robustness and plasticity are achieved in vivo (Petkova et al., 2019; Félix & Barkoulas, 2015).

1.1.7 The Bicoid Gradient as a Morphogenetic Input

Early anterior–posterior patterning in *Drosophila* starts with maternal morphogens—gradient-forming factors deposited in the egg. Bicoid (Bcd), translated from maternally supplied *bcd* mRNA, is concentrated at the anterior pole and forms a gradient by being produced at the front and lost farther away through diffusion and degradation (Driever & Nüsslein-Volhard, 1988a) (Figure 1A). As one of the first identified morphogens, Bcd provides positional information by controlling target-gene expression in a concentration-dependent way (Struhl et al., 1989). The shape and stability of the Bcd gradient are essential for correct spatial patterning, and its quantitative behavior is now a standard model for gradient-based gene regulation (Gregor et al., 2007; Little et al., 2013).



Figure 1. Early nc14 embryonic patterning readouts. Embryos are oriented anterior left, dorsal up; positions are reported as % embryo length (EL) (0 = anterior → 100 = posterior). Imaging settings were kept identical across comparable samples. Scale bars, 50 μ m. (A) Mid-sagittal optical section showing an anterior-to-posterior protein gradient with nuclear localization. Immunostaining used a rabbit primary antibody detected with Alexa Fluor 488–conjugated anti-rabbit IgG. Images were acquired on a Leica laser-scanning confocal microscope. (B) Whole-mount in situ hybridization for *hb* at early nc14. A digoxigenin-labeled *hb* antisense RNA probe was detected with an alkaline phosphatase–conjugated anti-DIG antibody and visualized with a chromogenic AP substrate (e.g., NBT/BCIP). The posterior boundary of the anterior *hb* domain (*hb* pbp) is indicated. Images were taken on a Zeiss microscope under brightfield illumination. (C) Whole-mount in situ hybridization for *eve* at early nc14. A digoxigenin-labeled *eve* antisense RNA probe was detected with an alkaline phosphatase–conjugated anti-DIG antibody and visualized with NBT/BCIP. The position of *eve* stripe 2 is indicated. Images were taken on a Zeiss microscope under brightfield illumination.

Quantitative measurements show that the Bcd profile is reproducible across embryos and carries enough information to specify position with high precision when the early gene network decodes it (Gregor et al., 2007; Dubuis et al., 2013; Tkačik & Gregor, 2021). These studies also define physical and noise-related limits on how gradients can be measured and interpreted in vivo, supporting the idea that Bcd acts as a probabilistic input that is nevertheless read out with striking accuracy.

A primary downstream target is *hb*. Bcd activates *hb* through a proximal enhancer that turns a graded input into a sharp anterior domain; cooperative activation together with opposing repression sets a steep posterior boundary that is highly consistent between embryos (Small et al., 1992; Perry et al., 2012; Petkova et al., 2019). Taking together, these features suggest that early patterning is robust at the level of morphogen decoding, even though transcription is broadly stochastic (Little et al., 2013).

In this work, we directly measure the Bcd gradient as an upstream readout. We fit anterior–posterior intensity profiles with a single-component exponential, $I(L) = A e^{(-L/\lambda)} + B$, and use the length scale (λ) as a compact descriptor of gradient shape. We analyze λ alongside positional outputs *hb* posterior boundary of the anterior domain *eve* stripe-2 position across temperatures and genetic backgrounds, including sensitized weak *bcd* mutants, to link upstream encoding to downstream patterning precision (Porcher & Dostatni, 2010; Gregor et al., 2007; Petkova et al., 2019).

1.1.8 *hunchback* PBP as a Measure of Positional Precision

One of Bicoid’s primary zygotic targets is *hb*, which forms a sharply defined anterior expression domain in the blastoderm shown in Figure 1B. Pbp of this domain marks the spatial limit of Bcd-dependent activation and is highly reproducible across embryos, even when the Bcd gradient’s shape or absolute level varies. This makes pbp a robust readout of morphogen interpretation (Driever & Nüsslein-Volhard, 1988b; Gregor et al., 2007).

Measurements of *hb* pbp are widely used to assess positional precision. The consistency of this boundary across embryos suggests that the early regulatory network buffers intrinsic transcriptional noise and external perturbations (e.g., temperature changes), keeping decoding of the maternal input accurate (Gregor et al., 2007; Perry et al., 2012; Petkova et al., 2019).

In this study, we define *hb* pbp at nuclear cycle 14 (nc14) and report it as percent embryo length (%EL; anterior = 0, posterior = 100). We use standardized staging and orientation and extract the boundary from intensity profiles using inflection-point or half-max criteria to reduce operator bias. Normalizing to %EL accounts for egg-size differences and lets us compare positions across temperatures and genetic backgrounds under constant-incubation conditions.

Classic precision studies mostly examined a single laboratory strain, so they measured embryo-to-embryo reproducibility but not cross-strain dispersion (Gregor et al., 2007; Perry et al., 2012). We will relate our positional analysis to published measurements of *hb* mRNA concentration variability, where available, and to natural-variation studies of early segmentation patterns, to connect concentration-level fluctuations with positional readouts such as *hb pbp*.

Within this framework, *hb pbp* serves as an upstream positional marker that samples decoding close to the maternal input. It complements downstream pair-rule readouts and allows tests of robustness and plasticity under constant-temperature regimes and in sensitized *bcd* backgrounds (Gregor et al., 2007; Petkova et al., 2019).

1.1.9 *eve* Stripe Positioning and Segmentation Accuracy

Further downstream in the gene regulatory cascade is *eve*, a pair-rule gene expressed as seven transverse stripes along the A–P axis shown in Figure 1C. Each stripe is set by the combined action of maternal and gap genes acting through multiple enhancers; for stripe 2, activation by Bicoid and *hunchback* is opposed by repression from *giant* and *Krüppel* (Frasch & Levine, 1987; Small et al., 1991; Small et al., 1992). Stripe 2 has been studied heavily because its position is precise and low-variance across embryos and because it is regulated directly by Bcd via enhancer logic (Frasch & Levine, 1987; Small et al., 1992; Surkova et al., 2008). Live imaging and reporter assays show that enhancer-specific transcriptional bursting and short-range repression sharpen and stabilize stripe 2, supporting high embryo-to-embryo precision despite globally stochastic transcription (Bothma et al., 2014; Little et al., 2013). This enhancer logic offers a concrete way the network filters intrinsic and small extrinsic fluctuations while keeping positional accuracy. Studies in natural populations found line-specific differences in *eve* stripe timing and spacing traced to regulatory variation (e.g., a deletion affecting *knirps*), yet adult segmentation remained normal due to downstream buffering—evidence that robustness can mask selectable variation (Jiang et al., 2015).

Eve stripe formation and spacing are essential for subdividing the embryo into proper parasegments, showing how graded inputs are turned into discrete, robust spatial outputs (Small et al., 1992; Surkova et al., 2008). Variability in stripe positions can

therefore signal weaker buffering of gene-expression noise or altered decoding of upstream inputs (Dubuis et al., 2013).

We use *eve* stripe-2 position as a positional marker that complements *hb* pbp. Stripe center (peak) is measured as % embryo length at nc14 with a fixed pipeline (peak of a smoothed intensity profile). Normalizing to %EL allows comparison across temperatures and genotypes under constant-incubation conditions. Because *eve* stripe 2 is more downstream in the gene regulatory cascade than *hb*, it can reveal whether downstream integration and cross-regulation keep positions precise when upstream inputs are weakened (e.g., sensitized *bcd* backgrounds) or when temperature is changed within constant-incubation regimes. A case where *hb* pbp stays stable but stripe-2 shifts would indicate where robustness is spent in the network (Dubuis et al., 2013).

1.2 Summary and Relevance to Robustness Studies

Together, the Bicoid gradient, *hunchback* (pbp), and *eve* stripe patterns form a hierarchical set of molecular markers that let us analyze gene-expression precision, noise tolerance, and patterning robustness in early development. Work in *Drosophila melanogaster* has established core principles of morphogen-based patterning and offers a clear framework for how organisms keep development on track under changing internal and external conditions (Driever & Nüsslein-Volhard, 1988a,b; Struhl et al., 1989; Small et al., 1992; Gregor et al., 2007; Little et al., 2013).

Within this hierarchy, *hb* pbp samples morphogen decoding close to the maternal input, while *eve* stripe 2 samples downstream integration by the gap and pair-rule networks. Measuring both readouts in the same embryos helps localize where variation enters or is buffered—either during initial decoding of Bcd (including gradient shape summarized by λ) or during later enhancer logic and cross-regulation (Small et al., 1992; Perry et al., 2012; Dubuis et al., 2013; Petkova et al., 2019).

Under constant-temperature incubation and standardized staging, we interpret embryo-to-embryo variance and temperature-dependent shifts in these positions as follows: robustness corresponds to small variance and minimal shifts; plasticity corresponds to systematic, bounded shifts that preserve the overall pattern. Comparing isogenic wild-type backgrounds with sensitized *bcd* lines tests whether weakening the

maternal input makes these positional metrics more sensitive, clarifying how buffering is distributed across the network (Porcher & Dostatni, 2010; Gregor et al., 2007; Perry et al., 2012). As a late morphological endpoint, we also score larval cuticle phenotypes only in sensitized weak *bcd* mutants, linking early readouts to final outcomes without relying on FA metrics.

Finally, studies of natural variation show that line-specific differences in early stripe organization can be compensated downstream to yield normal segmentation, indicating that robust development can mask hidden, potentially selectable diversity. We will compare our positional ranges with these reports in the Discussion (Jiang et al., 2015).

1.3 Experimental Strategy

To study how environment and developmental robustness interact, we will use a controlled experimental design in *Drosophila melanogaster*. Our goal is to quantify how variable early embryonic patterning is under different constant temperatures, and to compare this variation between wild-type lines and genetically sensitized backgrounds.

1.3.1 Model System: Isogenic Laboratory Strains

We used several well-established isogenic wild-type *D. melanogaster* strains Canton S, Oregon R, and Berlin K plus a body and eye color mutant background, *yw*. Because these lines are genetically homogeneous, background genetic noise is minimized. This lets us attribute embryo-to-embryo differences more precisely to environmental inputs and random developmental variation. Using these common “wild-type” genotypes also lets us compare canalization and plasticity across standard laboratory backgrounds (David et al., 2004; Pérez-Pereira et al., 2021).

Including multiple isogenic strains serves two purposes: (i) it establishes a baseline for within-line variance (developmental noise plus residual microenvironmental effects), and (ii) it provides a controlled way to assess line-specific robustness and plasticity by comparing positional readouts across constant temperatures. We included *yw*, a widely used background for transgenesis and marker screening, to sample a distinct, standardized genetic context alongside canonical wild-type stocks.

All stocks and embryos were kept in constant-temperature incubators under identical light, food, and density conditions to avoid handling artifacts. We used synchronized egg collections and matched maternal ages to limit cohort effects. These steps reduce extrinsic variability so that differences between lines more directly reflect buffering capacity or decoding thresholds.

In the analysis, we treat “line” as a factor to separate within-line variance (noise) from between-line shifts (background effects). We then compare *hb* pbps and *eve* stripe-2 positions between different lines and across temperatures to test whether some backgrounds are more canalized and robust than others (David et al., 2004; Pérez-Pereira et al., 2021).

1.3.2 Environmental Perturbation: Temperature Stress

We used temperature as a controlled environmental perturbation. Embryos from each isogenic line developed at three constant temperatures:

- 22 °C – mildly cool, slows development
- 25 °C – standard lab condition (control)
- 29 °C – mild heat stress, near the common upper limit for *Drosophila*

These conditions can change gene-expression dynamics, morphogen gradients, and basic cellular processes, while keeping embryos viable and patterned however survival rates and patterning differences were altered depending on temperature. (Cheung, Miles, Kreitman, & Ma, 2015)

We kept both adults and embryos at their assigned temperature in incubators. Egg collections were synchronized and handled the same way at all temperatures. This makes any shift in positional readouts reflect steady-state temperature, not handling.

In ectotherms, temperature changes reaction rates and timing. In *Drosophila* embryos, the timing of events scales uniformly across a broad range, and the order of events stays the same (Kuntz & Eisen, 2014). This lets us test whether early markers *hb* posterior boundary and *eve* stripe 2 are robust (means/variances unchanged) or show plastic but orderly shifts.

For each line and temperature, we measure embryo-to-embryo variance (precision) and mean position (sensitivity/robustness) for the two readouts, then compare the change in the variance and shift in the mean position to assess plasticity across temperatures and robustness against it across 22, 25 and 29°C. Small variance and minimal mean shifts = canalization; systematic but bounded shifts = plasticity (DeWitt et al., 1998).

1.3.3 Genetically Sensitized Background: Weak *bcd* Mutants

In addition to isogenic wild-type or near-wild-type lines (*yw*), we included weak *bicoid* (*bcd*) mutants with reduced maternal Bcd input. These stocks have been inbred for >5 years, giving near-identical genetic backgrounds. They show increased variability in segmentation and cuticle phenotypes and reduced survival, creating a sensitized context to test how maternal input affects patterning robustness—especially under temperature stress (Önal et al., 2021).

The CH(nt-H1-h2-RH) and CH(NT-h1-h2-RH) lines are chimeric homeodomain carrying Bicoid rescue lines created by replacing the parts of the ancestral Zerknullt (AncZB) sequence with corresponding ancestral Bicoid (AncBcd) substitutions. CH(NT-h1-h2-RH) uses the recognition helix (RH) and the N-terminal arm (NT) of AncBcd, while CH(nt-H1-h2-RH) uses AncBcd RH and helix 1 (H1). AncZB is the reconstructed ancestor of Zerknullt lacking Bicoid-like activity, AncBcd is the reconstructed ancestor of Bicoid.

Specifically, we use the CH(NT-h1-h2-RH) and CH(nt-H1-h2-RH) homeodomain-engineered rescue constructs from Önal et al. (2021). CH is chimeric homeodomain. We also include an AncBcd homeodomain rescue line (NT-H1-H2-RH) and a wild-type *bcd* rescue line as the control. The engineered constructs (all except the wild-type rescue) weaken or alter maternal Bcd input, including changes to DNA-recognition properties, and have been shown to increase variability in early patterning and reduce survival relative to fully rescuing controls (Önal et al., 2021).

When maternal Bcd input is weakened, the anterior *hb* domain shifts anteriorly, and stripe positioning becomes less permissive. In Önal et al. (2021), non-rescuing constructs placed the *hb* posterior boundary at ~18–33% embryo length (EL) (0 = anterior, 100 = posterior), whereas fully rescuing constructs positioned it at or

posterior to ~32% EL. They argued that ~35% EL is a minimal anterior-space requirement for robust downstream patterning and viability. By contrast, wild-type embryos and the AncBcd control formed *hb* pbp near ~46% EL (Önal et al., 2021; Chen et al., 2012).

We assay CH(NT-h1-h2-RH), CH(nt-H1-h2-RH), AncBcd, and the wild-type *bcd* rescue across constant 22/25/29 °C cohorts to test whether reduced maternal input amplifies embryo-to-embryo variance or shifts means in early positional readouts—*hb* posterior boundary and *eve* stripe-2—and, where measured, the Bcd gradient length scale (λ) from single-component exponential fits. As a late endpoint, we perform cuticle-based morphological classification only in sensitized weak *bcd* mutants to link early readouts to terminal phenotypes (Önal et al., 2021).

1.4 Extension to Natural Genetic Variation

How robustness and plasticity look in isogenic lines (near-zero genetic variation) is useful, but we also need to compare them to genetically diverse (“mass”) populations. Genetic variation is the raw material for evolution: different alleles can produce different responses to the same environment (Hendry, 2011). In outbred populations, heterozygosity often masks deleterious recessive and adds redundancy in developmental and stress-response pathways; in isogenic lines, homozygosity removes this buffering, which can reduce robustness and narrow plastic responses (Chen & Wagner, 2012).

Plasticity often reflects genotype-by-environment (G×E) interactions alleles differ in how they respond to the same change (Scheiner, 1993; Via & Lande, 1985). Canalized traits vary little across conditions, but canalization can weaken in homozygous lines if the fixed alleles are not optimal or if helpful epistasis is lost (Félix & Barkoulas, 2015).

Isogenic/isofemale lines minimize background genetic variance to isolate environmental or stochastic effects; outbred or reference panels capture segregating alleles and G×E for studying plasticity and robustness in a natural context (David et al., 2004; Mackay et al., 2012). Long-term inbreeding can purge some inbreeding load, but residual fixed alleles still shape baseline robustness and plasticity (Pérez-Pereira et al., 2021). Empirically, lines can differ in early *eve* stripe timing/spacing while

adults remain normal evidence that downstream buffering can mask selectable variation (Jiang et al., 2015).

Here we created a mass population by pooling five isofemale lines from wild-caught females in the Adana–Saimbeyli region (Turkey). Two founders were also analyzed individually under the same husbandry and constant-temperature conditions.

We use “line” for isogenic strains and “population” for outbred/mass-bred stocks. SB-1 and SB-2 are Saimbeyli-derived isogenic lines (recently isogenized; hence “Saimbeyli isogenic lines”). SB M is a Saimbeyli outbred, mass-bred population (hence “Saimbeyli population”). Abbreviations are Berlin K (BK), Canston S (CS), Oregon R (ORE), *yellow-white* (YW), Saimbeyli 1 (SB-1), Saimbeyli 2 (SB-2), Saimbeyli Mass (SB-M).

We analyzed the variance and shifts in the mean values of *hb* pbp, *eve* stripe-2 position, and where measured the Bicoid gradient length scale λ across 22, 25 and 29 °C. We test two predictions:

1. If heterozygosity buffers variation, the mass population should show equal or lower embryo-to-embryo variance (canalization) for robust developmental markers at a given temperature.
2. If allelic diversity broadens responses, the mass population should show smaller shifts across temperatures (robustness against temperature) by increasing plasticity while maintaining correct patterning.

1.5 Readouts of Developmental Robustness and Variability

In this study, we compared embryonic phenotypes across several isogenic lines and a genetically diverse mass population (limited variation; see Discussion). We focused on early spatial readouts and added a late endpoint only where it was informative.

Embryonic phenotypes were assessed through:

1. Bicoid gradient decay constant (λ) at nc14.
We fit anterior–posterior intensity profiles with a single-component exponential to summarize gradient shape: $I(L)=A e^{(-L/\lambda)}+B$.

2. Positional readouts of downstream genes at nc14.

- *hb pbp*, reported as % embryo length (anterior = 0 → posterior = 100), using a fixed inflection/half-max pipeline (e.g., Driever & Nüsslein-Volhard, 1988b; Gregor et al., 2007; Perry et al., 2012).
- *eve stripe-2* position, reported as %EL via a peak/centroid-calling pipeline (e.g., Frasch & Levine, 1987; Small et al., 1992; Surkova et al., 2008).

3. Cuticle-prep morphology (late endpoint).

Scored only in sensitized weak *bcd* mutants to link early patterning changes to terminal segmentation/structure.

4. Precision and variability estimates.

For each genotype and 22/25/29°C cohort, we quantified embryo-to-embryo variance (precision) and mean shifts (sensitivity/robustness) for *hb pbp*, and *eve stripe-2*. This enables reaction-norm comparisons across temperatures and genetic backgrounds (e.g., Gregor et al., 2007; Petkova et al., 2019).

Details of image acquisition, staging, boundary/peak extraction, %EL normalization, exponential fitting, and statistical analyses are provided in Methods.

1.6 Research Questions

This thesis asks how genetic background, temperature, and biological noise shape early embryonic patterning and survival. We measure one upstream readout the Bicoid gradient length scale (λ) from single-component exponential fits at nc14 and two positional readouts at nc14—*hb* posterior boundary of the anterior domain and *eve stripe-2* position—reported as % embryo length (0 = anterior → 100 = posterior) at 22, 25, and 29 °C. As a late endpoint, we score survival and larval cuticle morphology only in sensitized weak *bcd* mutants to connect early patterning to final outcomes

1. How variable are early readouts within genetically identical lines, and how does maternal-input sensitization (*bcd* variants) change this?

We compare embryo-to-embryo variance and means for λ , *hb pbp*, and *eve stripe-2* position in isogenic wild-type backgrounds (Canton S, Oregon R,

Berlin K, *yw*) versus sensitized *bcd* lines (RH–NT = CH(NT-h1-h2-RH), RH–H1 = CH(nt-H1-h2-RH), AncBcd, and a wild-type *bcd* rescue control). This tests whether weakening Bcd increases variance and/or shifts means, and whether *hb pbp* and *eve stripe-2* change in the same way or differently.

2. Does constant-temperature stress reveal hidden (cryptic) phenotypic variation? With identical handling (no pulses or transfers), we ask whether moving from 25 °C to 22 °C or 29 °C causes systematic, environment-dependent shifts (plasticity) or larger variance (lower precision) in λ , *hb pbp*, and *eve stripe-2*, and whether sensitivity/robustness depends on genetic background. This identifies which features are robust and which are temperature sensitive at different genetic backgrounds.

3. Are genetically diverse populations more robust to temperature changes than isogenic lines that appear normal under standard conditions? Using a mass population made by pooling five Adana–Saimbeyli isofemale lines (with two founders also analyzed individually for molecular comparisons), we test whether heterozygosity (i) buffers embryo-to-embryo variation at a given temperature (canalization) and/or (ii) produces more robust patterning across temperatures.

4. Does the moderately genetically diverse out-bred population (Saimbeyli Mass) show higher or lower within-group variation compared to isogenic lines?

To answer this, we analyzed the variation observed for *hb pbp* and *eve* second stripe positions, between Saimbeyli Mass and widely used isogenic strains as well as two founder lines Saimbeyli 1 and Saimbeyli 2.

2. MATERIALS

2.1 Materials for Fly Maintenance, Buffers and Glassware

Table 1: List of materials, buffers, and glassware used in fly maintenance, embryo fixation, cuticle preparation, *in situ* hybridization and immunostaining.

Product Name	Manufacturer	Catalog Number
Agar Agar	Benosen	E406
Corn Flour	Yazar	N/A
Granulated Sugar	Torku	N/A
Propionic Acid	Sigma Aldrich	800605-2500
Methyl 4-hydroxybenzoate	Carlo-Erba	354007
Hoyer's Medium	Home-Made	N/A
Narrow <i>Drosophila</i> Vials	Biologix	51-0500
60 mm x 15 mm Not TC-treated Bacteriological Petri Dish	Falcon	351007
Heptane	Sigma Aldrich	H2198-2.5L
NBT/BCIP Stock Solution	Roche	11681451001
Proteinase K	Genaxxon	M3036.0100
20 mL Glass Scintillation Vials	LLG-Wheaton	DWK986541
10x PBS	Home-made	N/A
20x SSC	Home-made	N/A
Anti-Fluorescein-AP, Fab Fragments	Roche	11426338910
Flypad, CO2 Anesthetizing Apparatus	FlyStuff	59-114
37% Formaldehyde Solution	Sigma Aldrich	8187081000
Lactic Acid	Merck	100366

Yeast	Pakmaya	N/A
Salmon Sperm DNA	Invitrogen	15632011
Heparin	Millipore	375095
Proteinase K	Genaxxon	M3036.0100

2.2 Materials for Food Fly and Embryo Maintenance

Table 2: Ingredients and recipe for making the fly food and apple juice agar used in all experiments.

Food	Ingredient	Amount
Fly Food (Classic Cornmeal Recipe)	Corn Flour	28.60 g/L
	Agar Agar	9.60 g/L
	Yeast	15.00 g/L
	Granulated Sugar	42.85 g/L
	Propionic Acid	3.80 mL/L
	10% Nipagin (in Ethanol)	7.15 mL/L
Apple Juice Agar	Apple Juice	70.00 mL/L
	Agar	25 g/L

2.3 Buffers for *in situ* Hybridization and Immunostaining

Table 3: List and the contents of buffers and reagents used in in situ hybridization and immunostaining.

Buffer	Contents
10X PBS	100 mM Na ₂ HPO ₄ 18 mM KH ₂ PO ₄ 1.37 M NaCl 27 mM KCl
PBTw	10 mM Na ₂ HPO ₄ 1.8 mM KH ₂ PO ₄ 13.7 mM NaCl 2.7 mM KCl 0.5% Tween-20

Hybridization Solution	150 mM NaCl 15 mM Sodium Citrate pH 8.0 50% Formamide 0.1 mg/mL Salmon Sperm DNA 0.05 mg/mL Heparin 0.5% Tween-20
<i>in situ</i> Buffer	13 mM Na ₂ HPO ₄ 23.4 mM KH ₂ PO ₄ 17.81 mM NaCl 3.51 mM KCl 67 mM EDTA pH 8.0
20x SSC	3 M NaCl 300 mM Sodium Citrate pH 8.0
AP Staining Solution	0.5 M NaCl 50 mM MgCl ₂ 100 mM Tris-HCl pH 9.5 0.1% Tween-20

3. METHODS

3.1 *Drosophila melanogaster* Fly Stock and Maintenance

Drosophila melanogaster stocks were maintained in 20 mm diameter narrow *Drosophila* vials containing standard fly food, Room is kept at 18°C with controlled humidity of 50-55% using a humidifier. Stocks were flipped every two-week depending on the condition of the food.

For the experiments, to increase the number of the flies, small vials were flipped to 250 ml bottles containing fly food, bottles were kept at 22°C.

Fly food is prepared mixing the dry ingredients with water, boiling the mixture to activate the agar. After letting it cool down below 70° C Propionic acid and 10% Nipagin were added to the solution. After 24 h of waiting for them to dry, they are plugged and stored at +4° C until further use.

3.2 Embryo Collection

Embryo collection cages are assembled by using sample collection containers. Bottoms of the containers were removed, and 106 mm stainless steel mesh is melted and covered the bottom of the container to allow air flow to the cage. Apple juice agars are prepared and used for an egg laying surface for the flies; after mixing 350 mL of dH₂O and 10.5 g of agar and boiling twice to activate the agar, 70 mL of apple juice were added to the mixture and mixed until its clear. Mixture is incubated at 60°C incubator until foam disappears and its poured into 60 mm cell culture dishes and let dry, they were placed in +4°C fridge until needed. They were used as a lid for the sample collection containers.

Flies were transferred into the collection cages one or two days prior to embryo collection to allow them to acclimate to the new environment. Cages were placed in incubators under 12-hour light/dark cycle. Agar plates were changed at specific intervals to collect certain stage embryos.

3.3 Embryo Fixation

Embryos were collected 0-3 hours after egg laying, after collection, the plates are incubated until embryos reach nuclear cycle 13-14. Due to temperature altering developmental time, incubation durations varied:

- 29°C: 0–2.5 hours

- 25°C: 0–2.75 hours
- 22°C: 0–3 hours

Embryos were dechorionated by treating the plates with 4% bleach for 2 minutes. Mixture was then poured through a metal mesh to collect the embryos; plates were rinsed with dH₂O and poured again through the mesh to collect the remaining embryos. The mesh was placed into a 20 mL scintillation vial that contains 8 mL of Fixation buffer (composed of 3 mL in situ buffer, 4 mL heptane, and 1 mL 37% formaldehyde). Vials were shaken vigorously for 25 minutes. After fixation, lower aqueous phase was removed, and 8 mL of methanol was added to the vial. After 1 minute of vigorous shaking, upper and middle phases were discarded. Then 4 mL of methanol is added for washing, Embryos left in the vial are transferred to a 1.5 mL Eppendorf's tube and washed twice with methanol and twice with ethanol, stored in -20°C until further use.

3.4 Cuticle Preparation

Larvae were obtained by collecting embryos for 5 hours and incubating at different durations depending on temperature: 16 hours at 29°C, 22 hours at 25°C, and 24 hours at 22°C. Larvae were dechorionated using 4% bleach for 2 minutes by gently shaking, mixture was passed through a metal mesh, and the mesh was rinsed with distilled water to remove residual bleach and yeast paste. Larvae were transferred to a 1.5 mL eppendorf's tube containing 1:1 mixture of heptane and methanol. Tubes were shaken vigorously for 30 seconds to devitellinize the larvae, after settling, top and middle phases were removed and they are washed twice with methanol, after the last wash larvae were rehydrated with 0.1% Triton X-100. For mounting larvae were transferred to a glass slide, 1:1 mixture of Hoyer's mounting medium and lactic acid was applied to the samples, a toothpick was used to separate them, a coverslip was placed over the samples for flattening. Slides were incubated at 60°C for 24 hours to allow the mounting medium to clear the insides, coins were placed on top of them Imaging was performed using a Zeiss Axio Imager.A1m upright microscope. For darkfield imaging, a 1 TL coin was manually placed over the light source. Images were acquired using a 10× objective lens.

3.5 Survival Analysis

Embryos were collected over ~5 hours and examined under a stereo microscope. Small agar pieces containing embryos were cut from the collection plates and transferred to fresh ones. Each plate was placed inside an empty tip box lined with moist paper tissue to maintain humidity and prevent desiccation. The tip boxes were then transferred to incubators set at 22°C, 25°C, or 29°C. Embryos were incubated until hatching, which occurred after ~14 h at 29°C, ~18 h at 25°C, and ~22 h at 22°C. Hatched larvae were counted under a stereo microscope and transferred to vials, with the number of larvae per vial recorded. Vials containing agar pieces were returned to the incubators, and pupae were monitored for 2 weeks until no additional larvae were observed. Survival rate was calculated by dividing the total number of pupae by the number of larvae originally transferred into each vial.

3.6 *In situ* Hybridization of *Drosophila* Embryos Using RNA Probes

The fixed embryos are washed twice in ethanol and washed twice in methanol. After that they were incubated by rocking on the shaker for 5 minutes in 1:1 methanol: PBTw + 5% formaldehyde solution. Then they are incubated while rocking for 25 minutes in PBTw + 5 % formaldehyde solution for 25 minutes. After that they are washed 3 times by rocking with PBTw. Then, they are incubated while rocking for 8 minutes with PBTw + 4µg/mL Proteinase K solution and washed again 3 times with PBTw its incubated by rocking for 25 minutes with PBTw + 5 % formaldehyde solution for 25 minutes again. Then, they are washed for 5 minutes 4 times with PBTw. Then, they are placed in 1:1 mixture of Hybridization solution:PBTw and washed while rocking for 5 minutes. Then they are incubated for 1-2 hours in 55° C waterbath inside hybridization solution. Then the probes are prepared by adding 0.3 µl of the probe of interest to 50 µl hyb solution, the mixture is heated at 80°C heatblock for 5 minutes and immediately on ice. Then, 50 µl of the probe is added to the tubes and they are placed in 55° C waterbath for at least 16 h overnight. After the incubation is completed, the probes are removed, and embryos are washed and incubated with hyb solution for 30 minutes twice. Then, they are washed while rocking for 5 minutes in 50/50 Hyb solution/PBTw. They are washed while rocking for 10 minutes with PBTw 4 times. After the final wash 400 ul of PBTw + anti-digoxigenin antibody is added to the tubes and incubated while rocking for 1-2 hours. After that the antibody is removed, and they are washed while rocking for 10 minutes with PBTw 4 times. After the last wash

they are washed with AP Staining Buffer 2 times while rocking for 5 minutes. The staining solution was prepared by adding 40 μ L NBT/BCIP solution into 1 mL AP Staining Buffer. Then, 250 μ L of staining solutions are added to the tubes, and they are let to stain in boxes to keep them dark. Embryos are watched until desired expression pattern appears, they are checked every 3 minutes. After that PBTw is immediately added to stop the staining reaction. They are washed with PBTw 2 times for 5 minutes. Then, they are washed with PBTw/Ethanol mixture for 5 minutes. After that, they were washed in ethanol for 25 minutes while rocking. Then, they are washed with PBTw/Ethanol mixture for 5 minutes. Then, they are washed with PBTw for 5 minutes 3 times. After the last wash they can be kept at +4 C fridge until they are mounted.

3.7 Immunostaining of *Drosophila* Embryos with Bcd Antibodies and DAPI

The fixed embryos are washed with ethanol twice and with methanol twice, after that they were washed with methanol while rocking for 5 minutes, and with 1:1 methanol PBTw:methanol solution. Then they were washed with PBTw while rocking for 10 minutes 4 times. Then, they are incubated in primary antibody. Primary antibody is 20640 Rabbit Bcd. Antibody is diluted 1:250 before use and BSA is added (Final BSA concentration is 4% in the mixture). Samples are incubated at +4°C while shaking overnight. The next day they were washed with PBTw by rocking for 10 minutes 4 times. Then, they are incubated with secondary antibody diluted in PBTw. Secondary antibody is Alexa Fluor 488 Goat Anti-rabbit diluted 1:400 in PBTw. They are incubated in secondary antibody at RT at least 1 hour in the dark, they should not be exposed to light. Then, they were washed with PBTw while rocking for 10 minutes each four times. Then they are mounted using Aqua Pol. Slides are kept at -20° C till they are visualized. Samples are visualized using Leica SP8 Confocal Microscope with 20x Dry objective using LASX software.

3.8 Hunchback pbp Analysis Protocol

First step is to open the .tif images using ImageJ, ImageJ>Open>Select the file. Use the Image>Transform tools to correct the orientation of the embryo, Dorsal up Anterior to the left. Draw a rectangle around the embryo and use Rotate function to correct the orientation. Then draw a rectangle along the embryo length from anterior to posterior, then carry it to the dorsal side (UP). It's important to draw the rectangle on full embryo length. Press “K” for obtaining the Data and the Graph. Click Data>Copy first Data

Set. Go to an empty excel and paste the data to columns A and B. Column A represents Embryo length and B represents the intensity. Label all the excel files in uniform, for example *HB_1* (This will be important in later steps). Open Spyder, before starting make sure the code files (.py) and the excel files are on the same file. First “Data_Point_Equalizer” code will be used, this code will reduce all data points for all embryos to 600.

This code will create “selected_files” according to how many files you insert. Second code is “Automize”. This code will automate the reversing of the intensity process and will generate files called “processed_selected_file”

Third code is called “pbp_Finder” this code will take the “processed_selected_file” and find the pbps of each embryo and will give you an excel called pbp_Analysis. After that you can use pbp values for further analysis. (Average, t.test etc.)

3.9 Eve Analysis Protocol

Upon loading into Fiji (ImageJ) software, the fluorescence pictures were rotated such that anterior was positioned to the left and dorsal side oriented upward. A rectangular region of interest (ROI) was created spanning the entire embryo and then manually adjusted to include the upper ~5% dorsal region while ensuring all 7 *eve* expression stripes remained within the ROI. By using the “Plot Profile” function under the *Analyze* menu, an intensity profile along the anterior-posterior (AP) axis was generated. The raw intensity dataset was imported into a Microsoft Excel file with the first column denoting spatial position (length) and the second column containing corresponding raw intensity values, saved with the correct names. These datasets were saved using standardized naming conventions within a folder structure compatible with downstream Python analysis scripts.

In the Python script, spatial position was normalized by dividing each value by the maximum length for that embryo and subsequently scaled to Percent Embryo Length (EL) by multiplying by 100. The raw intensity values were normalized by dividing the intensity by the maximum intensity observed in each embryo. Since the Fiji software considers the brightest (white) area as higher intensity, the normalized intensity was inverted by subtracting from 1 to obtain actual *eve* stripe signals (dark regions). To remove noise and preserve the structural features, including shapes and peak tendency,

a Savitzky-Golay filter (implemented using the Python function `Savgol_filter()` from `scipy.signal` library) was applied with a sliding window length of 11 and the local polynomial degree of 2. The first derivative (change rate) of the smoothed intensity profile was computed using `numpy.diff()` to capture the region of increasing or decreasing signal, facilitating the identification of local maxima and minima based on sign changes in the derivative. The sign change points from positive to negative were flagged and stored. To robustly detect strong and well-separated peaks, the `find_peaks()` function from `scipy.signal` library was employed with a minimum inter-peak distance of 10 and a minimum prominence threshold of 0.3. Then a filter was applied, retaining peaks located only between 25% EL to 85% EL, to exclude artifacts and background signals at embryo edges. The peaks were cross-validated with derivative-based peak detection using a nearest neighbor matching approach. Embryos (whole dataset) in which exactly seven peaks could not be identified were excluded from further analyses since without 7 peaks the gene expression is meaningless. To facilitate cross-embryo averaging, `numpy.linspace()` function was used to interpolate each embryo's intensity profile to 600 evenly spaced points along the AP axis. These interpolated datasets were averaged across biological replicates within each temperature condition group. While applying the closest match function, the stripe peak positions were stored in a separate Excel file for each embryo / replicate. The average and Standard Deviation of each stripe position were calculated. To determine the statistical significance of the spatial shift in stripe position across temperature conditions, a two-sample t-test was performed. Additionally, an F-test was applied to assess whether the variance in stripe position differs significantly across different temperature conditions.

3.10 Bicoid Analysis Protocol

3.10.1 Image Processing and Intensity Extraction

The raw two-channel confocal images (DAPI and Bcd) were initially processed in Fiji (ImageJ) software and rotated to align the dorsal side upward. Then, a MATLAB script was written to process these images and obtain raw intensity values. Subsequent image analysis was conducted in MATLAB using a live script partially adapted from the protocol of Xu et al., 2023. Both DAPI and Bcd channels were imported and converted to grayscale. Contrast enhancement was performed using the `adapthisteq()` function.

To isolate nuclei and remove the background DAPI signal, Otsu's model (implemented using `graythresh()` function) was applied, generating a binary nuclear mask in which pixels exceeding the threshold were assigned a value of 1, and the remainder 0. Embryos were then aligned along the major axis by identifying the angle between the x-axis and the longest axis within the mask. Following axis alignment, only the region containing a full embryo was cropped. The DAPI nuclear mask was applied to the *bcd* channel, and the edge of the dorsal embryo boundary was identified using the `edge()` function. Then, the outer embryo contour was smoothed using the robust locally weighted regression method ("rloess" method via the `smooth()` function) to obtain a continuous midline curve. Along the midline, the raw intensity was measured using 50 equal-distance bins, and the EL of 50 datapoints was scaled 0% to 100% EL. Sampling points were then defined at regular 50 equally spaced intervals along the midline, with a fixed perpendicular offset of 10 pixels. This approach enabled a reliable, geometry-aware sampling, resulting one-dimensional profile spanning from anterior to posterior. The spatial position data points were rescaled to range from 0% to 100% EL.

3.10.2 Gradient Modeling and Decay Constant Estimation:

The extracted Bcd intensity profiles were imported into RStudio using the `readxl` package. Each profile was modeled using a single-component exponential decay function via the `nlsLM` function from `minpack.lm` library.

$$I(L) = A * e^{-\frac{L}{\lambda}} + B$$

In the equation, $I(L)$ represent the Bcd fluorescence intensity at a given EL position L , A denotes the maximum intensity obtained in the anterior-most region, λ is the characteristic decay constant, and B represents the baseline offset due to posterior background signal. Since Bcd intensity never approaches 0 in the posterior part, B accounts for that background noise. The fitted decay constant λ was extracted from each embryo and used to characterize gradient steepness.

4. RESULTS

Phenotypic variation is often attributed to underlying genetic differences and environmental factors. However, phenotypic differences have also been reported among individuals with identical genomes, raising an intriguing question of the cause of the phenotypic variation among these individuals (Ayroles et al., 2015). We wondered how genetically identical *Drosophila* lines obtained from different populations would differ in their early embryonic gene expression patterns. We used 3 widely used *Drosophila* strains: Canton S, Oregon R, and Berlin K; 1 widely used eye and body color mutant *yw* and 2 isogenic lines that were recently generated from wild caught females from Adana Saimbeyli region, Turkey, which we named Saimbeyli 1 and Saimbeyli 2.

We first compared the early embryonic gene expression profiles of genetically identical (isogenic) populations. We also tested the robustness of these populations under temperature perturbation by comparing their survival rates in addition to the expression patterns of early embryonic gene expressions at 22, 25 and 29°C. For quantitative comparison of early gene expression patterns, we used *hb* and *eve*, which are under the regulation of maternal TF Bcd. Bcd forms a gradient that is instrumental in the patterning of these genes' expressions. For this reason, we also investigated weak *bcd* mutant isogenic lines and compared the Bcd gradients of mutant embryos at different temperatures explained in Figure 2. We then assessed the expression pattern differences between isogenic lines and a mass population which retains limited genetic variation. Finally, we analyzed the variation within and between population in genetically compromised isogenic populations by using transgenic lines that were generated previously to contain weak *bcd* mutants (Önal et al., 2021). In conclusion: there are patterning differences in the expression of *hb*, and *eve* stripes between different isogenic populations and they have different responses to temperature perturbations. Most isogenic populations have similar patterns at 22 and 25°C whereas at 29°C there was a posterior shift in all populations in *hb* and second stripe of *eve* shifted towards posterior in several lines (BK, CS, Ore, *yw*, SB1, SB2 and SB-M). As expected, genetically perturbed isogenic lines were all overall weak and this shown in their survival rates, expression pattern shifts at different temperatures as well as increased variation within the individual of the same lines at the same temperature, suggesting lowered canalization and decreased robustness in these strains. These

experiments showed for the first time the differences in the very early embryo patterning changes of essential genes in most widely used strains around the world. To our knowledge, they also present the first comparison of gene expression of these early markers between genetically identical lines and genetically variably mixed populations.

For the experiments explained in Figure 2, three approaches were mainly used. First, Molecular Analysis which consists of *in-situ* hybridization to find the expression profiles of genes *hb* and *eve*. Also, it includes immunostaining of *bcd* protein and DAPI staining to calculate the length constant of the *bcd* gradient. Survival Assay is used to find the viability and the variability of the viability of the lines upon facing different temperatures. Phenotypical Analysis to see the cuticle's formed by the embryos and calculate the number of mutant cuticle phenotypes, this experiment is only done for weak *bcd* mutants because they were the only ones to produce mutant cuticle phenotypes, Isogenic lines and Mass Population line did not produce any mutant cuticle phenotypes at any given temperature.

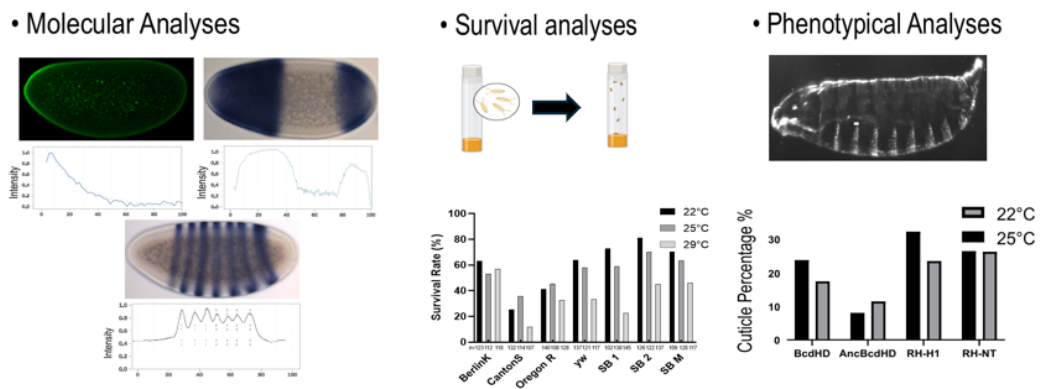


Figure 2: Experimental workflow to assess temperature effects on *Drosophila* embryogenesis. Embryos were raised at 22°C, 25°C, and 29°C and analyzed using three complementary approaches: (1) Molecular analyses of Bcd protein gradients (immunostaining, exponential λ fits), *hunchback* expression (in situ hybridization, posterior boundary quantification), and *eve* second stripe positioning (in situ hybridization, positional quantification). (2) Survival assays to evaluate viability across temperatures (3) Phenotypical analyses of cuticle preparations..

4.1 Embryonic Gene Expression of *hb* in Isogenic and Mass Populations of *D. melanogaster* at 25°C

To investigate the gene expression differences within and between isogenic laboratory strains we measured the anterior *hb* expression. The *hb* gene in *Drosophila* is a crucial gap gene and transcription factor involved in early embryonic segmentation and patterning. It is expressed maternally and zygotically in the embryo and helps define regions of the embryo that give rise to different segments, particularly specifying thoracic and anterior abdominal segments (Driever, W., & Nüsslein-Volhard, C. (1989)). Its early zygotic expression in the anterior is dependent on the maternal TF Bcd. This expression is driven by P2 enhancer and forms an anterior cap-like expression. *hb* expression is dynamic even in the early stages of embryonic development. It has a late middle stripe expression and a posterior expression driven by other enhancer elements (Driever, W., & Nüsslein-Volhard, C. (1989)). We quantified only the pbp of anterior cap expression Bcd dependent expression- in Berlin K, Canton S, Oregon R, *yw* and isogenic lines obtained from Adana region as well as the mass population from the same region with limited genetic variation. Within group variation was determined by variation.

Accordingly, at the optimal temperature 25°C, Berlin K within group variation was 1.7, in Canton S it was 5.3, 2.8 for Oregon R, 2.2 for *yw* embryos, in Saimbeyli 1 this was 2.9, in Saimbeyli 2 it was 2.5 shown in Table 4. In the mass population derived from 5 female founders from the same region, Saimbeyli Mass the variance was only 1.5 at 25°C (Table 4). In conclusion Canton S had the highest variation (5.3) and Saimbeyli Mass (1.5) had the lowest variation of *hb* pbp at 25°C.

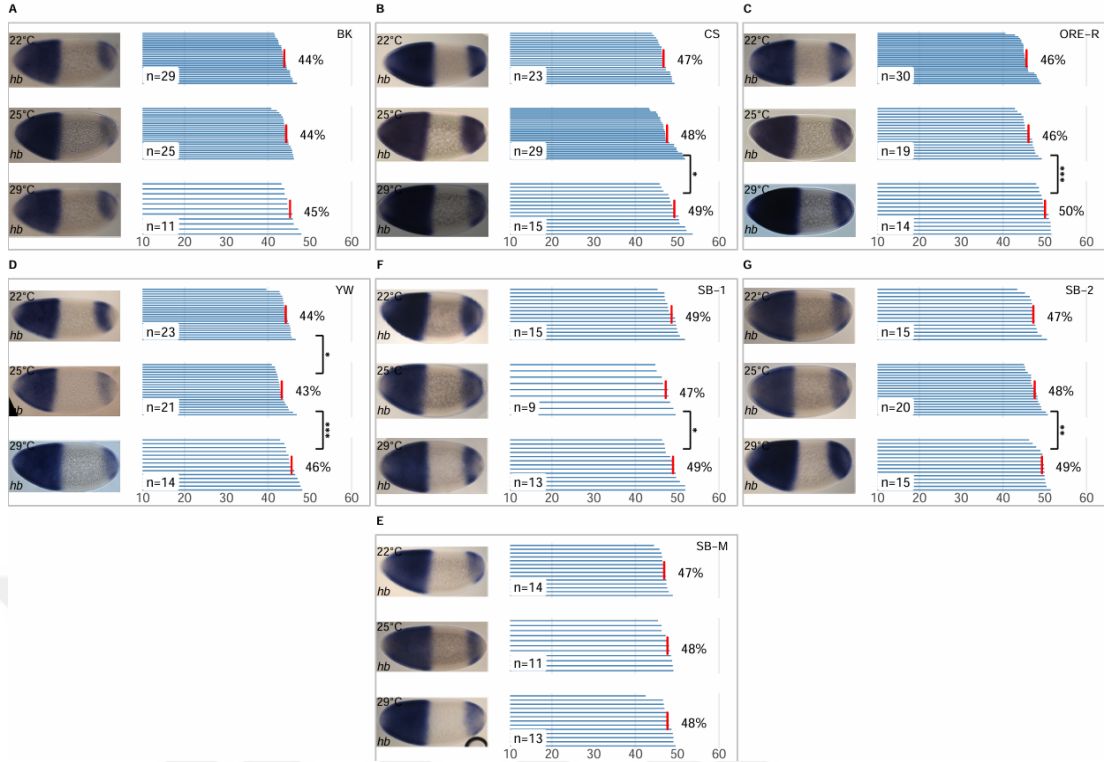


Figure 3: Average *hb* pbp of isogenic lines and out-bred *Drosophila* populations at 22°C, 25°C, and 29°C. BK = Berlin K, CS = Canton S, ORE = Oregon R, YW = *yw*, SB-1 = Saimbeyli 1, SB-2 = Saimbeyli 2, SB-M = Saimbeyli Mass. The vertical red line and the percentage above each panel indicate the mean *hb* pbp value for the corresponding strain at that temperature. Average values were calculated from individual embryo measurements, and horizontal blue lines in each panel represent the distributions.

Table 4: Mean and variance of *hb* pbp in isogenic and Saimbeyli *Drosophila* lines at 22 °C, 25 °C, and 29 °C. Pairwise comparisons between temperatures were performed using two-sample *t*-tests (22°C vs 25°C, 25°C vs 29°C), and equality of variances was assessed using *F*-tests. Reported *p*-values indicate whether mean positions (*t*-test) or variances (*F*-test) differ significantly between the compared conditions.

	22°C		25°C		29°C		t-test pval		F-test pval	
	Mean	Variance	Mean	Variance	Mean	Variance	22 vs 25	25 vs 29	22 vs 25	25 vs 29
Berlin-K	43.8967	2.0149	44.3327	1.6662	45.2644	2.1465	0.2427	0.0861	0.6407	0.5825
Canton-S	46.7084	2.1002	47.5484	5.2643	49.2963	5.0585	0.1144	0.0217	0.0303	0.9736
Oregon-R	45.6653	3.4017	46.1324	2.8247	50.0854	1.4829	0.3668	0.0000	0.6917	0.2406
Yellow-White	44.2351	1.9478	43.2727	2.1503	45.6437	2.5080	0.0316	0.0001	0.8175	0.7352
Saimbeyli-1	48.6410	3.0162	47.2451	2.8622	49.0001	3.2451	0.0692	0.0316	0.9810	0.8845
Saimbeyli-2	47.2979	2.6460	47.6073	2.5282	49.3580	1.7859	0.5781	0.0013	0.9081	0.5129
Saimbeyli-M	46.8627	1.1548	47.6681	1.5370	47.6484	3.3710	0.1031	0.9755	0.6181	0.2225

We then compared the average position of *hb* pbps between different isogenic lines and the mass population at 25°C. Accordingly, shown in Table 4 average *hb* pbp in

Berlin K was 44.3 % EL (0 is the most anterior and 100 is the most posterior tip), in Canton S it was 47.5 %, 46.1% in Oregon R, 43.2 % in *yw* embryos. In Saimbeyli 1 this was 47.2 %, in Saimbeyli 2 it was 47.6 %. In the mass population derived from 5 females from the same region Saimbeyli Mass the average *hb* pbp was 47.7 % at 25°C. In conclusion, Saimbeyli 2 had the most posteriorly shifted *hb* pbp average out of all the lines tested (47.6%). *yw* embryos had the most anteriorly shifted (43.2%) *hb* position in average, which was followed by Berlin K embryos with 44.3 %. These two populations are significantly different from the isogenic laboratory strains and wild caught lines as well as the Saimbeyli Mass. In Figure 4 at 25 °C, the analysis revealed significant differences in *hb* pbp among the isogenic and Saimbeyli lines (Table 4; Figure 4). Pairwise comparisons using the Tukey–Kramer test showed that several isogenic lines differed significantly from one another. For example, Canton S differed strongly from Berlin K (** $p < 0.001$), Oregon R from *yw* (** $p < 0.001$), and all three Saimbeyli lines from most isogenic backgrounds (** $p < 0.001$). In contrast, some comparisons, such as Oregon R vs Canton S or Saimbeyli-1 vs Saimbeyli-2, were not significant. Overall, the data indicates that *hb* boundary positioning varies significantly across genetic backgrounds, with Saimbeyli lines displaying marked divergence from isogenic lines Berlin K and *yw*.

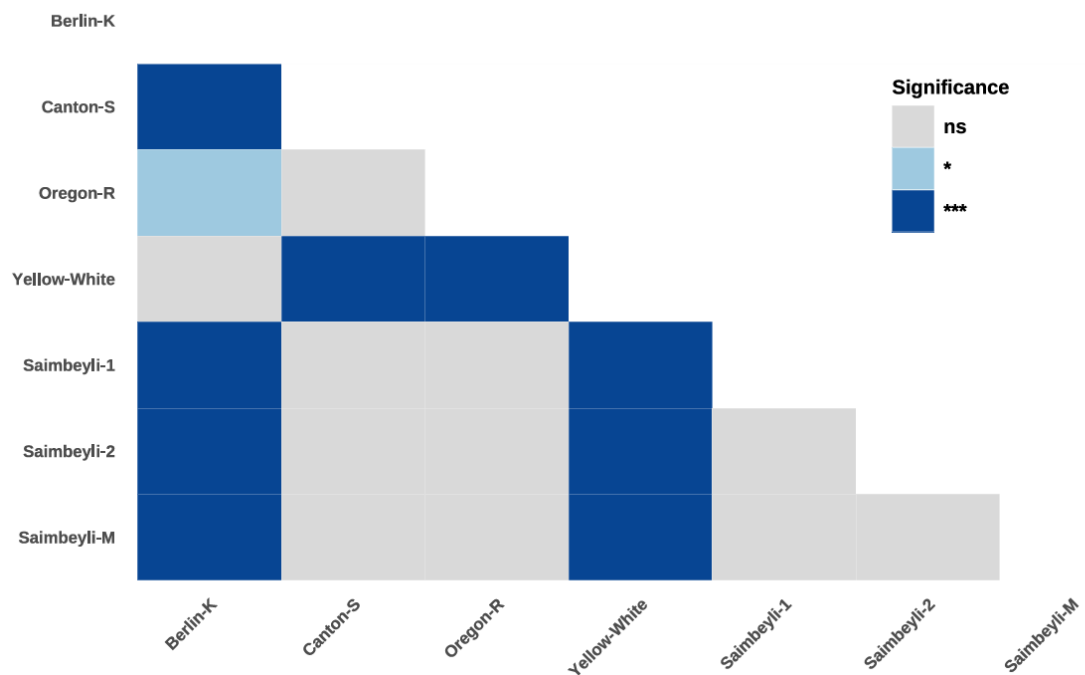


Figure 4: Statistical comparison of *hb* pbp averages among isogenic and out-bred *Drosophila* lines at 25°C. Pairwise comparisons were performed using Tukey–Kramer post-hoc test with a 95%

confidence interval. Color coding indicates the level of significance: ns = not significant, * = $p < 0.05$, *** = $p < 0.001$. Significant values indicate that the mean *hb* pbp differs between the compared lines.

4.2 Embryonic Gene Expression of *hb* in Isogenic and Mass Populations of *D. melanogaster* Against Temperature Perturbation

We next analyzed the *hb* pbp at different temperatures, both in terms of variances at different temperatures and robustness of *hb* expression against temperature change. For the temperature perturbation experiments we chose a mild temperature (22°C), and an extreme temperature (29°C).

4.2.1 Embryonic Gene Expression of *hb* in Isogenic and Mass Populations of *D. melanogaster* at 22°C

The variances of *hb* pbp at 22°C measured, the variation for Berlin K and Canton S can be seen in Table 4 were 2 and 2.1 respectively. The variance was 3.4 for Oregon R, and 1.9 for *yw*. Saimbeyli 1 and 2 were 3 and 2.6 respectively shown in Table 4. These variance values were much higher than the mass population, which was 1.1. Overall, the variance for Canton S decreased from 5.3 at 25°C to 2.1 at 22°C and Oregon R increased from 2.8 to 3.4 at 22°C. Variance in Berlin K increased from 1.6 to 2 at 22°C and *yw* decreased from 2.1 at 25°C to 1.9 at 22°C. Variances showed a little increase in Saimbeyli 1 and 2, from 2.9 to 3 and 2.5 to 2.6 respectively at 22 and 25°C. Mass population's variation decreased from 1.5 to 1.1 upon temperature change from 25°C to 22°C. In conclusion Oregon R had the highest variation (3.4) and mass population (1.15) had the lowest variation of *hb* pbp at 22°C. Canton-S shows the largest variance change, a drop from 5.3 at 25 °C to 2.1 at 22 °C and it's the only line with a significant variance shift by the F-test shown in Table 4.

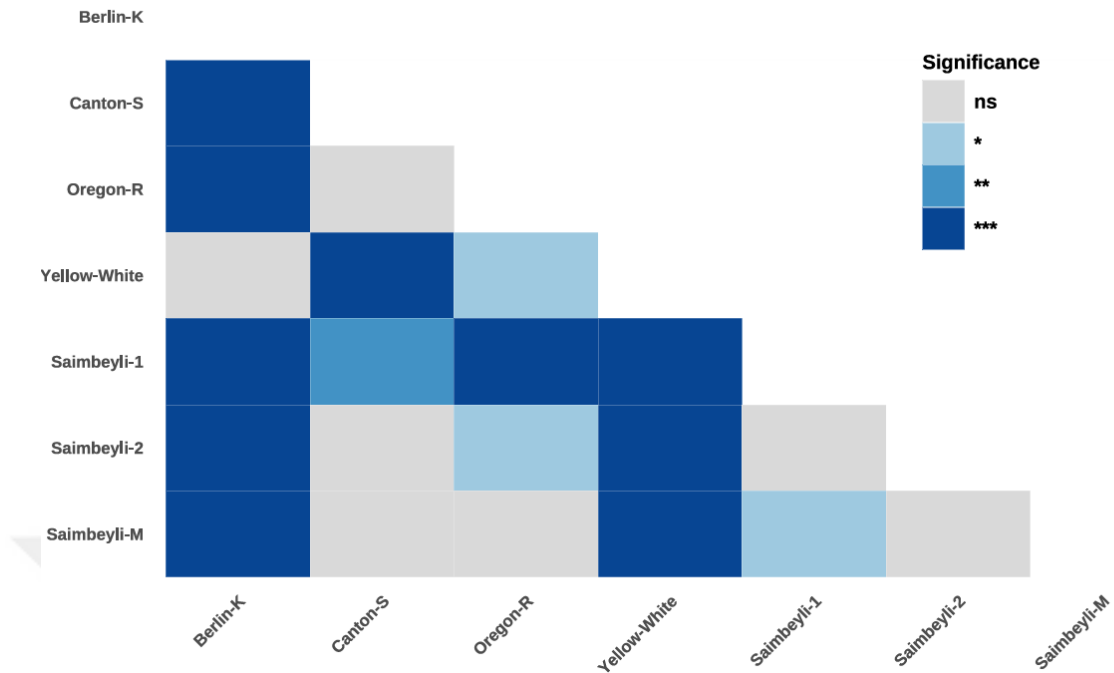


Figure 5: Statistical comparison of *hb* pbp averages among isogenic and out-bred *Drosophila* lines at 22°C. Pairwise comparisons were performed using Tukey–Kramer post-hoc test with a 95% confidence interval. Color coding indicates the level of significance: ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Significant values indicate that the mean *hb* pbp differs between the compared lines.

At 22°C shown in Figure 5 pairwise comparison, strong contrasts were detected compared to 25 °C. For example, Berlin K differed significantly from Canton S and all three Saimbeyli lines (** $p < 0.001$), while *yw* showed differences with Canton S and Oregon R ($p < 0.05$ to ** $p < 0.001$). In contrast, comparisons among the Saimbeyli lines themselves were mainly non-significant.

We then wondered if the average *hb* pbp showed any reaction to temperature perturbation to 22°C. The average *hb* pbp in Berlin K shown in Table 4 was 43.8 % EL in Canton S its 46.7%, 45.6 % in Oregon R and 44.2 % in *yw* embryos. In Saimbeyli 1 the average *hb* pbp was 48.6% and it was 47.2 % in Saimbeyli 2. The Saimbeyli Mass strain that derived from 5 different isofemales, showed an average *hb* pbp of 46.8%. In conclusion Berlin K had the most anterior *hb* pbp average and Saimbeyli 1 had the most posterior average *hb* pbp. Both are significantly different from other strains tested at 22°C.

4.2.2 Embryonic Gene Expression of *hb* in Isogenic and Mass Populations of *D. melanogaster* at 29°C

Then, we tested the temperature perturbation at a higher temperature compared to the optimal one using 29°C. The variance within the groups tested at 29°C for their *hb* pbp. Consistently, the variance shown in Table 4 in Berlin K was 2.1 and for Canton S it was 5.05. Oregon R and *yw* variations were 1.5 and 2.5 respectively. Saimbeyli 1 and 2 variations were 3.2 and 1.7 and Saimbeyli Mass strain showed a variation value of 3.4. Canton S had the highest and Oregon R had the lowest variation at 29°C. Variation levels are changed with the temperature perturbation shown in Table 4, variation in Berlin K changed from 1.6 to 2.1 29°C at when perturbed, Canton S changed from 5.2 to 5 at 29°C. Oregon R and *yw* changed from 2.8 and 2.1 at 25°C to 1.4 and 2.5 respectively at 29°C. Saimbeyli 1 and Saimbeyli 2 were 2.9 and 2.5 and they became 3.2 and 1.7 upon perturbation with 29°C. Saimbeyli Mass strain's variation was 1.5 and it changed to 3.3 at 29°C. In summary, out of the 7 shifts in the variation, Only Berlin K and Saimbeyli Mass were not significant, all the other changes in the variation were significant according to F-test shown in Table 4.

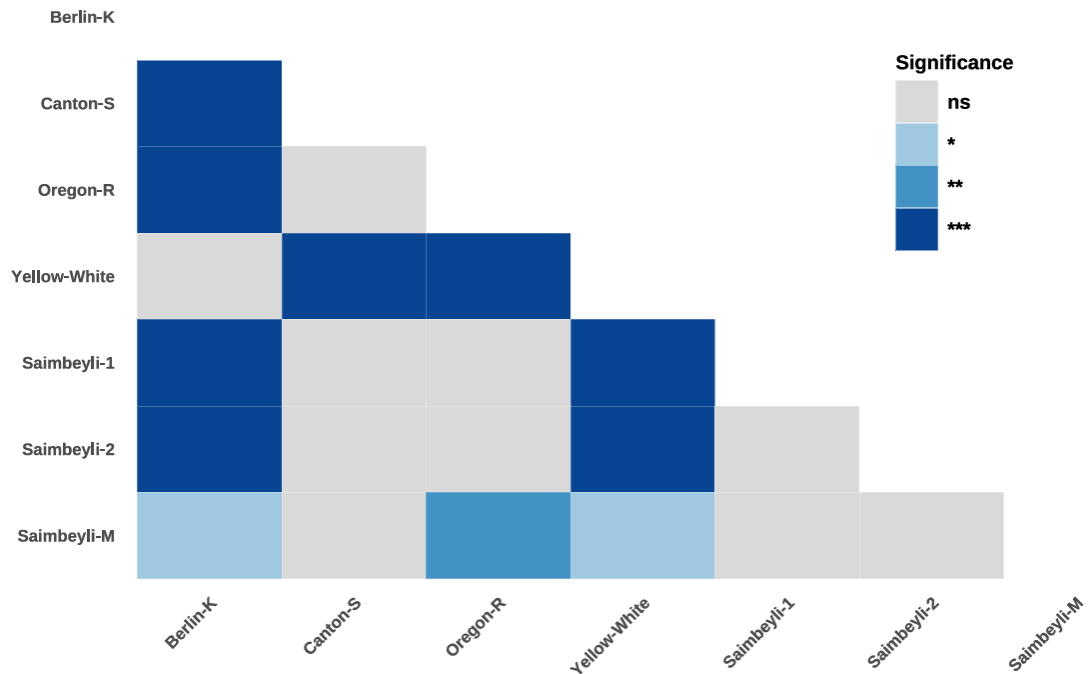


Figure 6: Statistical comparison of *hb* pbp averages among isogenic and out-bred *Drosophila* lines at 29°C. Pairwise comparisons were performed using Tukey–Kramer post-hoc test with a 95% confidence interval. Color coding indicates the level of significance: ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Significant values indicate that the mean *hb* pbp differs between the compared lines.

At 29°C, pairwise comparisons shown in Figure 6 revealed broad and robust differences among isogenic and Saimbeyli lines, with more significant contrasts than at 22°C but slightly fewer than at 25°C. For instance, Berlin K differed strongly from Canton S, Saimbeyli-1, and Saimbeyli-2 (** $p < 0.001$), while Oregon R and *yw* also showed marked divergence (** $p < 0.001$). In contrast, the out-bred line Saimbeyli M displayed weaker differences with other Saimbeyli backgrounds, reaching only moderate significance ($p < 0.05$ –* $p < 0.01$).

Their average *hb* pbps were also calculated at 29°C shown in Table 4 and Figure 3. Berlin K was 45.2% EL and Canton S was 49.2%. Oregon R and *yw* were 50% and 45.6% respectively. Saimbeyli 1 was 49% and Saimbeyli 2 was 49.3%. Mass strain showed a *hb* pbp of 47.6% at 29°C. Oregon R had the most posterior *hb* pbp and Berlin K had the most anterior one. Both of those lines were significantly different from the other isogenic lines tested and mass population.

4.2.3 The Average Position of *hb* pbp at 22°C and 29°C Compared to 25°C In Isogenic and Mass Population *Drosophila* Lines

We also compared the strains *hb* pbp averages of the temperature in the same groups to observe their robustness against temperature perturbations. The average *hb* pbp shown in Figure 3 in Berlin K decreased from 43.9% at 25°C to 44.3% at 22°C ($p > 0.05$). In Canton S it decreased from 47.5% to 46.7% ($p > 0.05$) at 25 and 22°C respectively. Oregon R and *yw* changed from 46.1% and 43.2% to 45.6% ($p > 0.05$) and 44.2% ($p < 0.05$) respectively from 25°C to 22°C. Saimbeyli 1 and 2 were 47.2% and 47.6% at 25°C and they shifted to 48.6% and 47.2% ($p > 0.05$) at 22°C respectively. Saimbeyli mass strain's average *hb* pbp changed from 47.6% to 46.8% ($p < 0.05$) at 22 and 25°C.

In conclusion none of the strains tested showed significant shift in their *hb* pbp under 22°C temperature perturbation except *yw*. Four out of six isogenic lines tested showed decreased *hb* pbp in EL, only *yw* and Saimbeyli 1 shifted towards posterior (increased EL) when perturbed at 22°C, but the changes were not significant in Saimbeyli 1.

After observing low number of significant changes under 22°C temperature perturbation, we wondered how the lines will react in terms of their *hb* pbp under 29°C temperature perturbation compared to 25°C. The average *hb* pbp shown in Figure 3 in

Berlin K increased from 44.3% at 25°C to 45.2% at 29°C ($p>0.05$) meaning a posterior shift, In Canton S average *hb* pbp increased from 47.5% at 25°C to 49.2% at 29°C ($p<0.05$). In Oregon R it significantly shifted from 46.1% at 25°C to 50% at 29°C ($p<0.05$) and *yw hb* pbp shifted from 43.2% to 45.6% at 25 and 29°C respectively. Wild caught Saimbeyli 1 and Saimbeyli 2 isogenic strains pbp were 47.2% and 47.6% at 25°C and they shifted significantly to 49% and 49.3% ($p<0.05$). Mass population, which derived from five different isogenic lines from Saimbeyli region, Saimbeyli Mass showed an average *hb* pbp in EL of 47.66% at 25°C, it showed a little to no change and became 47.64% ($p>0.05$) at 29°C.

In conclusion, the highest average *hb* pbp in EL at 29°C was Oregon R (50%) and the lowest was Berlin K (45.2%) meaning Berlin K has a significantly more anterior *hb* pbp than Oregon R. Both are significantly different from other lines tested (Berlin K and *yw*, Oregon R and Canton S, and Saimbeyli 1 and 2 are not significantly different). All six isogenic lines tested shifted in their average *hb* pbp towards posterior at 29°C compared to 25°C, five out of these six shifts were significant (except Berlin K). Interestingly, Saimbeyli Mass strain shifted little towards anterior from 47.66% to 47.64% ($p>0.05$).

In summary, temperature perturbation of 22°C didn't cause any significant change in average *hb* pbp of the six isogenic lines tested except *yw*, Saimbeyli mass population also didn't show any significant change in both perturbation temperatures. In contrast, temperature perturbation of 29°C, shifted the average *hb* pbp of five of the isogenic lines significantly towards posterior. Saimbeyli Mass strain's average *hb* pbb didn't change significantly under any temperature perturbation.

4.3 Embryonic Gene Expression of *eve* Second Stripe in Isogenic and Mass Populations of *D. melanogaster* at 25°C

The *eve* gene encodes a pair-rule transcription factor that plays a central role in *Drosophila* segmentation. Its seven transverse stripes emerge during the blastoderm stage as a readout of upstream maternal and gap gene gradients. Among these, the second stripe of *eve* is one of the most robustly characterized and widely studied. It is positioned in the anterior part of the embryo, where the Bicoid (Bcd) gradient and *hb* expression overlap to activate *eve*, while repression is mediated by *Giant* (*Gt*) and *Krüppel* (*Kr*) on the anterior and posterior sides, respectively (Small et al., 1992;

Fujioka et al., 1999). This combinatorial regulation makes the *eve* 2nd stripe a precise developmental readout of maternal-to-zygotic patterning inputs. Variation in the second Stripe of *eve* measured by one-way ANOVA summarized in Table 5 and visualized in the distribution of embryo stripe positions in Figure 7. It is observed that at the optimal temperature 25°C Berlin K had a variation of 0.3 and for Canton S it was 2.6. Oregon R and *yw* had the value of 0.7. Wild caught isogenic Saimbeyli 1 and 2 were 2.5 and 2.7 and Saimbeyli Mass had a relatively higher variation of 2.4 compared to most isogenic lines at 25°C. In summary, Saimbeyli 2 had the highest (2.7) variation among all while Berlin K (0.2) had the lowest amount followed by *yw* (0.7).

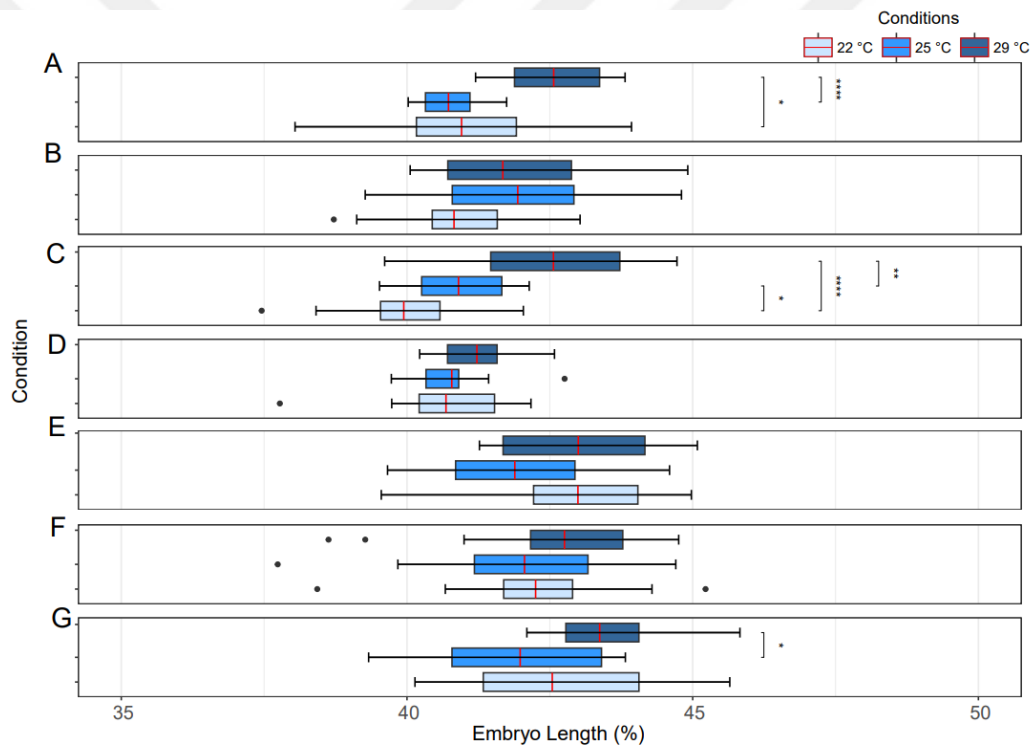


Figure 7: Variation in *eve* 2nd stripe position across temperatures in isogenic and out-bred *Drosophila* lines. A: Berlin K, B: Canton S, C: Oregon R, D: *yw*, E: Saimbeyli 1, F: Saimbeyli 2, G: Saimbeyli Mass. Stripe positions (expressed as % embryo length) are shown for 22°C (light blue), 25°C (medium blue), and 29°C (dark blue). Statistical comparisons between temperatures within each line were assessed by Tukey–Kramer post-hoc test; significance is indicated as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

Table 5: Mean and variance of *eve* stripe 2 position in isogenic and out-bred *Drosophila* lines at 22°C, 25°C, and 29°C Pairwise comparisons were performed using Welch's *t*-test (with 25 °C as the reference) to assess differences in mean stripe positions, and *F*-tests were applied to compare variances between temperatures. Reported *p*-values indicate whether mean positions (*t*-test) or variances (*F*-test) differ significantly between conditions.

Strain	22 °C		25 °C		29 °C		Welch's t-test p-values		F-test p-values	
	Mean	Variance	Mean	Variance	Mean	Variance	22 vs 25	29 vs 25	22 vs 25	29 vs 25
Berlin - K	40.955	2.682	40.726	0.293	42.568	0.800	0.6800	1.81e-06	0.00116	0.1030
Canton - S	40.823	1.405	41.939	2.662	41.677	2.154	0.0872	7.00e-01	0.30000	0.7290
Oregon - R	39.944	1.428	40.901	0.707	42.563	2.166	0.0295	1.69e-03	0.25400	0.0709
Yellow - White	40.683	1.507	40.784	0.695	41.223	0.542	0.8210	2.28e-01	0.25500	0.7160
Saimbeyli - 1	42.992	2.545	41.887	2.520	42.998	1.716	0.0943	7.33e-02	0.98700	0.4970
Saimbeyli - 2	42.251	2.453	42.057	2.703	42.756	2.834	0.7160	1.90e-01	0.87200	0.9030
Saimbeyli - M	42.542	2.880	41.981	2.445	43.372	1.133	0.4200	3.82e-02	0.84500	0.2370

We also calculated the average second stripe position on EL for the six isogenic and one mass population line at 25°C. Shown in Table 5 and Figure 7 for Berlin K it was 40.7% and it was 41.9% for Canton S. Oregon R had an average value of 40.9% and *yw* was 40.7%. Saimbeyli 1 and 2 were 41.8% and 42.0% respectively. Saimbeyli Mass average *eve* second stripe was 41.9% at 25°C. In summary, Saimbeyli Mass (41.9%) and Saimbeyli 2 (42%) had the most posterior average second stripe position and Berlin K and *yw* (40.7%) had the most anterior one.

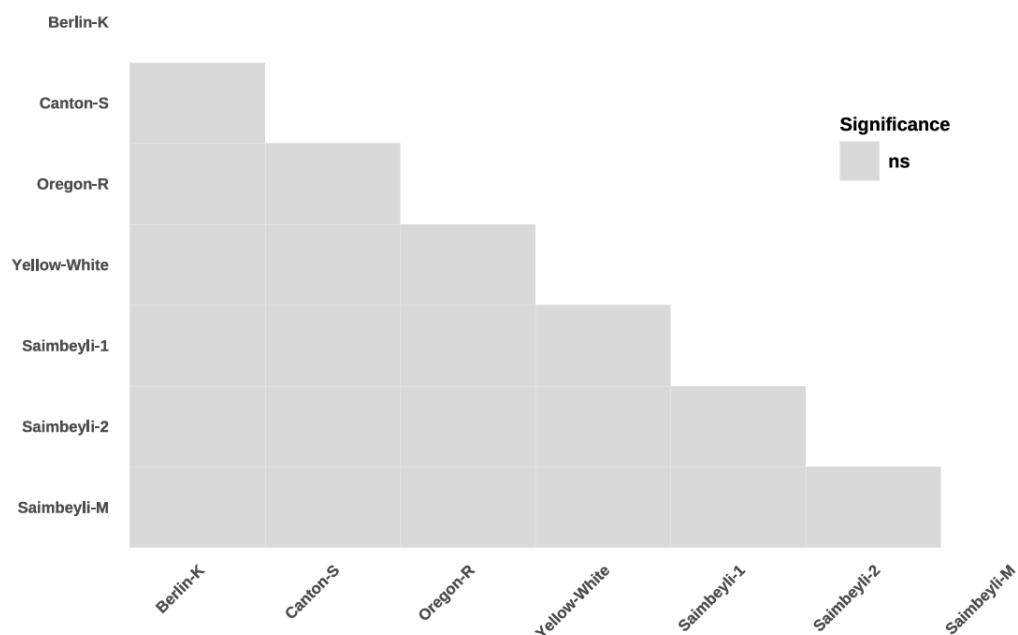


Figure 8: Statistical comparison of *eve* 2nd stripe positions among isogenic and out-bred *Drosophila* lines at optimal 25°C. Pairwise comparisons were performed using Tukey–Kramer post-hoc test with a 95% confidence interval. Color coding indicates the level of significance: ns =

not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Significant values indicate that the mean *eve* second stripe differs between the compared lines.

At 25 °C, no significant differences were detected in *eve* 2nd stripe positions among the isogenic and Saimbeyli lines, as shown by the Tukey–Kramer test shown in Figure 8. This indicates that under optimal temperature conditions, stripe positioning is highly conserved across genetic backgrounds.

4.4 Embryonic Gene Expression of *eve* in Isogenic and Mass Populations of *D. melanogaster* Second Stripe Under Temperature Perturbation

Now, just like we did for *hb pbp*, we put the strains under 22°C and 29°C temperature perturbation to see if their average positions and variance will change.

4.4.1 Embryonic Gene Expression of *eve* in Isogenic and Mass Populations of *D. melanogaster* Second Stripe Under Temperature Perturbation of 22°C

Following the variation seen in the second *eve* stripe at 25°C, we wanted to see its behavior under a 22°C temperature perturbation. The amount of change in the variation is also assessed by using F-test (Table 5) and is illustrated in the spread of stripe positions across conditions shown in Figure 7. Berlin K had a variation of 2.6 and Canton S was 1.4. Oregon R and *yw* had 1.4 and 1.5 respectively. Saimbeyli 1 was 2.5 and Saimbeyli 2 was 2.4. Saimbeyli Mass strain showed variation level of 2.8. Saimbeyli Mass had the biggest variation and Canton S and Oregon R were the lowest among strains. The amount of change in the variation is also assessed by using F-test, the results are shown in Table 5. The variance in Berlin K increased from 0.3 to 2.7 at 22°C. Canton S changed from 2.7 to 1.4 at 22°C. Oregon R and *yw* were 0.7 and they increased to 1.4 and 1.5 respectively at 22°C. Saimbeyli 1 and 2 were 2.6 and 2.5 and they changed to 2.5 and 2.7 respectively. Saimbeyli Mass were 2.4 at 25°C and its variation has increased to 2.8 at 22°C. In conclusion, only significant change in variation was observed in Berlin K.

The average positions of the second stripe at 22°C were also calculated. Consequently, average second stripe position of Berlin K was 40.9%, Canton S had the EL of 40.8%. Oregon R and *yw* were 39.9% and 40.6% respectively. Saimbeyli 1 43% and Saimbeyli 2 was 42.2%. Saimbeyli Mass showed an average value of 42.5% for its *eve* second

stripe position at 22°C. In summary, Saimbeyli 1 (42.9%) had the most posterior second stripe position while Oregon R (39.9%) had the most anterior one at 22°C.

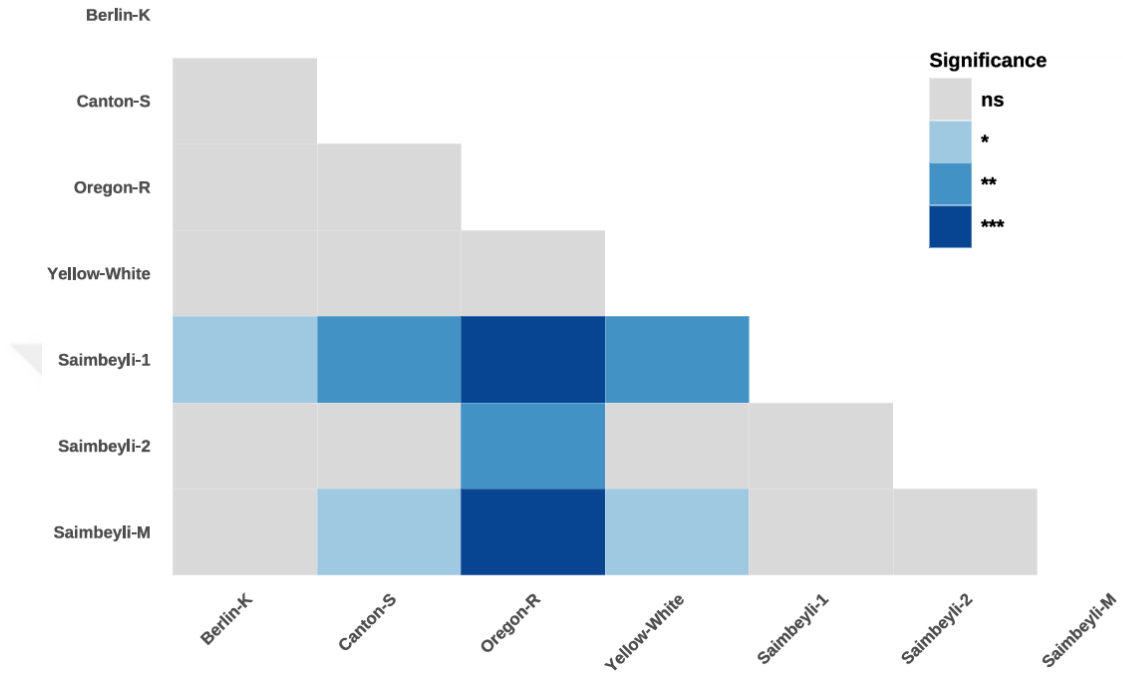


Figure 9: Statistical comparison of *eve* 2nd stripe positions among isogenic and out-bred *Drosophila* lines at 22°C perturbation. Pairwise comparisons were performed using Tukey–Kramer post-hoc test with a 95% confidence interval. Color coding indicates the level of significance: ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Significant values indicate that the mean *eve* second stripe differs between the compared lines.

At 22 °C, significant differences in *eve* 2nd stripe positioning were mainly detected among the Saimbeyli lines and their comparisons with isogenic strains shown in Figure 9. For instance, Saimbeyli-1 showed strong divergence from Oregon R and *yw* (**, **), while Saimbeyli M differed significantly from Canton S and Oregon R (***). By contrast, most isogenic strains (Berlin K, Canton S, Oregon R, *yw*) did not differ significantly from one another. These results indicate that *even* under mild perturbation, Saimbeyli lines already exhibit greater variability in stripe positioning compared to isogenic lines.

4.4.2 Embryonic Gene Expression of *eve* In Isogenic and Mass Populations of *D. melanogaster* Second Stripe Under Temperature Perturbation of 29°C

After testing the second stripe position of *eve* under optimal temperature of 25°C and a mild perturbation of 22°C, we used 29°C perturbation to see if its variation among the group and its average position on EL will change. Variation patterns at 29°C compared to 22°C and 25°C are shown in Table 5 and in the distribution of stripe positions shown in Figure 7. Thus, we observed at 29°C Berlin K had a variation of 0.7, Canton S had 2.1. Oregon R and *yw* were 2.1 and 0.5 respectively. Saimbeyli 1 and 2 were 1.7 and 2.8 respectively. Saimbeyli Mass had relatively lower value of 1.1 compared to isogenic lines. In summary, Saimbeyli 2 had the highest variation (2.8) and *yw* (0.5) had the lowest for their *eve* second stripe positions on EL at 29°C.

Then we went on to calculate their average second stripe positions under 29°C temperature perturbation. Berlin K had a value of 42.5% and Canton S had 41.6%. Oregon R and *yw* were 42.5% and 41.2% respectively. Saimbeyli 1 was 42.9% and Saimbeyli 2 was 42.7%. Mass population derived from five isogenic lines, Saimbeyli Mass had an average of 43.3% EL. In conclusion Saimbeyli 1 had the most posteriorly shifted second stripe position from 41.9% to 42.9% and *yw* had the most anterior shifted one from 40.7% to 41.2% at 29°C.

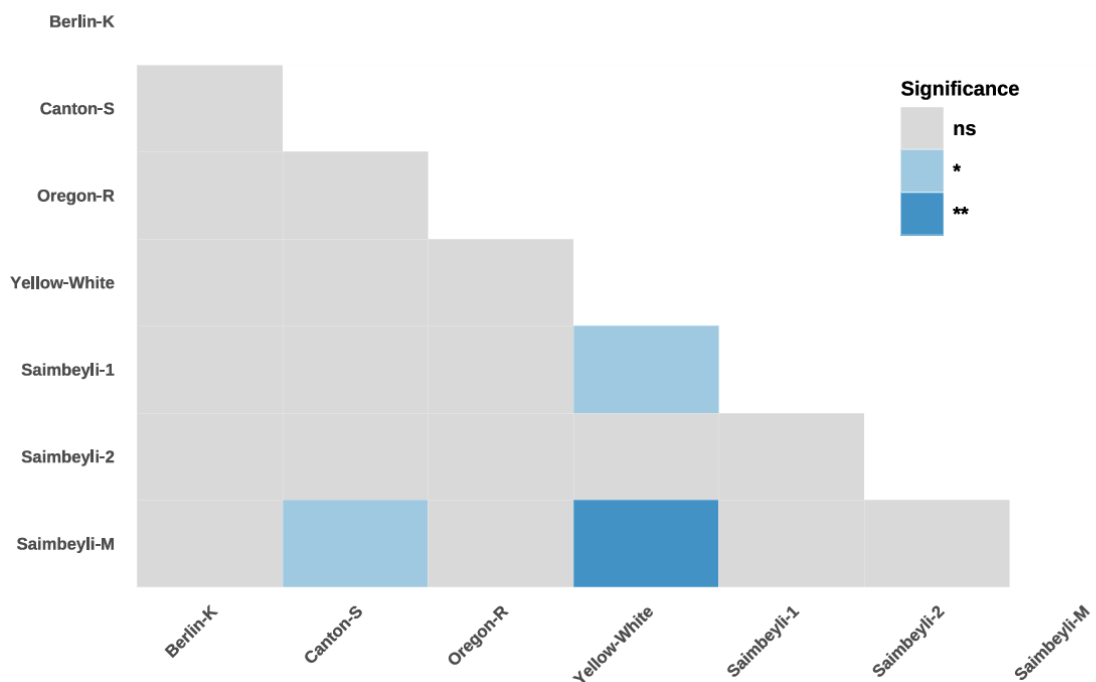


Figure 10: Statistical comparison of *eve* 2nd stripe positions among isogenic and out-bred *Drosophila* lines at 29°C perturbation. Pairwise comparisons were performed using Tukey–Kramer post-hoc test with a 95% confidence interval. Color coding indicates the level of significance: ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Significant values indicate that the mean *eve* second stripe differs between the compared lines.

At 29 °C, only a few significant differences emerged, mainly involving Saimbeyli-M (e.g., vs Canton S and *yw*), whereas at 22 °C broader divergence among Saimbeyli lines was observed, and at 25 °C no significant differences were detected shown in Figure 10.

4.4.3 The Average Position of *eve* Second Stripe at 22°C and 29°C Compared to 25°C In Isogenic and Mass Population *Drosophila* Lines

We also compared the strains *eve* second stripe position averages between the temperature in the same groups to observe their robustness against temperature perturbations. The average *eve* second stripe position in Berlin K increased from 40.7% at 25°C to 40.9% at 22°C ($p > 0.05$). In Canton S it decreased from 41.9% to 40.8% ($p > 0.05$) at 25 and 22°C respectively. Oregon R and *yw* changed from 40.9% and 40.8% to 39.9% ($p < 0.05$) and 40.7% ($p > 0.05$) respectively from 25°C to 22°C. Saimbeyli 1 and 2 were 41.9% and 42.1% at 25°C and they shifted to 42.9% and 42.3% ($p > 0.05$) at 22°C respectively. Saimbeyli mass strain's average *eve* second stripe position changed from 41.9% to 42.5% ($p > 0.05$) at 22 and 25°C. In conclusion, under 22°C temperature perturbation, only one significant shift was observed (Oregon, shifted towards anterior). Three out of seven shift events were towards posterior and four of them were towards anterior. Saimbeyli Mass Line did not observe any significant shift for its *eve* second stripe position.

After observing low ratio of significant changes under 22°C temperature perturbation, we wondered how the lines will react in terms of their average second stripe positions under 29°C temperature perturbation compared to 25°C shown in Table 5 and Figure 7. The average *eve* second stripe positions in Berlin K increased from 40.7% at 25°C to 42.5% at 29°C ($p < 0.05$), In Canton S average *eve* second stripe position decreased from 41.9% at 25°C to 41.7% at 29°C ($p > 0.05$). In Oregon R it significantly shifted from 40.9% at 25°C to 42.5% towards posterior at 29°C ($p < 0.05$) and *yw* *eve* second stripe position shifted from 40.7% to 41.2% ($p > 0.05$) at 25 and 29°C respectively. Wild caught Saimbeyli 1 isogenic strain was 41.9% at 25°C and it shifted to 42.9%.

Saimbeyli 2 was 42% 25°C and it shifted to 42.7% ($p>0.05$) at 29°C. Mass population derived from five different isogenic lines from Saimbeyli region, Saimbeyli Mass, showed an average second stripe position in EL of 41.9% at 25°C, it shifted towards posterior to 43.3% ($p<0.05$) at 29°C.

In conclusion, under 29°C temperature perturbation, out of seven shift events three of them were significant (Berlin K, Oregon R and Mass, towards posterior). Six out of seven shift events were towards posterior (exception being Canton S).

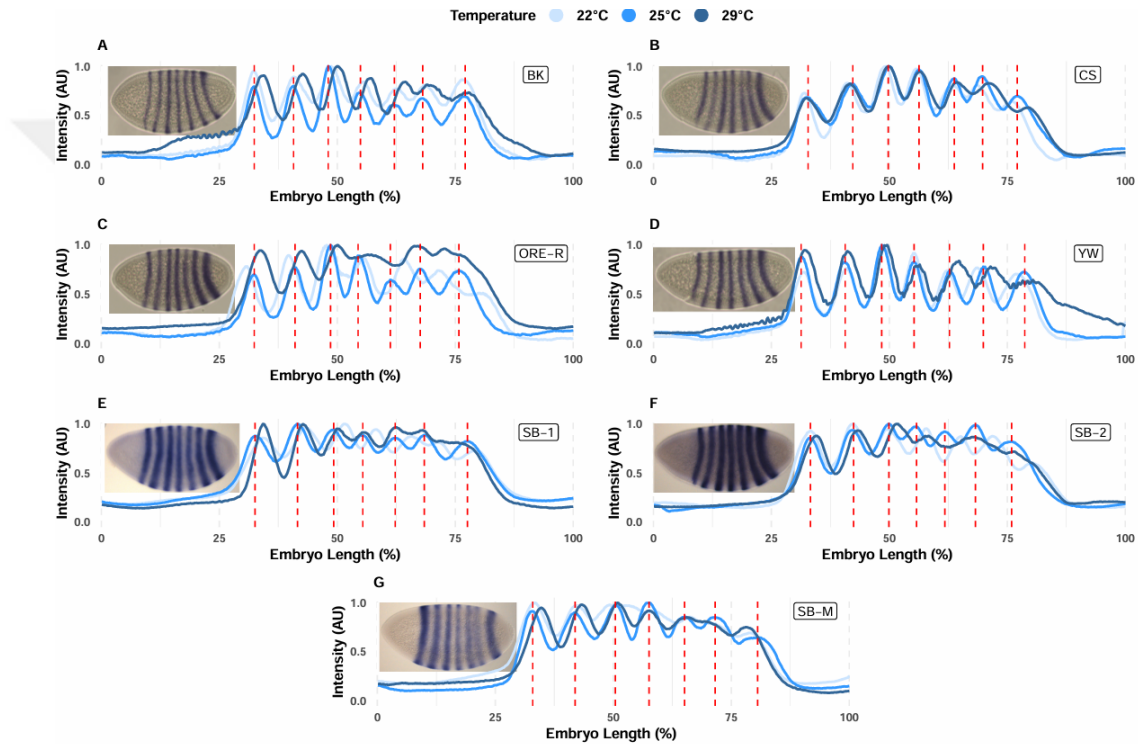


Figure 11: Average eve stripe positions on embryo length of isogenic and out-bred *Drosophila* lines at 22°C, 25°C, and 29°C. BK = Berlin K, CS = Canton S, ORE = Oregon R, YW = *yw*, SB-1 = Saimbeyli 1, SB-2 = Saimbeyli 2, SB-M = Saimbeyli Mass. Blue curves represent mean intensity along the anterior–posterior axis; 0 being the most anterior 100 being the most posterior, red dashed lines provide approximate stripe references. Insets show representative embryos at 25 °C.

Overall, this figure provides a visual representation of the average stripe positions compared with each other across temperatures and strains, complementing the statistical comparisons in Table 5 and Figure 11.

Finally, the isogenic lines and the Saimbeyli lines are tested for their survival rates to see how temperature perturbation affects their viability.

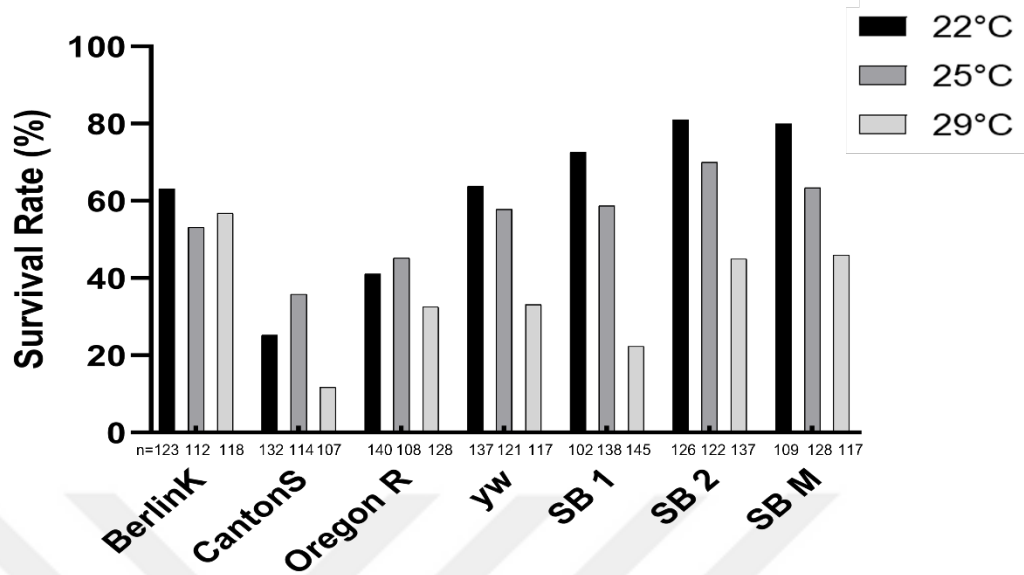


Figure 12: Survival rates of isogenic and out-bred *Drosophila* lines across temperatures. Survival rates (%) of isogenic lines (Berlin K, Canton S, Oregon R, *yw*) and Saimbeyli lines (Saimbeyli-1, Saimbeyli-2, Saimbeyli-M) at 22 °C (solid bars), 25 °C (gray bars), and 29 °C (open bars). Sample sizes (*n*) are shown below each bar.

Survival rates of both isogenic and Saimbeyli *Drosophila melanogaster* lines decreased with increasing temperature Figure 12. At 22 °C, survival was highest in SB-2 (81.1%), SB-M (80.0%), and SB-1 (72.7%), while Canton S had the lowest survival (25.3%). At 25°C, survival generally declined across strains, with SB-2 (70.0%) and SB-M (63.5%) maintaining relatively high viability, whereas Canton S improved slightly to 35.9%. At 29°C, survival dropped most dramatically, especially in Canton S (11.8%) and SB-1 (22.4%), while isogenic strains like Oregon R (32.6%) and *yw* (33.2%) also showed reduced survival. In contrast, Berlin K exhibited relatively stable survival across temperatures (63.2% at 22 °C, 53.2% at 25 °C, 56.9% at 29 °C).

Overall, wild-caught lines (SB-1, SB-2, SB-M) displayed higher survival at moderate temperatures (22–25°C) compared to isogenic lines, but at 29°C their survival decreased substantially, with SB-1 showing the steepest drop. These results suggest that while Saimbeyli lines provide an advantage under mild conditions, they may be more vulnerable under extreme heat stress.

4.5 The Impact of Temperature Perturbations on Genetically Compromised Developmental Systems: Weak *bcd* Mutants

The Bicoid (Bcd) protein gradient is an essential organizer of the anterior-posterior axis during early *Drosophila* embryogenesis. It is a maternal effect gene whose mRNA is localized at the anterior tip of the egg during oogenesis (Driever & Nüsslein-Volhard, 1988). After fertilization, the mRNA is translated into the protein, which diffuses and forms a concentration gradient that decreases exponentially from the anterior to the posterior of the embryo. This gradient functions as a morphogen, providing positional information that directs cells to adopt specific fates along the body axis. This gradient is instructive in the expression and positioning of several gap and pair rule genes such as *hb* and *eve*.

For the next part, weak *bcd* mutants are tested against 22°C temperature perturbation, these lines are mentioned in the introduction part, and they will be referred to as BcdHD (control), AncBcdHD(rescue) and the mutants RH-H1 and RH-NT.

These rescue lines are known to have low survival rates and manifest variable mutant cuticle phenotypes at the optimal 25°C temperature, to make sure, they are tested again for their survival rates and mutant cuticle phenotype counts those experiments are repeated. None of these lines were viable at 29 °C, therefore, this perturbation was not used for them.

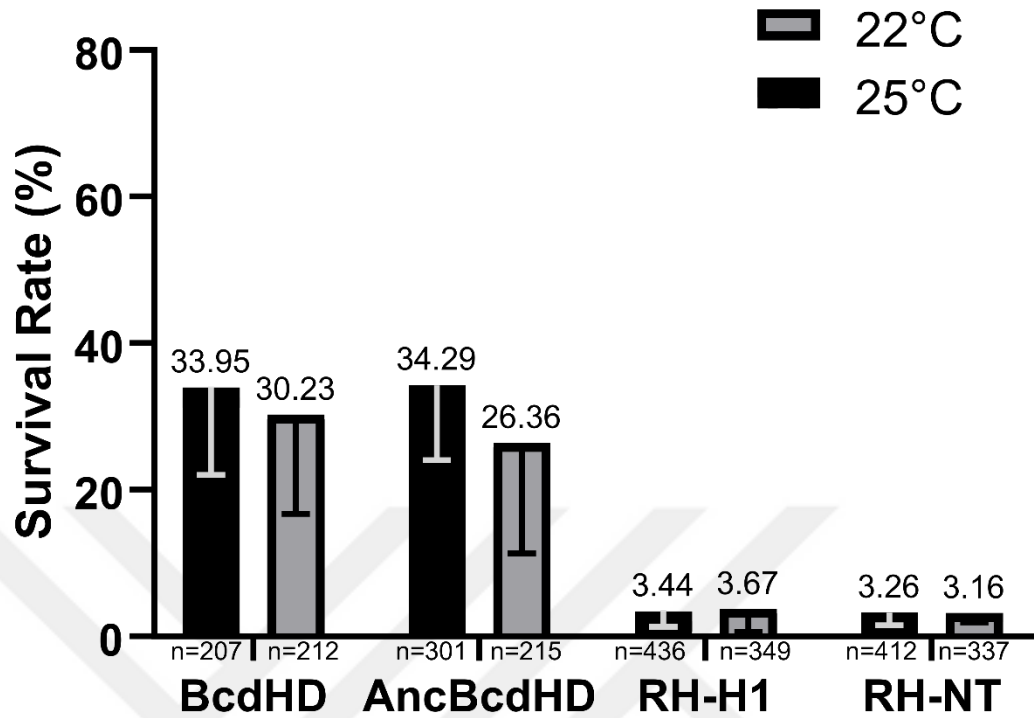


Figure 13: Survival rates of weak *bcd* mutant lines at 22 °C and 25 °C. Bar plots show the percentage of surviving embryos for BcdHD, AncBcdHD, RH-H1, and RH-NT genotypes. Gray bars represent 22 °C, and black bars represent 25 °C. Mean survival rates are indicated above each bar, with sample sizes (*n*) shown below. Error bars represent standard error of the mean (SEM).

Survival analysis of weak *bcd* mutants showed marked differences between genotypes. Both BcdHD and AncBcdHD lines exhibited moderate survival rates, with BcdHD surviving at ~34% at 25 °C and ~30% at 22 °C, while AncBcdHD showed a similar trend (34% at 25°C vs. 26% at 22°C). By contrast, survival in RH-H1 and RH-NT was dramatically reduced, with average rates below 4% at both temperatures shown in Figure 13.

Next, their mutant cuticle phenotypes are analyzed by counting all the cuticles and dividing the mutant phenotype count by the total number of cuticles counted to obtain the ratio of mutant phenotypes. The frequency of mutant cuticle phenotypes varied across weak *bcd* mutants and temperatures Figure 14. BcdHD embryos showed ~24% mutant cuticles at 25 °C, which decreased slightly to ~17% at 22 °C. Similarly, AncBcdHD displayed low mutant frequencies (8% at 25 °C, 11% at 22 °C). In contrast, RH-H1 mutants had the highest defect rates, with ~32% at 25 °C and ~23% at 22 °C.

RH-NT embryos showed consistently elevated mutant frequencies at both temperatures (~26%).

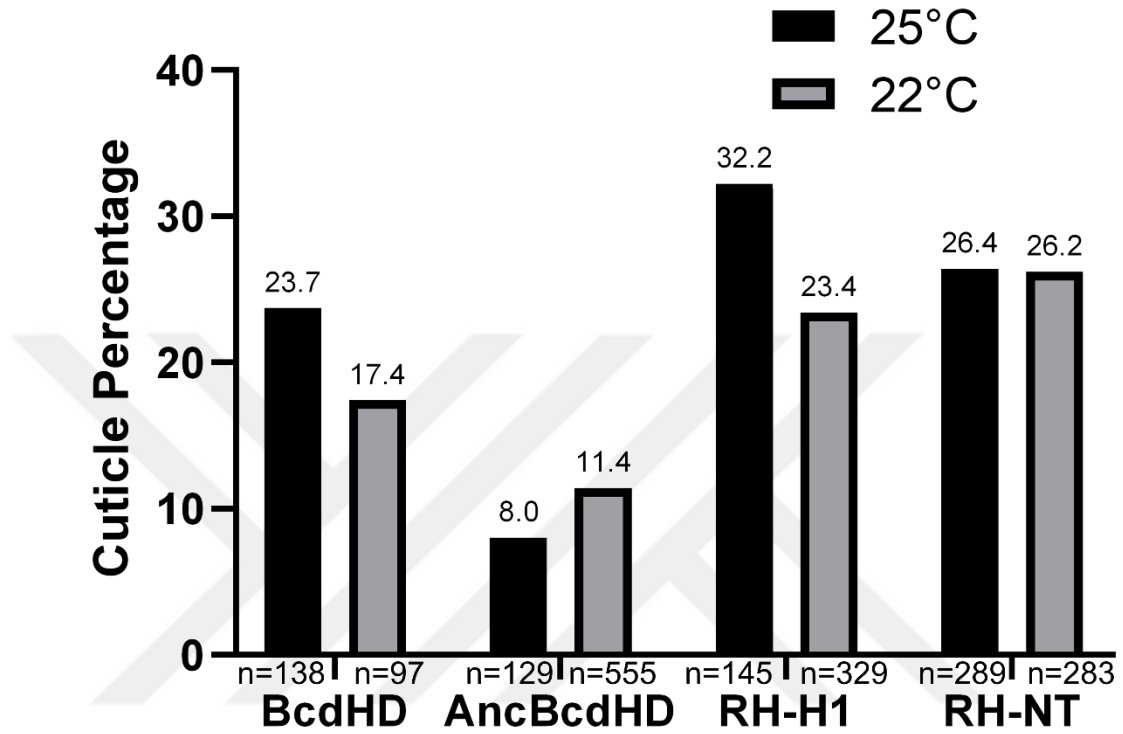


Figure 14: Mutant cuticle phenotype percentages among all cuticles counted in weak *bicoid* lines at 22 °C and 25 °C. Bar plots show the proportion of cuticles displaying mutant phenotypes in BcdHD, AncBcdHD, RH-H1, and RH-NT genotypes. Gray bars represent 22 °C and black bars represent 25 °C. Values above the bars indicate mean percentages, and sample sizes (*n*) are shown below each genotype.

After confirming low survival rates and variable mutant cuticle phenotypes in weak *bcd* mutant lines, we turned our attention to embryonic gene expression starting with *hb* due to it being the direct target of *Bcd*.

4.5.1 Embryonic Gene Expression of *hb* in weak *bcd* Mutant lines of *D. melanogaster* at 25°C

To investigate the variation in the embryonic gene expression profiles at different temperatures *hb* pbp of weak *bcd* mutants are calculated the same way we did for isogenic and Saimbeyli lines. These weak *bcd* mutants were already known to have *hb*

pbp that are shifted towards anterior (Önal et al., 2021), on that article it states on Figure 4, that RH-H1 had an average *hb* pbp of 35% EL and RH-NT was 32% EL.

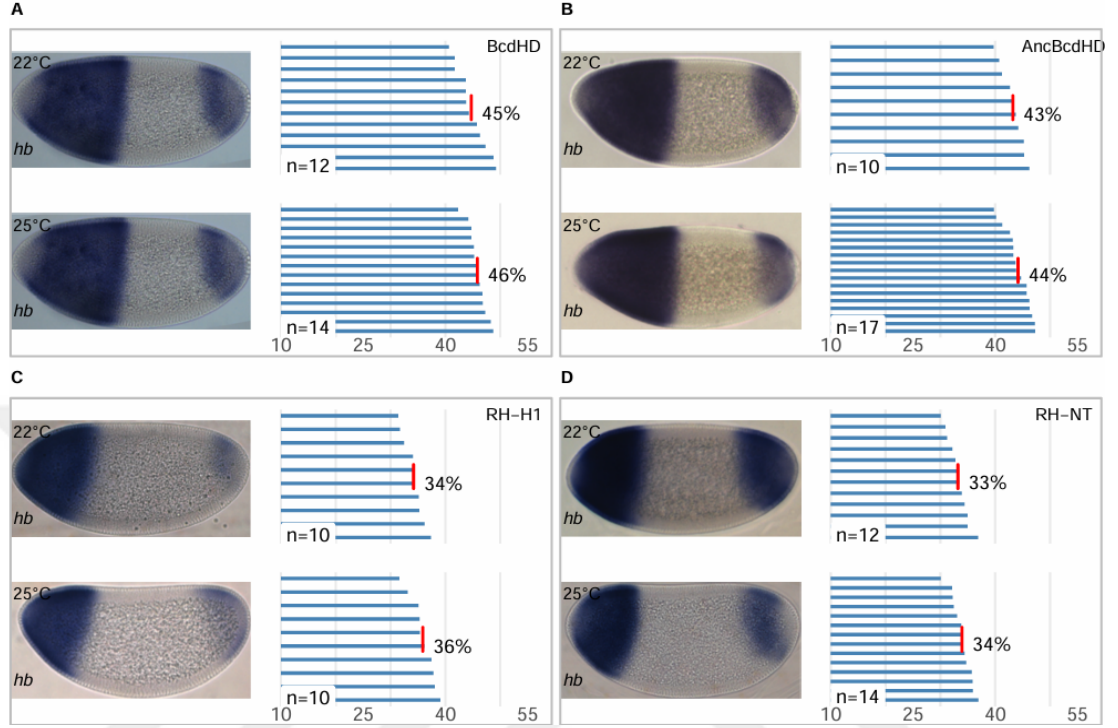


Figure 15: Pbp of *hb* expression in weak *bicoid* mutant lines at 22 °C and 25 °C. Representative embryos (left) and averaged posterior boundary positions (right) are shown for (A) BcdHD, (B) AncBcdHD, (C) RH-H1, and (D) RH-NT. Each horizontal line represents an individual embryo; the red vertical line indicates the mean boundary position, with values expressed as percentage of embryo length from the anterior. Sample sizes (*n*) are indicated for each condition.

Table 6: Mean positions and variances of the *hb* pbp in weak *bcd* mutant lines at 22 °C and 25 °C. The table shows mean *hb* pbp positions (as percenta embryo length) and corresponding variances for BcdHD, AncBcdHD control and RH-H1, and RH-NT mutant lines. Comparisons between 22°C and 25°C were performed using Welch's *t*-test (mean position) and F-test (variance).

	22°C		25°C		22°C vs 25°C	
	Mean	Variance	Mean	Variance	t-test	F-test
BcdHD	44.7239	7.7226	45.8323	2.8144	0.2438	0.0870
AncBcdHD	43.2232	4.5739	44.1635	5.5113	0.2999	0.8015
RH-H1	34.1895	3.6894	35.8706	5.4068	0.0955	0.5782
RH-NT	33.2013	3.8295	33.9332	3.4694	0.3412	0.8545

The *hb* pbp variation levels at 25°C were variable as shown in Table 6, for BcdHD it was 2.8 and AncBcdHD showed a within group variation of 5.5. For the mutant RH-H1 it was 5.4 and for RH-NT it was 3.5. At 25°C AncBcdHD showed the highest variation (5.5) and BcdHD (2.8) was the lowest in terms of within group variation. We then calculated the average *hb* pbp on these lines at 25°C shown in Figure 15 and Table 6. BcdHD's average *hb* pbp were 45.9% and AncBcdHD were 44.1%. The mutant lines were known to have anteriorly shifted *hb* pbp compared to WT and control groups, and our results were consistent with the previous studies. RH-H1 showed an average *hb* pbp of 35.8% and RH-NT was 34% shown in Figure 15.

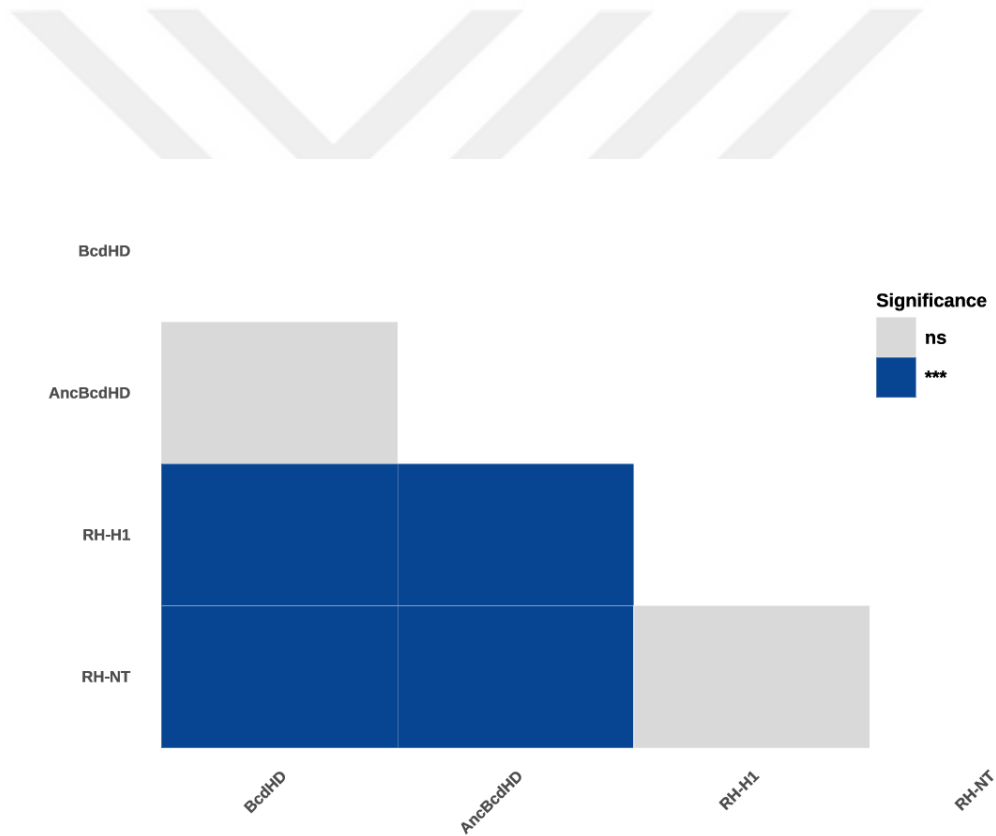


Figure 16: Statistical comparison of *hb* pbps among weak *bicoid* mutant lines at 25 °C. Pairwise comparisons were performed using Tukey–Kramer post-hoc test with a 95% confidence interval. Color coding indicates significance: ns = not significant, *** = $p < 0.001$. Significant values indicate differences in mean *hb* boundary position between the compared mutant lines.

At 25°C shown in Figure 16, statistical comparisons revealed that RH-H1 and RH-NT mutants showed significantly different *hb* pbp positions compared to both BcdHD and AncBcdHD. In contrast, BcdHD and AncBcdHD did not differ significantly from each other (ns).

4.5.2 Embryonic Gene Expression of *hb* In Weak *Bcd* Mutants of *D. melanogaster* Against Temperature Perturbation of 22°C

Next, we wanted to observe how 22°C temperature perturbation would affect the pbp of *hb* expression and the variation within the group. As shown in Table 6, BcdHD showed a variation of 7.7 and AncBcdHD was 4.5. RH-H1 and RH-NT were 3.7 and 3.8 respectively. In summary at 22°C highest variation was observed in BcdHD (7.7) and the lowest variation was observed in RH-H1 (3.6).

Then we compared their average *hb* pbp at 22°C as they are shown in Table 6 and Figure 15 A and B. BcdHD showed a *hb* pbp of 45% and AncBcdHD was 43%. RH-H1 had an average *hb* pbp of 34% and RH-NT was 33% shown in Figure 15 C and D. In summary BcdHD had the most posterior (45%) average *hb* pbp and RH-NT (33%) had the most anterior average *hb* pbp at 22°C.

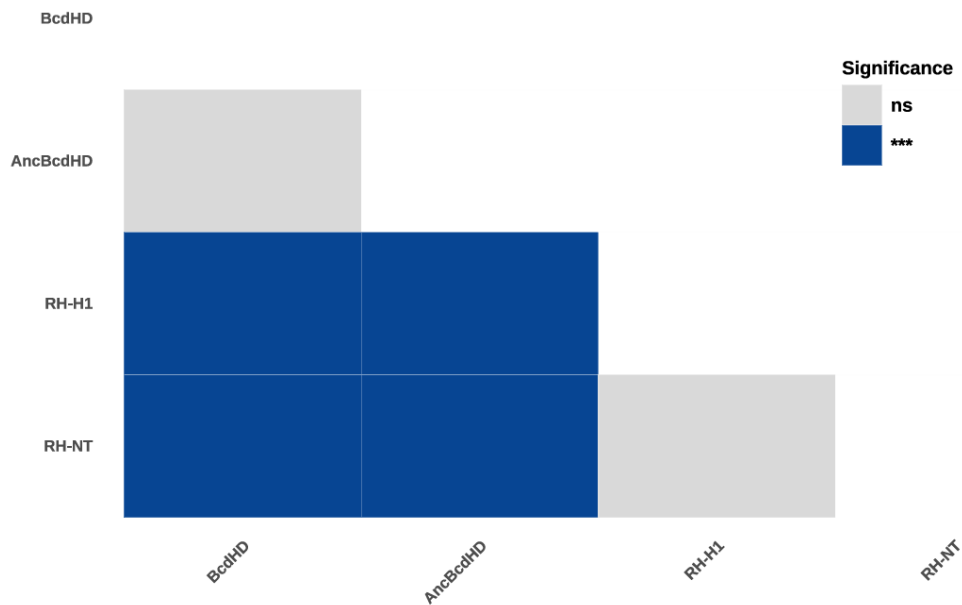


Figure 17: Statistical comparison of *hb* pbp among weak *bicoid* mutant lines at 22°C. Pairwise comparisons were performed using Tukey–Kramer post-hoc test with a 95% confidence interval. Color coding indicates the level of significance: ns = not significant, * = $p < 0.05$, *** = $p < 0.001$. Significant values indicate that the mean *hb* pbp differs between the compared lines.

At 22°C shown in Figure 17 statistical comparisons showed that RH-H1 and RH-NT were significantly different from BcdHD and AncBcdHD, in contrast BcdHD and AncBcdHD were not significantly different from each other in terms of their *hb* pbps.

4.5.3 The Average Position of *hb* pbp and Variation at 22°C Compared to 25°C of Weak *bcd* Mutants in *Drosophila* Lines.

We compared the change in the variation observed under 22°C temperature perturbation, for BcdHD variation value changed from 2.8 to 7.7. AncBcdHD's variation level has changed from 5.5 at 22°C to 4.5 at 25°C. RH-H1 and RH-NT had a variation of 5.4 and 3.4 and these variations changed to 3.7 and 3.8 respectively.

Next, we compared the average *hb* pbp of weak *bcd* mutant lines at 22°C temperature perturbation shown in Figure 15 to check if they are resistant against temperature perturbation, BcdHD *hb* pbp showed a little to no change (ns) towards anterior, from 46% at 25°C to 45% at 22°C. AncBcdHD's *hb* pbp was 44% at 25°C and it shifted towards anterior to 43% (ns). RH-H1 had an average *hb* pbp value of 36% at 25°C and it shifted towards anterior to 34% again this change was also not significant. RH-NT was 34% at 25°C and it again shifted towards anterior to 33% (ns). **In conclusion**, none of the shifts observed under 22°C temperature perturbation were significant.

4.5.4 Embryonic Gene Expression of *eve* Second Stripe In Weak *bcd* Mutants of *D. melanogaster* at 25°C

Investigating the expression of *eve* second stripe in weak *bcd* mutants is important in understanding how temperature effects the variation and average position of this stripe because that stripe is indirectly regulated by maternally deposited *bcd* through it being the regulatory element of the gap gene's expression such as *hb*, *Krüppel* and *giant*. (Frasch & Levine, 1987; Small et al., 1991)

Investigation of the second stripe of *eve* is done the same way as the isogenic and mass population explained previously. First their average second stripe positions and their variations are calculated at 25°C. The variation levels for *eve* second stripe at 25°C are calculated and shown in Table 7. BcdHD had a variation of 0.8 and AncBcdHD was 1.2. RH-H1 and RH-NT were 8 and 2.5 respectively at 25°C. In summary, BcdHD had the lowest (0.8) amount variation in its second stripe position and RH-H1 had the highest (8).

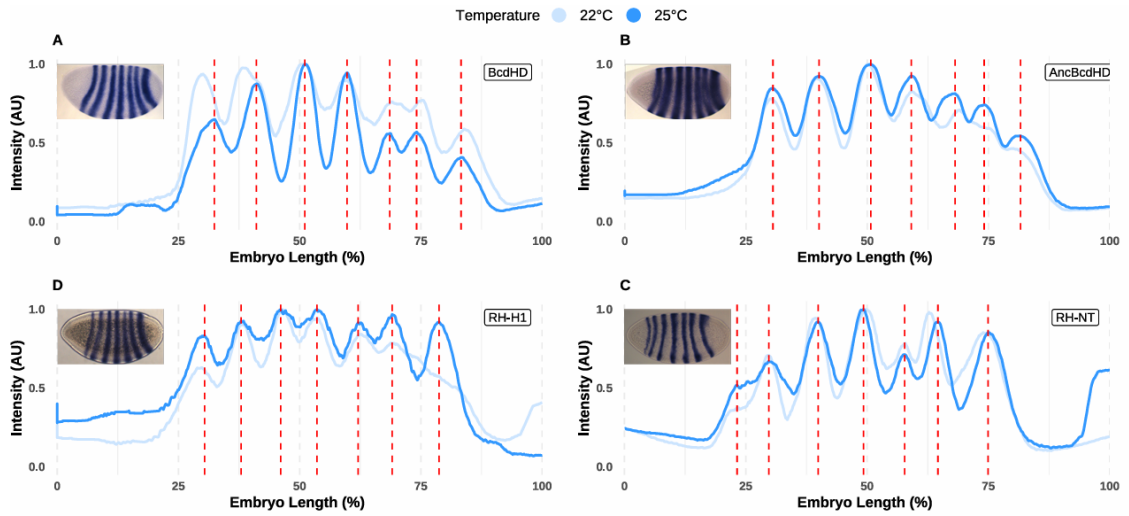


Figure 18: Average *eve* stripe intensity profiles in weak *bicoid* mutant lines at 22 °C (light blue) and 25 °C (dark blue). Blue curves represent mean intensity along the anterior–posterior axis; 0 being the most anterior 100 being the most posterior, red dashed lines provide approximate stripe references. Insets show representative embryos at 25 °C profiles were generated by averaging multiple embryos per condition to assess temperature-dependent variation in stripe patterning.

Table 7: Mean and variance of *eve* stripe 2 position across weak *bcd* mutants *Drosophila melanogaster* strains at 22 °C and 25 °C. Pairwise comparisons were performed using Welch’s *t*-test (with 25 °C as the reference) to assess differences in mean stripe positions, and *F*-tests were applied to compare variances between temperatures. Reported *p*-values indicate whether mean positions (*t*-test) or variances (*F*-test) differ significantly between conditions.

Strain	22 °C		25 °C		22°C vs 25°C	
	Mean	Variance	Mean	Variance	t-test	F-test
BcdHD	40.623	5.293	40.741	0.837	0.928	0.1070
AncBcdHD	39.813	1.159	40.231	1.280	0.461	0.8980
RH-H1	36.116	10.999	36.083	8.081	0.982	0.6980
RH-NT	32.724	15.458	30.737	2.547	0.189	0.0196

We also found average second stripe positions of weak *bcd* mutants, they are shown in Figure 18 and Table 7. BcdHD had an average second stripe position of 40.7 % and AncBcdHD had 40.2%. RH-H1 and RH-NT were 36% and 30.7% respectively. Next, we checked if they were significantly different from each other at 25°C. In summary

BcdHD had the most posterior (40.7%) second stripe position and RH-NT had the most anterior (30.7) one.

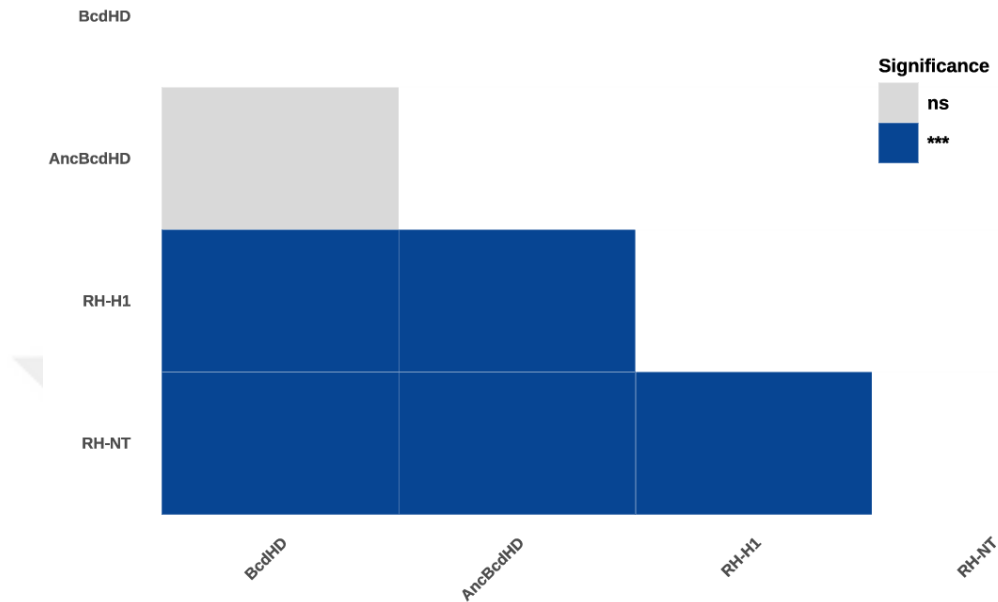


Figure 19: Statistical comparison of *eve* 2nd stripe positions among weak *bicoid* mutant lines at 25 °C. Pairwise comparisons were performed using Tukey–Kramer post-hoc test with a 95% confidence interval. Color coding indicates significance: ns = not significant, *** = $p < 0.001$. Significant values indicate differences in mean *eve* second stripe position between the compared mutant lines.

At 25°C differences among weak *bcd* mutant lines were observed for the second stripe of *eve* shown in Figure 19, both mutant lines RH-H1 and RH-NT were significantly different from BcdHD and AncBcdHD whereas the control lines did not significantly differ from each other.

4.5.5 Embryonic Gene Expression of *eve* Second Stripe In Weak *Bcd* Mutants of *D. melanogaster* Against Temperature Perturbation of 22°C.

After 25°C, we used 22°C temperature perturbation to see how it will change the variation and average values of *eve* second stripe positions, results are shown in Table 7 Figure 19 and Figure 22. At 22°C BcdHD had variation level of 5.2 and AncBcdHD was 1.1. The mutant lines RH-H1 and RH-NT were 11 and 15.4 respectively, none of the changes in variation were significant. AncBcdHD (1.1) was the lowest and RH-NT (15.4) had the highest variation.

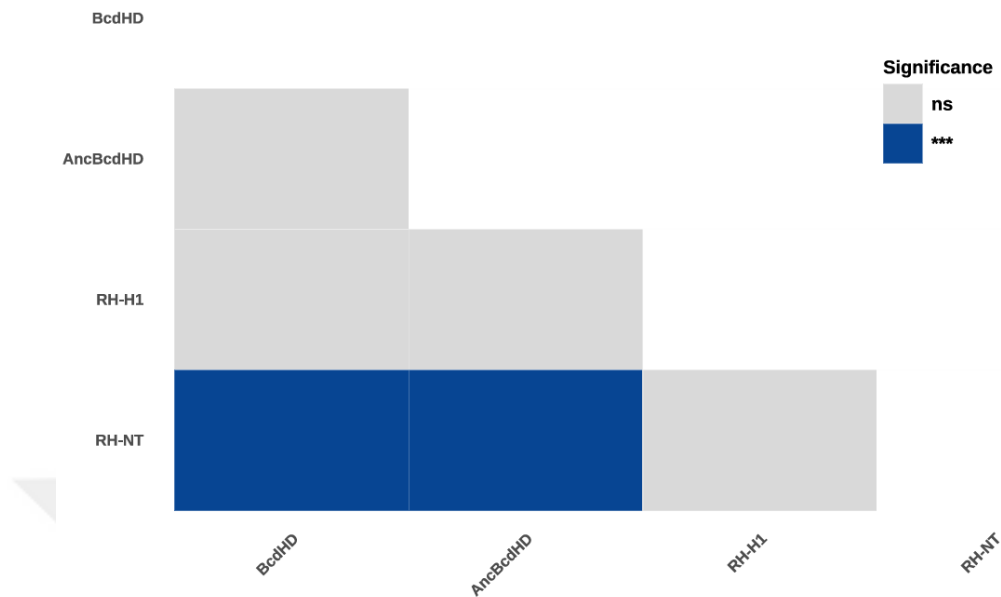


Figure 20: Statistical comparison of *eve* 2nd stripe positions among weak *bicoid* mutant lines at 22°C. Pairwise comparisons were performed using Tukey–Kramer post-hoc test with a 95% confidence interval. Color coding indicates the level of significance: ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Significant values indicate that the mean *eve* second stripe differs between the compared lines.

At 22°C only RH-NT showed a significant difference from BcdHD and AncBcdHD.

4.5.6 The Average Position of *eve* Second Stripe Position and Variation at 22°C Compared to 25°C of Weak *bcd* Mutants in *Drosophila* Lines

We compared average second stripe positions at 22°C Figure 7 and Table 7. BcdHD's average second stripe position showed a little to no change, it was 40.7% at 25°C and it became 40.6% at 22°C. AncBcdHD *eve* 2 position was 40.2% at 25°C and it shifted slightly towards anterior to 39.9% at 22°C. RH-H1 was 36% at 25°C and it changed to 36.1% at 22°C. RH-NT shifted towards posterior with perturbation, from 30.7% at 25°C to 32.7% at 25°C ($p > 0.05$). The variation levels also changed with the temperature perturbation shown in Figure 22 BcdHD's variation level changed from 0.8 at 25°C to 5.2 at 22°C shown in Figure 21. AncBcdHD had a variation level of 1.3 at 25°C and it became 1.1 at 22°C. RH-H1 and RH-NT had a variation of 8 and 2.5 and they changed to 11 and 15.4. Only the change in variation of RH-NT was significant according to the F-test shown in Table 7.

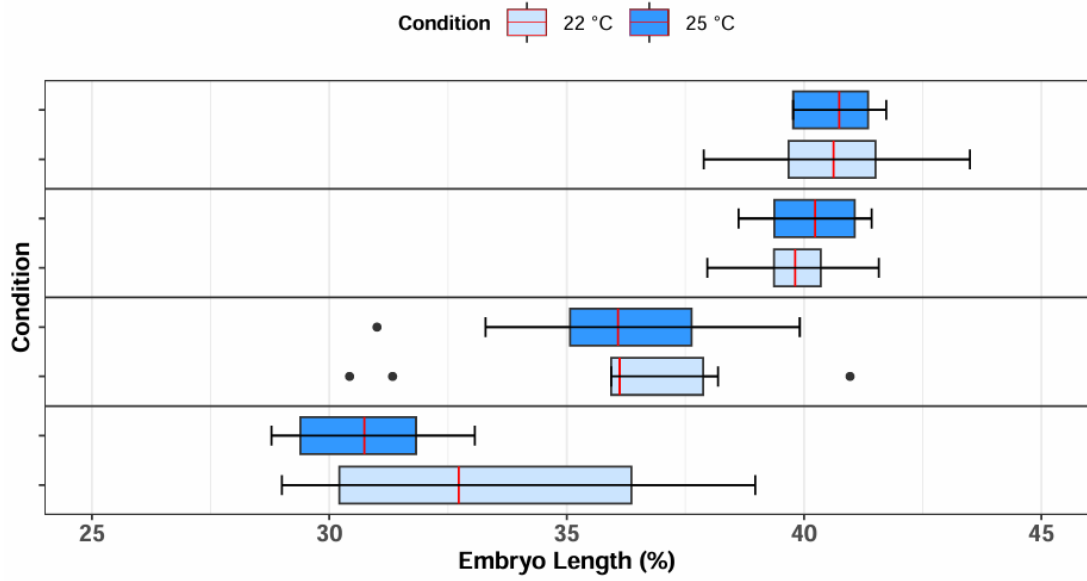


Figure 21: Variation in *eve* 2nd stripe position across temperatures in weak *bcd* mutants of *Drosophila* lines. Lines in the figure are as follows: BcdHD, AncBcdHD, RH-H1 and RH-NT. Stripe positions (expressed as % embryo length) are shown for 22 °C (light blue), 25 °C (medium blue). Statistical comparisons between temperatures within each line were assessed by Student's *t*-test; significance is indicated as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

4.6 Bcd Gradient's Length Constant Comparison at 25 and 22°C in Weak *bcd* Mutants

The Bcd protein gradient is an essential organizer of the anterior-posterior axis during early *Drosophila* embryogenesis. It is a maternal effect gene whose mRNA is localized at the anterior tip of the egg during oogenesis. After fertilization, the mRNA is translated into the protein, which diffuses and forms a concentration gradient that decreases exponentially from the anterior to the posterior of the embryo. This gradient functions as a morphogen, providing positional information that directs cells to adopt specific fates along the body axis. This gradient is instructive in the expression and positioning of several gap and pair rule genes such as *hb* and *eve*.

To investigate how temperature perturbations influence the shape and the distribution of *Bicoid* protein gradient, we performed immunofluorescent staining of Bcd protein and quantified the signal intensity data along anterior-posterior axis of the *D. melanogaster* embryo. For each embryo raw fluorescence intensity was extracted and fitted with an exponential decay function, this resulted in a decay length constant (λ). Where smaller λ value means a steeper rapidly decaying gradient whereas larger λ means a shallower gradient which is extending further through the posterior on the

embryo, by comparing λ values among embryos across temperatures we were able to assess whether Bcd gradient stays robust against temperature perturbation.

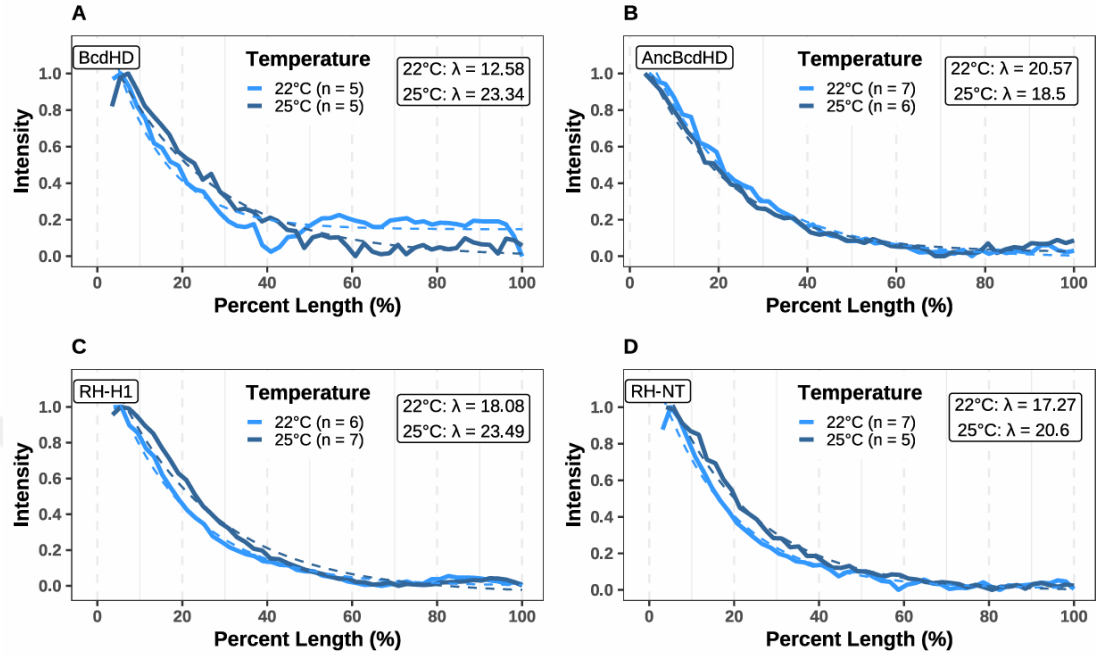


Figure 22: Bicoid (Bcd) protein gradients in weak *bicoid* mutant lines at 22 °C and 25 °C visualized by immunostaining. Intensity profiles along the anterior–posterior axis are plotted, with solid lines representing raw intensity measurements and dashed lines showing fitted exponential decay curves. λ values denote the exponential decay length constant of the Bcd gradient: smaller λ corresponds to a steep gradient that decays rapidly, while larger λ indicates a shallow gradient extending further into the embryo. Sample sizes (n) are indicated for each condition.

Results are shown in Figure 22, The gradient length constant (λ), which reflects how steeply Bcd concentration decays along the embryo (anterior-posterior axis) decreased under lower temperature conditions a higher λ shows us a shallow gradient with having a faster decay. BcdHD had a λ value of 23.3 at 25°C and this decreased to 12.6 at 22°C. AncBcdHD was 18.5 and 20.6 for 25 and 22°C respectively. RH-H1 had a λ of 23.5 at 25°C and it decreased to 18 at 22°C. RH-NT's λ value has decreased from 20 at 25°C to 17.3 at 22°C.

5. DISCUSSION

While early *Drosophila* development is often described as robust, the extent to which environmental perturbations and genetic homozygosity contribute to this robustness has been less investigated. Little is known about the genetic background and how it influences the equilibrium between robustness and plasticity when conferred with environmental stress. In this study we investigated how exposure to fluctuating temperature affects the positional information provided to early *Drosophila* embryo by early developmental decisions and mechanisms, for this we focused on three molecular readouts of the segmentation network, *hb* pbp, *eve* second stripe position and the steepness of Bcd. We used widely used laboratory strains, moderately genetically diverse mass population line and two of its constituents and sensitized weak *bcd* mutants. With this design, we were able to tell the difference between genetic variation from the non-genetic variability caused by the environmental stress and find the answer to which markers behave more plastic, and which are more robust across conditions.

Generally used wild-type laboratory strains look similar compared to perturbed conditions (with some significant differences between some lines such as Saimbeyli lines and Berlin K significantly differs at 25°C) at the optimal 25°C for their mean *hb* pbps and their differences are increased at 22°C and 29°C mainly variation is increased under any of the perturbation conditions shown in Figure 4 and 5. The statistical difference between groups at the same temperature is increased upon temperature perturbation this is clearly the case for *eve* second stripe positions shown in Figure 8 and 9. This might suggest environmental stress such as temperature fluctuations could increase the variation and reduce the robustness of these strains for a given trait and it tells us that the change in their robustness against temperature perturbation is differential, and this might be coming from non-adaptive allelic differences they each possess.

Another observation was, Canton S showed the highest variance at *hb* pbp level at 25°C (5.4) for Canton S average *hb* pbp interval was from 43% EL to 51% EL whereas Saimbeyli Mass showed the lowest variance (1.5) and the interval was 45% EL and 49% EL. For example, a 5% shift in *hb* pbp in EL means approximately 300 nuclei (given an estimated 12 nuclei per um and a total embryo length of 500 um) (Foe & Alberts, 1983; Lecuit & Wieschaus, 2000). This highlights that robustness is not

simply coming from the mechanisms on the segmentation network, but it comes from the many interactions between diversity (genetic variability) and buffering, long term inbreeding could cause the eruption of canalization of a trait whereas genetic diversity, even when its moderate, might be partially restoring it. This observation explains the classical description of canalization, explained by Waddington (1957), where it states that developmental systems buffer against environmental or genetic perturbations to produce consistent phenotypes. When the same lines are exposed to temperature perturbation the differences that once were hidden became more apparent shown in Figure 3, Oregon R variance for *hb pbp* increased when perturbed with 22°C and its mean *hb pbp* shifted significantly at 29°C whereas the Saimbeyli Mass remained more stable against the same perturbation. These findings indicate that environmental perturbation, such as temperature, can trigger average shifts of the markers and changes in variation in isogenic lines while Saimbeyli Mass remained relatively unaffected by this perturbation due to having genetic variability. Same perturbation (22°C or 29°C) condition is met with divergent reactions in magnitude (some of them are significant and some of them are not) and different directions (some shifts towards posterior, some towards anterior). This suggests that robustness is not a universal mechanism rather depends on the specific allelic combinations on a given strain, for instance Oregon R showed a strong reaction by significantly shifting its *hb pbp* while Berlin K only shifting slightly. These contrasting responses shows that robustness is unique on the specific allelic background of each different strain (Gibson & Dworkin, 2004; Paaby & Rockman, 2014).

Another important comparison was the isogenic wild type lines and isogenic weak *bcd* mutants, weak mutants were much more variable both for *hb pbp* and *eve* second stripe shown in (Table 4,5,6 and 7), for example, *hb pbp* variation at 25°C were varying between 1.6 in Berlin K to 5.3 in Canton S, whereas it was 5.4 in RH-H1 at 25°C, indicating that weakened maternal input increased the embryo to embryo variability. More importantly this excess variation was not limited to molecular signatures in weak *bcd* mutants, they also affected the downstream processes of development, their viability was low and they manifested mutant cuticle phenotypes shown in Figure 13 and 14. These results demonstrate that maternal Bcd gradient is very sensitive against changes in the environment, once the maternal input is compromised with mutations, as in the sensitized *bcd* mutants, early molecular markers became highly variable and

lose canalization towards successful development into adulthood. For instance RH-NT embryos showed a dramatic increase in *eve* second stripe variance even at benign temperatures showing how changes in the maternal input destabilize the entire developmental system and compromise robustness against stress.

Upon comparing all the molecular readouts in this study, *hb pbp* strikes as the most sensitive among the three. In the Isogenic and Mass Population comparisons five out of six isogenic lines responded to 29°C by shifting towards posterior significantly, Saimbeyli Mass remained stable, ($p > 0.05$). In contrast to *hb pbp*, *eve* second stripe was more robust against this temperature perturbation shown in Figure 7 and Figure 11, only Berlin K, Oregon R and Saimbeyli Mass shifted towards posterior, other shifts were not significant. This difference between *hb pbp* and *eve* second stripe is most likely resulting from the enhancer logic; where *hb* is a direct target of Bcd which is sensitive to alterations in the threshold of the protein, whereas *eve* second stripe uses multiple activators and repressors which are buffering positional precision against environmental fluctuations (Small et al., 1992; Jaeger, 2011). This conclusion answers the second research question (Does temperature alterations reveal hidden phenotypic variation?), showing constant perturbation of 29°C, reveals variation otherwise hidden at optimal temperatures. In addition, the analysis of the length constant (λ), showed us that λ values decreased almost consistently at 22°C when compared to 25°C, this produced a steeper gradient. A steeper gradient results in a reduced range of positional information provided by the Bcd protein, which as a result may reduce the effective activation of downstream target genes at the proper positions.

A key interest in this study is how variation levels differ between genetically heterozygous Saimbeyli Mass and other isogenic lines across temperatures. For the *hb pbp*, Saimbeyli Mass consistently showed lower variance values than most isogenic lines at perturbation temperatures 22°C and 29°C. This means that moderate genetic diversity in the Saimbeyli Mass contributes to canalization. In contrast *eve* second stripe position showed a different pattern at 25°C, isogenic lines such as Canton S and Oregon R showed elevated variation values compared to Saimbeyli Mass but at 29°C some isogenic lines experienced reduction in variance while Saimbeyli Mass remained relatively stable, this suggests that unlike *hb pbp*, *eve* second stripe variation is less consistently buffered by genetic diversity and its variance can even decrease upon temperature stress. All together these results highlight that genetic diversity in

Saimbeyli Mass, help regulate and buffer the variation in traits that are tightly linked to maternal input like *hb pbp* but other traits such as *eve* second stripe which are under complex regulation, may respond in more unexpected ways against environmental perturbations. These results directly addresses our fourth research question (Is the within-group variation higher or lower in the moderately genetically diverse out-bred population Saimbeyli Mass?).

Previous studies has shown that temperature can influence the dynamics in development in different ways, Kuntz and Eisen (2014), stated that embryogenesis is scaling uniformly with temperature, saying that developmental timing stays accurate despite environmental fluctuations. Cheung et al. (2015) stated that temperature variations alters the Bicoid gradient but the dependant activation of *hb* transcription remains robust. Building on these findings, our study showed that even when viability and major patterning boundaries are maintained across temperatures, additional features such as *eve* second stripe showed strain specific sensitivity to temperature.

One of the most important question in this all comparative study was the, comparison between long-inbred isogenic lines (Berlin K, Canton S, Oregon R and *yw*) and newly formed genetic variability retaining Saimbeyli Mass. At 25°C Saimbeyli Mass showed the least amount of variance for its *hb pbp* (1.5) whereas Canton S showed the highest (5.3) (Table 4). Supporting the idea that heterozygosity (genetic variability) is the mechanism that is enhancing developmental precision while repeated inbreeding erodes it, the same effect continued while under 29°C temperature perturbation, when five out of six isogenic lines shifting significantly for their *hb pbp*, Saimbeyli Mass remained robust against temperature change. Suprisingly, Saimbeyli Mass experienced a significant shift in its *eve* second stripe when perturbed under 29°C, from 41.9% to 43.3% EL ($p < 0.05$), whereas its two founders Saimbeyli 1 and Saimbeyli 2 did not show such significant change, hence the idea that same genetic variability (found moderately) in Saimbeyli Mass, helped to canalize *hb pbp*, showed plasticity in *eve* second stripe. This division of labor might suggest that heterozygosity does not buffer all traits the same, rather it might stabilize and canalize some traits while letting other traits act more plastic and flexible. These findings are in line with the theory of robustness-evolvability balance, which states that robustness and evolvability is regulated by molecular systems and developmental mechanisms under fluctuating environments. Robusntess does not mean complete invariance, some traits remain

canalized while some others are allowed to act plastic. In our case Saimbeyli Mass canalized *hb* pbp but let *eve* second stripe to vary, showing how different traits can be selectively buffered or exposed to variation depending on the developmental network. (Wagner, 2005; Félix & Wagner, 2008). Another issue is, these molecular changes and fluctuations did not jeopardize the ability of the organism to survive, survival rates (some dropped at 29°C shown in Figure 12) stayed high for most strains, both isogenics and Mass Population, this results raises the idea that *hb* pbp shifts in isogenic lines are met with buffering mechanisms happening downstream. This links back to our third research question (Are non-isogenic populations of *D. melanogaster* more robust against temperature perturbations?). The moderately genetically diverse Saimbeyli Mass population showed canalization of *hb* while allowing *eve* to remain more plastic.

Our pairwise tests show the strongest between-line separation at 22 °C (for *hb* PBP, and to a lesser extent for *eve*-2). At 25 °C most pairs are less significantly different, consistent with this being the optimal condition where lines converge. At the cooler 22 °C, slower kinetics likely lengthen the patterning window, making the system more permissive to positional variation and exposing genotype specific differences in maternal input and downstream regulation. At 29 °C, divergence reappears in some lines, but not as broadly as at 22 °C, heat imposes common constraints and reduces viability, yielding selective, line-dependent departures. Overall, temperature tunes how much genetic background is expressed: 22 °C unmasks variation, 25 °C buffers it, and 29 °C produces targeted differences rather than a global spread.

In contrast to these, weak *bcd* mutants showed what would be the case when this buffering capacity is sensitized by compromised maternal input, the Bcd gradient's steepness increased, with temperature shifts and variance increased greatly (for *eve* second stripe Table 5) and downstream *hb* and *eve* boundaries became noisy, these inconsistencies directly correlated with organismal failure both mutant lines survived less than 4% shown in Figure 12.

6. CONCLUSION AND FUTURE DIRECTIONS

In conclusion, robustness and plasticity of the early patterning in *Drosophila* is not evenly distributed, *hb pbp* appears to act more plastic because it's a direct target of the gradient formed by Bcd and it reacts more to the fluctuations occurring in the threshold, (Driever & Nüsslein-Volhard, 1988; Gregor et al., 2007). In contrast, *eve* second stripe uses many different activators and repressors in its regulation which helps buffering against fluctuations. Saimbeyli Mass line shows us how genetic variability brings about robustness that is distributed, it canalizes *hb* but allows *eve* to be more plastic in comparison. On the other hand weak *bcd* mutants shows us how robustness is broken down when the maternal input is destabilized, shortening of the Bcd length constant (steeper gradient), noisy *eve* and *hb* boundaries, increased mutant cuticle phenotype and finally lowered viability. Hence, the genetic background and the stability of maternal inputs act together to decide which developmental pathways are buffered against fluctuations and which are allowed to remain more plastic, ultimately ensuring the organism's adaptability.

While this study brings new insights to how genetic background shapes an organism's robustness and plasticity levels, many limitations of the study should be acknowledged, some cases the number of the embryos that are analyzed were low, that's because when working with different temperatures, the maturation time of the embryos should be taken strongly into consideration, and all analysis require embryos that are at the same stage of their development. Small number of embryos reduces the statistical power of the analysis. Another limitation is, although we quantified the length constant in weak *bcd* mutants, we had trouble while repeating the same experiment for the isogenic and mass population lines due to problems with DAPI staining. This analysis is important because it connects maternal input to downstream readouts of the development such as *hb pbp* and *eve*. Third limitation is, Saimbeyli Mass line was generated from five different isogenic founders, but we were only able to use two of them for comparison and other analysis. The other three founders are also necessary to understand each founder's individual contribution to the plasticity and robustness of the strain, for a complete comparison we need to include them to the study but that would still not change the fact that the variability is still moderate. To fix that in the future, the major goal is to put together a mass population strain that has 15-20 founders, to increase the genetic variability (in contrast to Saimbeyli Mass

which had only five founders and had moderate genetic variation) and provide a further comparison between purely inbred isogenic strains and a mass bred line possessing high levels of genetic variation. We measured pattern positions only (Bcd shape, hb boundary, eve-2 position) and did not quantify RNA abundance, so we could not relate expression levels to positional variance, future studies include quantifying the *hb* and *eve* mRNA across lines and temperatures (e.g., smFISH or RT-qPCR) and test whether transcript level predicts boundary/stripe variance. These steps would not only enhance our conclusions, but they would also put together a foundation to further investigate how increased genetic variation helps developmental stability, when faced with environmental perturbation.



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APPENDIX

Appendix 1: Codes Used

Appendix 1a:Data_Point_Equalizer

```
import pandas as pd
import numpy as np

# Number of files to process
# Change the number according to the number of files
num_files = 13

for i in range(1, num_files + 1):
    # Construct input and output file names
    input_file = f'BK_{i}.xlsx'
    output_file = f'output{i}.xlsx'
    selected_file = f'selected_file{i}.xlsx'

    # Read the input file
    df = pd.read_excel(input_file)

    # Normalize the first column by its maximum value
    max_value = df.iloc[:, 0].max()
    df.iloc[:, 0] = df.iloc[:, 0] / max_value

    # Save the modified data to an output file
    df.to_excel(output_file, index=False)
    print(f'The modified data has been saved to {output_file}')

    # Read the modified file
    df = pd.read_excel(output_file)

    # Ensure 200 samples are selected
    num_samples = 600
    indices = np.linspace(0, len(df) - 1, num_samples, dtype=int)
    selected_df = df.iloc[indices]

    # Save the selected samples to a new file
    selected_df.to_excel(selected_file, index=False)
    print(f'The selected data has been saved to {selected_file}')
```

Appendix 1b: Automize

```
import pandas as pd
import os

# Get the current working directory where the script is located
directory_path = os.path.dirname(os.path.realpath(__file__))

# Define a function to process each Excel file
def process_excel_file(file_path):
    # Load the Excel file into a DataFrame
    data = pd.read_excel(file_path)

    # Multiply the values in the first column by 100
    data.iloc[:, 0] = data.iloc[:, 0] * 100

    # Find the maximum value in the second column
    max_value_col2 = data.iloc[:, 1].max()

    # Normalize the second column by dividing by its maximum value
    data['Normalized_Column2'] = data.iloc[:, 1] / max_value_col2

    # Apply a transformation to invert (Since ImageJ counts the gray value as intensity but we need the colored
    # value, It needs to be reversed) the normalized values (1 - x)
    data['Normalized_Column2'] = 1 - data['Normalized_Column2']

    # Drop the original second column from the DataFrame
    data = data.drop(data.columns[1], axis=1)

    # Reorder columns: move 'Normalized_Column2' to the position of the dropped column (second column)
    columns = list(data.columns)
    columns.insert(1, columns.pop(columns.index('Normalized_Column2')))
    data = data[columns]

    # Return the processed DataFrame
    return data

# Loop through all files named 'selected_file1.xlsx' to 'selected_file20.xlsx' in the same directory
for i in range(1, 21): # Adjust this range depending on how many files you have
    file_name = f'selected_file{i}.xlsx' # Construct the file name
    file_path = os.path.join(directory_path, file_name) # Construct the full path to the file

    # Check if the file exists before processing
    if os.path.exists(file_path):
        # Process the file using the function defined above
```

```

processed_data = process_excel_file(file_path)

# Construct the output file name
output_file = f'processed_{file_name}'

# Save the processed data to a new Excel file WITHOUT headers
processed_data.to_excel(os.path.join(directory_path, output_file), index=False, header=False)

print(f"Processed data saved to {output_file} without headers")
else:
    # If the file is not found, skip and print a message
    print(f"File {file_name} not found. Skipping...")

```

Appendix 1c:pbp_Finder

```

import pandas as pd
import numpy as np
import matplotlib.pyplot as plt
from scipy.signal import savgol_filter, find_peaks
import os
from openpyxl import Workbook

# Define a simpler method to detect the point where signal stabilizes
def find_stabilization_point(y_section, threshold=0.002, min_length=5):
    # Calculate the gradient (slope) of the intensity values
    intensity_gradient = np.gradient(y_section)
    # Find points where the slope is small (signal is stable)
    stable_indices = np.where(np.abs(intensity_gradient) < threshold)[0]
    # Look for a continuous stable region of minimum length
    for i in range(len(stable_indices) - min_length + 1):
        if stable_indices[i + min_length - 1] - stable_indices[i] == min_length - 1:
            return stable_indices[i]
    return len(y_section) - 1 # Return last point if no stable section is found

# Process a single file to detect peaks and determine the change point
def process_sample(file_path):
    # Load the Excel file into a DataFrame
    data = pd.read_excel(file_path)

    # Separate columns into x (egg length) and y (fluorescence intensity)
    x = data.iloc[:, 0].values
    y = data.iloc[:, 1].values

    # Smooth the intensity signal using Savitzky-Golay filter

```

```

window_length = min(len(y) - 1, 51) if len(y) >= 51 else len(y) // 2 * 2 + 1
polyorder = 3
y_smooth = savgol_filter(y, window_length, polyorder)

# Find all significant peaks in the smoothed signal
peaks, _ = find_peaks(y_smooth, prominence=0.05, height=0.05)

# If at least two peaks are found, detect the midpoint of the decreasing section between them
if len(peaks) >= 2:
    prominences = y_smooth[peaks]
    sorted_indices = np.argsort(prominences)[-2:] # Get top two peaks
    major_peaks = peaks[sorted_indices]
    major_peaks.sort()

    first_peak_idx, second_peak_idx = major_peaks[0], major_peaks[1]

    if second_peak_idx - first_peak_idx < 3:
        print(f"Section between peaks too small in {file_path}")
        return None

    # Analyze the section between the two major peaks
    x_section = x[first_peak_idx:second_peak_idx + 1]
    y_smooth_section = y_smooth[first_peak_idx:second_peak_idx + 1]
    intensity_gradient = np.gradient(y_smooth_section)
    threshold = -0.002
    decreasing_indices = np.where(intensity_gradient < threshold)[0]

    if len(decreasing_indices) > 0:
        groups = np.split(decreasing_indices, np.where(np.diff(decreasing_indices) > 1)[0] + 1)
        longest_group = max(groups, key=len)
        start_idx = longest_group[0]
        end_idx = longest_group[-1]
        midpoint_idx = (start_idx + end_idx) // 2
        change_point_x = x_section[midpoint_idx]
    else:
        change_point_x = None

else:
    # If less than 2 peaks: use the highest one or fallback to steepest descent
    if len(peaks) == 1:
        first_peak_idx = peaks[0]
    else:
        print(f"Not enough peaks in {file_path}, using max value.")
        first_peak_idx = np.argmax(y_smooth)

```

```

x_section = x[first_peak_idx:]
y_smooth_section = y_smooth[first_peak_idx:]
intensity_gradient = np.gradient(y_smooth_section)
steepest_idx = np.argmin(intensity_gradient)
decreasing_indices = np.where(intensity_gradient < -0.005)[0]

if len(decreasing_indices) > 0:
    start_idx = decreasing_indices[0]
    end_idx = decreasing_indices[-1]
    midpoint_idx = (start_idx + end_idx) // 2
    change_point_x = x_section[midpoint_idx]
else:
    change_point_x = x_section[steepest_idx]

# Plot the results
plt.figure(figsize=(10, 6))
plt.plot(x, y, label='Raw Data', color='grey')
plt.plot(x, y_smooth, label='Smoothed Data', color='black')
if len(peaks) >= 1:
    plt.axvline(x=x[first_peak_idx], color='blue', linestyle='--', label='First Peak')
if len(peaks) >= 2:
    plt.axvline(x=x[second_peak_idx], color='green', linestyle='--', label='Second Peak')
if change_point_x is not None:
    plt.axvline(x=change_point_x, color='red', linestyle='--', label='Midpoint of Decrease')
plt.xlabel('% of Egg Length')
plt.ylabel('Fluorescence Intensity')
plt.title(f'Fluorescence Profile for {os.path.basename(file_path)}')
plt.legend()
plt.grid(True, linestyle='--', alpha=0.7)
plt.savefig(os.path.splitext(file_path)[0] + '_plot.png')
plt.close()

return change_point_x

# Function to process multiple samples and save results to Excel
def process_multiple_samples(num_samples, output_excel_file):
    wb = Workbook()
    ws = wb.active
    ws.title = "Midpoints"
    ws.append(["Sample", "Midpoint (% Egg Length)"])

    for i in range(1, num_samples + 1):
        file_name = f'processed_selected_file{i}.xlsx'
        if os.path.exists(file_name):
            print(f'Processing {file_name}...')

```

```

        change_point = process_sample(file_name)
        if change_point is not None:
            print(f'Midpoint found at {change_point:.2f}')
            ws.append([file_name, change_point])
        else:
            print(f'No clear decrease in {file_name}')
            ws.append([file_name, "N/A"])
        else:
            print(f'{file_name} not found. Skipping...')

    # Save final Excel summary
    wb.save(output_excel_file)
    print(f'Midpoints saved to {output_excel_file}')

# Run the script if executed directly
if __name__ == '__main__':
    num_samples = int(input("Enter the number of samples to process: "))
    output_excel_file = 'PBP_analysis.xlsx'
    process_multiple_samples(num_samples, output_excel_file)

# -----
# SECOND PART: Simple signal smoothing and peak detection
# -----

import pandas as pd
import numpy as np
from scipy.signal import savgol_filter

# Load fluorescence signal data from Excel
try:
    data = pd.read_excel(r"C:\Users\tunahan\Desktop\Protocol\Eve_Analysis.xlsx")
    print("Data loaded successfully.")
except Exception as e:
    print("Failed to load data:", e)
    exit()

# Assign length and intensity from the DataFrame
length = data.iloc[:, 0]
intensity = data.iloc[:, 1]

# Define smoothing parameters
window_length = 11 # Should be odd and smaller than the number of data points
polyorder = 2 # Second order polynomial

# Apply Savitzky-Golay filter for smoothing

```

```

smoothed_intensity = savgol_filter(intensity, window_length, polyorder)
print("Smoothing applied.")

# Calculate the first derivative of the smoothed signal
differences = np.diff(smoothed_intensity)
differences = np.append(differences, 0) # Match original array length
print("Differences calculated.")

# Find points where slope changes from positive to negative (potential peaks)
change_points = np.diff(np.sign(differences))
change_points = np.append(change_points, 0)
print("Change points identified.")

# Add results back into the DataFrame
data['Smoothed Intensity'] = smoothed_intensity
data['Differences'] = differences
data['Change Points'] = (change_points < 0).astype(int) # Only mark negative peaks (going down)

# Save the updated data to Excel
data.to_excel("Smoothed_Output.xlsx", index=False)
print("Data saved to Smoothed_Output.xlsx.")

```

Appendix 1d: Eve Analysis Python

```

###-----Onal_lab-----###
##-----Bilkent University-----##
#-----Ankara, Turkey-----#

import pandas as pd
import numpy as np
import os
import re
import glob
import matplotlib.pyplot as plt
from scipy.signal import savgol_filter, find_peaks
from openpyxl.drawing.image import Image as XLImage
from openpyxl import load_workbook
from scipy.stats import ttest_ind
import warnings
warnings.simplefilter(action='ignore', category=FutureWarning)

# Constructing folder and file capture (Automation)
parent_folder = "/Users/shaheenkabir/ISO2" # Change the folder name accordingly.
FOLDER_PATTERN = "ISO*" # Change the pattern accordingly.

```

```

log_path = os.path.join(parent_folder, "pipeline_log.txt")
with open(log_path, "w") as log:
    log.write("=== Analysis Log -- Failures ===\n")
matched_folders = sorted([
    os.path.join(parent_folder, d)
    for d in os.listdir(parent_folder)
    if os.path.isdir(os.path.join(parent_folder, d)) and re.fullmatch(FOLDER_PATTERN.replace("*", "."), d)
])
# Starting parent-level analysis.
for input_folder in matched_folders:
    print(f"\n📁 Starting analysis in: {input_folder}")
    output_folder = os.path.join(input_folder, "results")
    os.makedirs(output_folder, exist_ok=True)

    summary_rows = []

    for filename in os.listdir(input_folder):
        if filename.endswith(".xlsx"):
            input_path = os.path.join(input_folder, filename)
            output_path = os.path.join(output_folder, f"processed_{filename}")
            temp_plot_path = "temp_plot.png"
            try:
                data = pd.read_excel(input_path)
                length = data.iloc[:, 0]
                intensity = data.iloc[:, 1]

                # Normalize and invert
                norm_intensity = intensity / intensity.max()
                inverted_intensity = 1 - norm_intensity
                norm_length = length / length.max()
                percent_length = norm_length * 100

                # Smooth and calculate derivatives
                smoothed = savgol_filter(inverted_intensity, 11, 2)
                diff = np.diff(smoothed, prepend=smoothed[0])
                change_points = np.diff(np.sign(diff), prepend=0)
                change_point_flags = (change_points < 0).astype(int)

                # Detect peaks
                peaks, _ = find_peaks(smoothed, distance=10, prominence=0.03) # Optimal is 0.03 prominence.
                peak_percent_lengths = percent_length.iloc[peaks].values
                peak_values = smoothed[peaks]
                valid_peak_mask = (peak_percent_lengths > 28) & (peak_percent_lengths < 85) # based on mutation.
                peak_percent_lengths = peak_percent_lengths[valid_peak_mask]
                peak_values = peak_values[valid_peak_mask]

```

```

data['Normalized Intensity'] = norm_intensity
data['Inverted Intensity'] = inverted_intensity
data['Normalized Length'] = norm_length
data['Percent Length'] = percent_length
data['Smoothed'] = smoothed
data['Difference'] = diff
data['Change Point'] = change_point_flags
data.to_excel(output_path, index=False)

plt.figure(figsize=(5, 3))
plt.plot(percent_length, smoothed, label="Smoothed", color='blue')
plt.scatter(peak_percent_lengths, peak_values, color='red', s=50, label="Peaks > 25%")
plt.title("Smoothed Inverted Intensity")
plt.xlabel("Percent Length (%)")
plt.ylabel("Inverted Intensity")
plt.legend()
plt.tight_layout()
plt.savefig(temp_plot_path, dpi=200)
plt.close()

wb = load_workbook(output_path)
ws = wb.active
img = XLImage(temp_plot_path)
img.width = 360
img.height = 220
ws.add_image(img, "K2")

# Add Peaks sheet
if "Peaks" in wb.sheetnames:
    del wb["Peaks"]
peak_ws = wb.create_sheet("Peaks")
peak_ws.append(["Percent Length", "Inverted Intensity"])
for x, y in zip(peak_percent_lengths, peak_values):
    peak_ws.append([x, y])

# Match peaks to change points
change_df = data[(data["Change Point"] == 1) & (data["Percent Length"] > 28) & (data["Percent
Length"] < 85)]
closest_matches = []
for px in peak_percent_lengths:
    if not change_df.empty:
        closest = change_df.iloc[(change_df["Percent Length"] - px).abs().argsort()[:1]]
        closest_val = closest["Percent Length"].values[0]
        closest_matches.append(closest_val)

```

```

# Using a cutoff to discard mismatched files.
if len(closest_matches) != 7:
    with open(log_path, "a") as log:
        log.write(f"✗ {filename}: Found {len(closest_matches)} stripes, not 7 → skipped.\n")
    print(f"⚠ Skipping {filename}: found {len(closest_matches)} stripes, not exactly 7.")
    continue # Skip rest of the loop

# First 3 stripes
stripes = closest_matches[:3]
wb.save(output_path)
os.remove(temp_plot_path)

# Add Macro sheet
macro_df = pd.DataFrame({
    "Percent Length": percent_length,
    "Inverted Intensity": inverted_intensity
})
with pd.ExcelWriter(output_path, engine='openpyxl', mode='a', if_sheet_exists='replace') as writer:
    macro_df.to_excel(writer, sheet_name='Macro', index=False)

# Add Macro_600points
try:
    indices = np.linspace(0, len(macro_df) - 1, 600, dtype=int)
    squeezed_macro_df = macro_df.iloc[indices].reset_index(drop=True)
    with pd.ExcelWriter(output_path, engine='openpyxl', mode='a', if_sheet_exists='replace') as writer:
        squeezed_macro_df.to_excel(writer, sheet_name='Macro_600points', index=False)
except Exception as e:
    print(f"✗ Error creating Macro_600points sheet for {filename}: {e}")

summary_rows.append({
    "File": filename,
    "Stripe-1": stripes[0],
    "Stripe-2": stripes[1],
    "Stripe-3": stripes[2]
})
except Exception as e:
    print(f"✗ Error processing {filename}: {e}")

# Sort summary_rows by numeric suffix in filenames
def extract_numeric_suffix(filename):
    nums = re.findall(r'(\d+)', filename)
    return tuple(map(int, nums)) if nums else (0,)
summary_rows_sorted = sorted(summary_rows, key=lambda x: extract_numeric_suffix(x["File"]))
summary_df = pd.DataFrame(summary_rows_sorted)
summary_output_path = os.path.join(output_folder, "stripe_summary.xlsx")

```

```

summary_df.to_excel(summary_output_path, index=False)

all_matched_rows = []
for row in summary_rows_sorted:
    file = row["File"]
    result_path = os.path.join(output_folder, f"processed_{file}")
    try:
        wb = load_workbook(result_path, data_only=True)
        peaks_ws = wb["Peaks"]
        peaks = []
        for i, r in enumerate(peaks_ws.iter_rows(min_row=2, values_only=True), start=2):
            if r[0] is not None:
                peaks.append(r[0])
        df = pd.read_excel(result_path)
        change_df = df[(df["Change Point"] == 1) & (df["Percent Length"] > 28)]

        matched_stripes = []
        for px in peaks:
            if not change_df.empty:
                closest = change_df.iloc[(change_df["Percent Length"] - px).abs().argsort()[:1]]
                matched_val = closest["Percent Length"].values[0]
                matched_stripes.append(matched_val)
            else:
                matched_stripes.append(None)

        all_matched_rows.append({
            "File": file,
            "Stripe-1": matched_stripes[0],
            "Stripe-2": matched_stripes[1],
            "Stripe-3": matched_stripes[2],
            "Stripe-4": matched_stripes[3],
            "Stripe-5": matched_stripes[4],
            "Stripe-6": matched_stripes[5],
            "Stripe-7": matched_stripes[6]
        })
    except Exception as e:
        print(f"⚠ Skipping peak matching for {file}: {e}")
matched_all_df = pd.DataFrame(all_matched_rows)
with pd.ExcelWriter(summary_output_path, engine='openpyxl', mode='a') as writer:
    matched_all_df.to_excel(writer, sheet_name="7_Stripes", index=False)

wb = load_workbook(summary_output_path)
df_7 = pd.read_excel(summary_output_path, sheet_name="7_Stripes")
stripe_groups = {
    "Stripes_1-2": ["Stripe-1", "Stripe-2"],

```

```

    "Stripes_3-4": ["Stripe-3", "Stripe-4"],
    "Stripes_5-7": ["Stripe-5", "Stripe-6", "Stripe-7"]
}
with pd.ExcelWriter(summary_output_path, engine="openpyxl", mode="a", if_sheet_exists="replace") as
writer:
    for sheet_name, cols in stripe_groups.items():
        group_df = df_7[["File"] + cols].copy()
        avg_row = pd.DataFrame(["AVERAGE"] + group_df[cols].mean().tolist(),
                                columns=group_df.columns)
        stdev_row = pd.DataFrame(["STDEV.S"] + group_df[cols].std(ddof=1).tolist(),
                                columns=group_df.columns)
        blank_row = pd.DataFrame([None] * len(group_df.columns), columns=group_df.columns)
        final_df = pd.concat([group_df, blank_row, avg_row, stdev_row], ignore_index=True)
        final_df.to_excel(writer, sheet_name=sheet_name, index=False)

wb = load_workbook(summary_output_path)
ws = wb.active
cols = list(range(2, 5)) # Columns B, C, D
data = []
n = ws.max_row
for col in cols:
    col_data = []
    for row in range(2, n + 1):
        val = ws.cell(row=row, column=col).value
        try:
            val = float(val)
            col_data.append(val)
        except (TypeError, ValueError):
            pass
    data.append(col_data)
averages = [round(np.mean(col_data), 4) if len(col_data) > 0 else None for col_data in data]
stdevs = [round(np.std(col_data, ddof=1), 4) if len(col_data) > 1 else None for col_data in data]
ws.insert_rows(n + 1)
ws.cell(row=n + 2, column=1, value="AVERAGE")
ws.cell(row=n + 3, column=1, value="STDEV.S")
for idx, col in enumerate(cols):
    ws.cell(row=n + 2, column=col, value=averages[idx])
    ws.cell(row=n + 3, column=col, value=stdevs[idx])
wb.save(summary_output_path)
print(f"\n 🇧🇷 Stripe summary written to: {summary_output_path}")

# Combine all Macro_600points into one Excel
macro_percent_cols = []
macro_intensity_cols = []
sample_names = []

```

```

processed_files = sorted(
    [f for f in os.listdir(output_folder) if f.startswith("processed_") and f.endswith(".xlsx")],
    key=lambda x: int(re.findall(r'(\d+)', x)[-1])
)
for idx, fname in enumerate(processed_files, start=1):
    fpath = os.path.join(output_folder, fname)
    try:
        df = pd.read_excel(fpath, sheet_name="Macro")
        percent_col = df.iloc[:, 0] / df.iloc[:, 0].max()
        intensity_col = df.iloc[:, 1]
        indices = np.linspace(0, len(df) - 1, 600, dtype=int)
        percent_squeezed = percent_col.iloc[indices].reset_index(drop=True)
        intensity_squeezed = intensity_col.iloc[indices].reset_index(drop=True)
        label = os.path.basename(input_folder) + f"_{idx}"
        sample_names.append(label)
        macro_percent_cols.append(percent_squeezed.rename(label))
        macro_intensity_cols.append(intensity_squeezed.rename(label))
    except Exception as e:
        print(f"✖ Skipping {fname}: {e}")

percent_df = pd.concat(macro_percent_cols, axis=1)
intensity_df = pd.concat(macro_intensity_cols, axis=1)
percent_df[""] = ""
intensity_df[""] = ""
percent_df["Average"] = percent_df.select_dtypes(include=[np.number]).mean(axis=1)
intensity_df["Average"] = intensity_df.select_dtypes(include=[np.number]).mean(axis=1)
combined_macro_path = os.path.join(output_folder, "combined_macro_600points.xlsx")
with pd.ExcelWriter(combined_macro_path) as writer:
    percent_df.to_excel(writer, sheet_name="Percent Length", index=False)
    intensity_df.to_excel(writer, sheet_name="Inverted Intensity", index=False)

# Creating all average summary file.
output_combined_file = os.path.join(parent_folder, "all_avg_summary.xlsx")
length_averages = []
intensity_averages = []
labels = []
for subdir in matched_folders:
    combined_path = os.path.join(subdir, "results", "combined_macro_600points.xlsx")
    if os.path.exists(combined_path):
        try:
            percent_df = pd.read_excel(combined_path, sheet_name="Percent Length")
            intensity_df = pd.read_excel(combined_path, sheet_name="Inverted Intensity")
            avg_length = percent_df["Average"]
            avg_intensity = intensity_df["Average"]

```

```

        name = os.path.basename(subdir)
        labels.append(name)
        length_averages.append(avg_length.rename(name))
        intensity_averages.append(avg_intensity.rename(name))
    except Exception as e:
        print(f"⚠️ Could not extract from {subdir}: {e}")
    else:
        print(f"❌ Missing file in: {subdir}")

length_avg_df = pd.concat(length_averages, axis=1)
intensity_avg_df = pd.concat(intensity_averages, axis=1)
output_combined_file = os.path.join(parent_folder, "all_avg_summary.xlsx")
if os.path.exists(output_combined_file):
    mode = "a"
    if_sheet_exists = "replace"
else:
    mode = "w"
    if_sheet_exists = None
with pd.ExcelWriter(output_combined_file, engine="openpyxl", mode=mode, if_sheet_exists=if_sheet_exists) as writer:
    length_avg_df.to_excel(writer, sheet_name="Average Percent Length", index=False)
    intensity_avg_df.to_excel(writer, sheet_name="Average Intensity", index=False)

# Plot all average curves
plt.figure(figsize=(7, 5))
for label, x, y in zip(labels, length_averages, intensity_averages):
    plt.plot(x, y, label=label)
plt.xlabel("Percent Length (%)")
plt.ylabel("Inverted Intensity")
plt.title("Average Intensity Curves")
plt.legend()
plt.tight_layout()
plot_path = os.path.join(parent_folder, "average_plot.png")
plt.savefig(plot_path, dpi=300)
plt.close()

# Folder map
folders = glob.glob(os.path.join(parent_folder, FOLDER_PATTERN))
folder_map = {}
for folder in folders:
    folder_name = os.path.basename(folder)
    key = folder_name.split("_")[-1]
    stripe_file = os.path.join(folder, "results", "stripe_summary.xlsx")
    folder_map[key] = stripe_file
print(f"✅ folder_map:", folder_map)

```

```

def load_stripe_column(folder_key, stripe_n):
    path = folder_map[folder_key]
    if not os.path.exists(path):
        print(f"⚠️ File missing for folder {folder_key}: {path}")
        return np.array([])
    try:
        df = pd.read_excel(path, sheet_name="7_Stripes")
    except Exception as e:
        print(f"⚠️ Could not load '7_Stripes' sheet from {path}: {e}")
        return np.array([])

    col_name = f"Stripe-{stripe_n}"
    if col_name in df.columns:
        return df[col_name].dropna().values
    else:
        print(f"⚠️ Column '{col_name}' not found in '7_Stripes' sheet of {path}")
        return np.array([])

required_groups = ["22", "25", "29"]
missing = []

for grp in required_groups:
    pattern = os.path.join(parent_folder, f"*_{grp}")
    matches = glob.glob(pattern)
    if not matches:
        missing.append(f"No match for pattern: {pattern}")

if missing:
    print("❌ One or more required groups are missing!")
    for m in missing:
        print(f"➡️ Missing: {m}")
    print("🛑 Aborting comparisons.")
    exit(1) # or sys.exit(1)

# Comparison
comparisons = [("22", "25"), ("25", "29"), ("22", "29")]
for group1, group2 in comparisons:
    output_path = os.path.join(parent_folder, f"{group1}_vs_{group2}.xlsx")
    sheets_added = 0
    with pd.ExcelWriter(output_path, engine='xlsxwriter') as writer:
        for stripe_n in range(1, 8):
            data1 = load_stripe_column(group1, stripe_n)
            data2 = load_stripe_column(group2, stripe_n)

```

```

if len(data1) < 2 or len(data2) < 2:
    print(f"⚠️ Not enough data for Stripe-{stripe_n} comparison {group1} vs {group2}, skipping")
    continue
df = pd.DataFrame({
    f'{group1}': pd.Series(data1),
    f'{group2}': pd.Series(data2)
})
blank_row = pd.DataFrame([[None, None]], columns=df.columns)
t_stat, p_val = ttest_ind(data1, data2, equal_var=False, alternative='two-sided')

t_stat_row = pd.DataFrame([["t-stat", t_stat]], columns=df.columns)
p_val_row = pd.DataFrame([["p-value", p_val]], columns=df.columns)
df = pd.concat([df, blank_row, t_stat_row, p_val_row], ignore_index=True)
df.to_excel(writer, sheet_name=f'Stripe-{stripe_n}', index=False)

workbook = writer.book
worksheet = writer.sheets[f'Stripe-{stripe_n}']
sig_fmt = workbook.add_format({'font_color': 'green'})
not_sig_fmt = workbook.add_format({'font_color': 'red'})
sig_text = "Significant" if p_val < 0.05 else "Not Significant"
sig_format = sig_fmt if p_val < 0.05 else not_sig_fmt
sig_row = len(df) + 2
worksheet.write(sig_row-1, 1, sig_text, sig_format)
sheets_added += 1

if sheets_added == 0:
    print(f"❌ No sheets added for {group1}_vs_{group2}, deleting empty file.")
    try:
        os.remove(output_path)
    except FileNotFoundError:
        pass

print("🎉 All analyses done!")
print("Good Luck!")

```

Appendix 1e: Bcd Matlab

```

Matlab:
%% == SETUP ==

parent_folder = '/Users/shaheenkabir/Bcd_analysis/yw/yw_22';
output_parent = '/Users/shaheenkabir/Bcd_analysis/yw/yw_22';

% == Pick single file ==

filename = fullfile(parent_folder, 'yw_22_5.tif'); % Change this
[~, baseName, ~] = fileparts(filename);

```

```

%Factor2 = 80/80; % exposure correction
%Factor = 0.6431; % micron/pixel

fprintf('Processing file: %s\n', filename);

% === Create output folder ===
output_folder_pic = fullfile(output_parent, 'masks')
if ~exist(output_folder_pic, 'dir')
    mkdir(output_folder_pic)
end
output_folder = fullfile(output_parent, 'results');
if ~exist(output_folder, 'dir')
    mkdir(output_folder);
end

%% === Load channels ===
DapiI = imread(filename, 1);
BcdI = imread(filename, 2);
figure; imshow(DapiI);
figure; imshow(BcdI);
imwrite(DapiI, fullfile(output_folder_pic, strcat(baseName, '_DapiI_raw.tif')));
imwrite(BcdI, fullfile(output_folder_pic, strcat(baseName, '_BcdI_raw.tif')));

%% === DAPI mask ===
DapiI = adapthisteq(DapiI);
%treshold = graythresh(imadjust(DapiI));
treshold = 0.40
mask = imbinarize(DapiI, treshold);
figure; imshow(mask);
%imwrite(mask, fullfile(output_folder_pic, strcat(baseName, '_mask.png')));

% === Largest embryo ===
stats = regionprops(mask, 'all');
embryoArea = [stats.Area];
[~, embryoID] = max(embryoArea);

% === Nuclear mask ===
nmask = zeros(size(mask));
nmask(stats(embryoID).PixelIdxList) = 1;
figure; imshow(nmask);
imwrite(nmask, fullfile(output_folder_pic, strcat(baseName, '_nmask.png')));

% === Apply nuclear mask ===
DapiI = DapiI .* uint8(nmask);
figure; imshow(DapiI);

```

```

%imwrite(DapiI, fullfile(output_folder_pic, strcat(baseName,'_DapiI_masked.png')));

% === Rotate and crop ===
theta = -stats(embryoID).Orientation;
DapiI = imrotate(DapiI, theta);
BcdI = imrotate(BcdI, theta);
W = imrotate(nmask, theta);

figure; imshow(DapiI);
figure; imshow(BcdI);
figure; imshow(W);
%imwrite(DapiI, fullfile(output_folder_pic, strcat(baseName,'_DapiI_rotated.tif')));
%imwrite(BcdI, fullfile(output_folder_pic, strcat(baseName,'_BcdI_rotated.tif')));
%imwrite(W, fullfile(output_folder_pic, strcat(baseName,'_nmask_rotated.tif')));

% Anterior-left, dorsal-up
BW = edge(W, 'canny');
imwrite(BW, fullfile(output_folder, strcat(baseName,'_nmask_edge.png')));
m0 = find(sum(BW, 1) > 0);
n0 = find(sum(BW, 2) > 0);

DapiI1 = imcrop(DapiI, [min(m0), min(n0), max(m0)-min(m0), max(n0)-min(n0)]);
BcdI1 = imcrop(BcdI, [min(m0), min(n0), max(m0)-min(m0), max(n0)-min(n0)]);
%theta2 = 180 * double(max(max(BcdI1(:,1:50))) < max(max(BcdI1(:,end-49:end))));
%DapiI1 = imrotate(DapiI1, theta2, 'crop');
%BcdI1 = imrotate(BcdI1, theta2, 'crop');
%imwrite(DapiI1, fullfile(output_folder_pic, strcat(baseName,'_DapiI_final.png')));
%imwrite(BcdI1, fullfile(output_folder_pic, strcat(baseName,'_BcdI_final.png')));

figure; imshow(DapiI1);
figure; imshow(BcdI1);
figure; imshow(W);

% === Find nuclear edge from DAPI ===
BW1 = edge(DapiI1, 'canny');
figure; imshow(BW1);
%imwrite(BW1, fullfile(output_folder_pic, strcat(baseName,'_DapiI_edge.png')));

m1 = find(sum(BW1, 1) > 0);
n1 = zeros(1, length(m1));
for j = 1:length(m1)
    n1(j) = min(find(BW1(:, m1(j)) > 0));
end
n2 = smooth(m1, n1, 0.07, 'rloess');

```

```

%% == Midline ==
Bins = 50;
EL = max(m1) - min(m1) + 1;
L = floor(EL / Bins);
gama = atan(gradient(n2));
radius = 10;
centroidx = round(m1 - sin(gama) * radius);
centroidy = round(n2 + cos(gama) * radius);

centroidx1 = zeros(1, Bins);
centroidy1 = zeros(1, Bins);
z = zeros(1, Bins);

for j = 1:Bins
    idx = ceil(0.5*EL - (Bins+1)/2*L + j*L);
    centroidx1(j) = centroidx(idx);
    centroidy1(j) = centroidy(idx);
    z(j) = mean2([BcdI1(centroidy1(j), centroidx1(j)-4:centroidx1(j)+4), ...
        BcdI1(centroidy1(j)-1, centroidx1(j)-4:centroidx1(j)+3), ...
        BcdI1(centroidy1(j)+1, centroidx1(j)-3:centroidx1(j)+4), ...
        BcdI1(centroidy1(j)-2, centroidx1(j)-3:centroidx1(j)+3), ...
        BcdI1(centroidy1(j)+2, centroidx1(j)-3:centroidx1(j)+3), ...
        BcdI1(centroidy1(j)-3, centroidx1(j)-2:centroidx1(j)+3), ...
        BcdI1(centroidy1(j)+3, centroidx1(j)-3:centroidx1(j)+2), ...
        BcdI1(centroidy1(j)-4, centroidx1(j)-2:centroidx1(j)+2), ...
        BcdI1(centroidy1(j)+4, centroidx1(j)-2:centroidx1(j)+2)]);
end

% == Save sampling scatter ==
figure; imshow(BcdI1); hold on;
scatter(m1, n2, 'r'); hold on;
scatter(centroidx1, centroidy1, 'b');
f = figure; imshow(BcdI1); hold on;
scatter(m1, n2, 'r');
scatter(centroidx1, centroidy1, 'b');

saveas(f, fullfile(output_folder_pic, strcat(baseName, '_sampling_points.png')));
close(f);

%% == Absolute profile ==
L2 = round(900 / Bins);
Bins2 = floor(EL / L2);
z2 = zeros(1, Bins2);
centroidx2 = zeros(1, Bins2);
centroidy2 = zeros(1, Bins2);

```

```

for j = 1:Bins2
    idx = j*L2 - ceil(L2/2) + 1;
    centroidx2(j) = centroidx(idx);
    centroidy2(j) = centroidy(idx);
    z2(j) = mean2([BcdI1(centroidy2(j), centroidx2(j)-4:centroidx2(j)+4), ...
        BcdI1(centroidy2(j)-1, centroidx2(j)-4:centroidx2(j)+3), ...
        BcdI1(centroidy2(j)+1, centroidx2(j)-3:centroidx2(j)+4), ...
        BcdI1(centroidy2(j)-2, centroidx2(j)-3:centroidx2(j)+3), ...
        BcdI1(centroidy2(j)+2, centroidx2(j)-3:centroidx2(j)+3), ...
        BcdI1(centroidy2(j)-3, centroidx2(j)-2:centroidx2(j)+3), ...
        BcdI1(centroidy2(j)+3, centroidx2(j)-3:centroidx2(j)+2), ...
        BcdI1(centroidy2(j)-4, centroidx2(j)-2:centroidx2(j)+2), ...
        BcdI1(centroidy2(j)+4, centroidx2(j)-2:centroidx2(j)+2)]));
end

%% === Store ===
Data.EL = EL;
Data.relx = centroidx1 - min(m1) + 1;
Data.rely = centroidy1 - min(n2) + 1;
Data.relBcd = double(z);
Data.absx = centroidx2 - min(m1) + 1;
Data.absy = centroidy2 - min(n2) + 1;
Data.absBcd = double(z2);
Data.name = baseName;

%% === Save tables ===
relX = Data.relx / Data.EL;
absX = Data.absx;
relBcd = Data.relBcd;
absBcd = Data.absBcd;

Trel = table(relX, relBcd, 'VariableNames', {'AP_Position', 'Intensity'});
Tabs = table(absX, absBcd, 'VariableNames', {'AP_Position', 'Intensity'});

writetable(Trel, fullfile(output_folder, [baseName '_Bcd_profile_rel.xlsx']));
%writetable(Tabs, fullfile(output_folder, [baseName '_Bcd_profile_abs.xlsx']));

fprintf('✅ Saved results for: %s\n', baseName);
disp('Done ✅');

```

Appendix 1f: Bcd R

```
library(readxl)
library(tidyverse)
library(gridExtra)
library(minpack.lm)

data <- read_excel("/Users/shaheenkabir/Bcd_analysis/mass/mass_25/results/mass_25_22_Bcd_profile_rel.xlsx")
colnames(data) <- c("Length", "intensity")
data$norm_length <- (data$Length/max(data$Length))*100

#data$norm_intensity <- ((data$intensity - min(data$intensity))/(max(data$intensity) - min(data$intensity)))*100
#data$norm_length <- data$Length
#max(data$Length) * .25
plot1 <- ggplot(data, aes(x = norm_length, y = intensity))+
  geom_line()+
  labs(x = "Embryo Length (%)", y = "Bicoid concentration (AU)")
plot2 <- ggplot(data, aes(x = norm_length, y = intensity))+
  geom_line()+
  labs(x = "Embryo Length (%)", y = "Bicoid concentration (AU)")
# plot2 <- ggplot(data, aes(x = norm_length, y = intensity))+
#   geom_point()+
#   labs(x = "Position (%)", y = "Bicoid concentration")
plot1
plot3
ggsave(filename = "bcd_raw.pdf", plot = plot1, device = "pdf",
        path = "final", width = 3.56, height = 2.56, units = "in")
ggsave(filename = "bcd_fit.pdf", plot = plot3, device = "pdf",
        path = "final", width = 3.56, height = 2.56, units = "in")
#data <- data %>% dplyr::filter(norm_length <= 50)
#data <- data %>%
#   dplyr::filter(norm_length < 0.60)

fit <- nlsLM(intensity ~ A * exp(-norm_length / lambda) + B, data = data, start = list(A = max(data$intensity),
lambda = 30, B = min(data$intensity)))
summary(fit)
coef(fit)["lambda"]
coef(fit)["B"]
data$fit <- predict(fit, newdata = data)

plot3 <- ggplot(data, aes(norm_length, intensity)) +
  geom_line() +
  geom_line(aes(y = fit), color = "red") +
  labs(x = "Embryo Length (%)", y = "Bicoid concentration (AU)",
        title = "Exponential fit of Bicoid gradient")
```

```
plot4 <- ggplot(data, aes(norm_length, intensity)) +  
  geom_point() +  
  geom_line(aes(y = fit), color = "red") +  
  labs(x = "Position (%)", y = "Bicoid concentration",  
       title = "Exponential fit of Bicoid gradient")  
grid.arrange(plot1, plot2, plot3, plot4, ncol = 2)
```