The Little Flies and their Genes:

How Research Exploiting *Drosophila* has led to Prize-wining Advances in Genetic and Biological Knowledge, all the way to uncovering Mysteries of the Circadian Clock

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THIS ESSAY WILL PAY HOMAGE to basic research. Context: The 2017 Nobel Prize in Physiology or Medicine (P or M). For this, the word "or" alludes to the other noun (P) meaning essentially *anything* biological, irrespective of any apparent M relevance. All bio-phenomena, at base, are "physiological," which is so even for genetics (how cellular processes mediate replication of the genetic material and transmission of it from one generation to another: cellular and parent/offspring).

The subject matter that follows will have much to do with a subset of the basic-research enterprise: genetics, in part from historical perspectives, both generic and personal. The former adjective alludes to putting rhythm-related genetic studies in contexts extending, at times, beyond "the little flies;" the latter adjective points to 20th and 21st century developmental-, behavioral-, neural-, and molecular-genetic matters revolving round the fruit-fly *Drosophila melanogaster.*

With regard to the foregoing introductory passages, the 2017 trio of P or M awardees performed research that was deeply rooted in genetic and molecular-biological studies of *Drosophila*. The deep background of research in those related arenas led to knowledge and materials that were crucial for establishing and sustaining research enterprises in what came to be called "chrono-genetics:" studies of daily biological rhythms from genetic (mutational and molecular) perspectives.

I invoked the adjective "basic" up-top in order to claim that a Nobel Prize in the biological field need not necessarily have any practical significance, at least early on, such as impacts connected with "Medicine". That said, huge numbers of advances made on behalf of basic research (in scientific areas well beyond biology) turned out to be extra-interesting, in the sense that *applications* of fundamental findings can be exemplified and re-exemplified.

The immediately foregoing remark prompts a mention of "model systems," such as the *Drosophila* one. To decode the phrase just quoted: Potentially studying something or other in a "lower" organism, for example, might be a "model" for elucidating processes manifested other species, including "higher" ones such as mammals and us. In this light (or cynical dark), research-grant-proposal boilerplate often includes passages about hoped-for *relevance* of studying whatever in a given model system. Another point of view: Research findings can be appreciated for their own sake, involving new knowledge about an Earth-based life form in and of itself. This attitude belies any necessity that basic-research findings will somehow be applicable somewhere else, organismically. Thus, with regard to oh-so-many times I have been asked how and why is *Drosophila* a good model system, I eventually came to respond: "The genetics of *D. melanogaster* is a fine model system for studying hereditary phenomena in *Drosophila*."

Studies that purport, in contrast, to point toward practical applications come under the heading of what is called, nowadays, "translational research:" Can certain findings be rather quickly translated into scientific activities that promote human welfare, for example? I cannot claim that "non-translational" research is just as good, in terms of promoting even eventual applications. I do claim, with no compunction and high-mindedly, that essentially all categories of research have the positive potential to generate new knowledge per se, whether or not such is quickly (or ever) perceived to be especially interesting or useful beyond the newness itself.

Approaching matters revolving around daily biological rhythms in *Drosophila*, studied genetically, I have firm memories of the early days during which such research was initiated then limped along for a fair number of subsequent years. Few, perhaps none, of the relevant researchers could

have predicted the broadly and deeply based implications of such chrono-genetic findings. Such expansions were based in part on ways that "non-drosophilists" went to school on the fruit fly factors and phenomena, thus extending the ostensible "insect-only" significance of what the 2017 awardees contributed, by applying *Drosophila*-based potential to analyze non-insect phenomena via genetic and molecular approaches.

Backing up to elements of my own career and how it connected, however loosely, with the history of genetic research performed using *Drosophila*, these endeavors turned out to generate biological findings with robust significance for understanding many ways that animals are formed, then function. Such accomplishments, harking back to the first half of the 20th century and unrelated to yours truly, were even deemed worthy of Prize awardings. (Stay tuned.) As for this one of the 2017 awardees, I began to get my feet wet in *Drosophila* genetics as an undergraduate. During about 40% of that stint, I performed a pair of low-level genetic projects. They had to with what-I-call "genetic genetics:" studying hereditary phenomena per se, with essentially zero general biological implications. I carried out these projects under the supervision of a *Drosophila* geneticist named Phillip T. Ives. As I learned later, Ives was a direct descendant of Thomas Hunt Morgan and Alfred Sturtevant, two of the giants in genetic history (Stay tuned.) Accordingly, as I registered in retrospect, Ives not only genetically instructed his handful of undergrad charges, he also imbued them with "the lore of *Drosophila*:" how that system was founded, expanded, and sustained over several decades, meaning from about the 1910s to what was then the mid/late 1960s.

As previewed, Ives performed his PhD-earning research at the California Institute of Technology (CalTech, a.k.a. C.I.T., Pasadena, CA, U.S.) under Prof. Alfred Sturtevant. The latter was an undergraduate then graduate student under the aforementioned Morgan, whose small lab – then at Columbia University, New York City, U.S. – had single-handedly founded *Drosophila* as a system in which one could elucidate genetic factors and events. This came down initially during the 1910s; then and later Morgan and his students, with them further functioning as alumni, generated vast amounts of generally important genetic knowledge. Not for nothing, they also discovered and created large numbers of genetic variants. Many of them have been maintained ever since, and several of them became crucially applicable by subsequent geneticists and bio-geneticists (For the latter, again stay tuned.) Owing to all this pioneering research, and supervision thereof, Morgan was awarded the Nobel Prize in 1933.

Several grad students working under him earned their PhDs via fruit-fly genetics. A trio of Morgan's "F1" (genetic jargon) became the best-remembered members of his group at Columbia and then CalTech: Sturtevant, along with Calvin Bridges and Hermann Muller. Who cares? Or why

am I going on about these investigator-based histories? Well, elements of what Morgan and his F1 achieved turned out to be monumentally relevant to the performance of many subsequent studies, notably in the aforenoted area of bio-genetics (taking a genetic approach to study any number of biological processes), including *chrono*-genetics. Such instances of historical significance, catalyzed by Morgan and crew, extended well beyond the meaning of their genetic-genetic findings, hugely impactful though they were (e.g., Sturtevant, 1913; Bridges, 1916; Muller, 1927). It was an aspect of Muller's genetic career that caused him to be a Prize awardee (1946). Yet what Sturtevant and Bridges achieved was equally meaningful, at least in terms of ways that materials they established had whopping heuristic value down the decades. For example, the latter geneticist and *cyto*-geneticist observed and analyzed the chromosomes (viz. "cyto-") of D. *melanogaster* and thus established highly-resolved descriptions of those sub-cellular organelles. These findings (e.g., Bridges, 1935) held many drosophilists in good stead later on, as will be exemplified later in a chrono-molecular-genetic context.

Such chromosome-level observations often occurred in the context of Muller and his labmates generating genetic variants at, indeed, the chromosomal level. One category of such came to be called "balancer" chromosomes (Lindsley and Grell, 1968a). By definition – yet with apology for now diving into the cyto-genetic weeds, but importantly so I think – a balancer (here abbreviated *Bal*) harbors inverted (*In*) chromosomal segments along with a dominant (*Dom*) marker mutation. Now *In*'s, over an homologous chromosome in normal (*norm*) sequence, block meiotic recombination between such homologs. Thus, an offspring from a *Bal/norm* parent that does *not* exhibit effects of the *Dom* marker must carry the entire "norm" (normal) chromosome. The latter, however, might not an environmental agent having mutagenic effects (e.g., Muller, 1927; with other later, germane examples to follow).

Balancer chromosomes – which are essentially unique to *Drosophila* – are far from the only type of chromosome-level anomaly extant in the fruit fly system. Another example: the "attached-X," meaning two X chromosomes in a *D. melanogaster* female attached to one centromere, vs. standard "free Xs," each attached to their own centromere. The first case of an attached-X was discovered, as a spontaneously occurring sport (read: anomalous organism, possibly a genetic variant) by Thomas Hunt Morgan's better half (Morgan, 1922). Owing to principles & practices established previously by Bridges (1916), attached-X-over-Y females (the Y chromosome being *not* sex-determining), mated to standard males (XY), is a "true-breeding" situation: All live offspring are attached-X/Y females and XY males. The latter receive their one X from *fathers*, the reverse of "criss-cross" inheritance occurring in a standard free-Xs x XY mating (cf.

Morgan, 1922). Other types of zygotes produced from an attach-X x standard-male mating – triple-X and YY – carry *lethal* genotypes; more about those, more generally, later.

The attached-X deal established a most powerful cyto-genetic tool, applied again and again later to initiate various bio-genetic enterprises, including a chrono-genetic one. (Stay tuned.) In this regard, Muller himself designed and generated one of the now-classical and hugely-useful attached-X "strains." He published this accomplishment, aimed at establishing a particularly stable attached-X type and item, in a tiny research note (mid-1940's, albeit unrelated to the Prize that came his way then; see Lindsley and Grell, 1968b). The Mullerian strain in question – formally named *C(1)DX, y f* – got scattered all over the *Drosophila* community and held several subsequent investigators in good stead, as previewed briefly above.

An insert and disclaimer about *Drosophila*-genetic nomenclature: *C* (previous paragraph) always stands for Compound Chromosome, meaning 2 usually free chromosome "arms" attached to one centromere; *(1)* refers to the X, that is the first chromosome; *DX* means double-X; *y* and *f* are, respectively, abbreviations for the recessive X-chromosomal mutations *yellow* (body color) and *forked* (bristle morphology), with regard to which Muller's attached-X is homozygous (viz. *y f / y f*). It *might* be believed that there is some method to this nomenclature madness, whereby genotypic designators are invoked according to putatively comprehensible norms; those include designating inverted chromosomes in part with *In*, followed by the X or autosome number in question.

Back to me and early stages of my career. My grad student supervisor was Larry Sandler (LS), who like Ives was a superb mentor. Examples: (1) I learned from LS how well-regarded a geneticist was Ives. (2) Sandler taught his mentees about the deep history of *Drosophila* genetics, involving and specifically invoking the noun "lore", both in terms of *genic* variants and chromosome-level ones. (3) For the latter genotypes, we were informed about not only the properties of chromosome aberrations, but also how they were designed, constructed, and analyzed.

One thing that I learned from Sandler – the academic grandson of Sturtevant, by the way – was how well his "grandfather" had analyzed the meiotic behavior of inverted chromosomes. Elements of those analyses were co-performed by another to-become-famous geneticist. (See below.) It was notable that, when Morgan moved from New York to Southern California during the late 1920s, Sturtevant along with Bridges (but not Muller) moved with him. This move promoted founding of the "Division of Biology" at CalTech, whose ever-enhancing fame was to come, including with respect to pioneering chrono-genetics.

Speaking of the intellectual, research-related "line" at hand, another

student under Sturtevant at CalTech in addition to Phil Ives was Edward (Ed) Lewis. He is long-remembered in bio-genetic and Nobel Prize winning history (Lewis, 1978, 1995). In order to recite elements of Lewis's estimable career, a piece of background information that is on-point (lore-wise): Back when I was "under" Ives (1960s), he mentioned that his CalTech labmate Bridges (1930s) had "the best eye" for recognizing novel sports. One that Bridges detected (1910s, back at Columbia) got named *bithorax* (*bx*), for a posterior-thorax structure was subtly modified in the direction of a more anteriorly located structure (Lindsley and Grell, 1968c). *bx* was demonstrated by Bridges and labmates to be a heritable mutant, hence the italicized name and abbreviation. And the *bx* single-locus mutation contributed to establishing an autosomal "linkage group" (cf. that for the X chromosome, e.g., Sturtevant, 1913). The autosome that harbors *bx* is one of the large non-sex chromosomes in *D. melanogaster*. This chromosome, number 3, was later realized to contain some of the all-time famous fruit fly genes (e.g., as a conceit, recounted by Villella and Hall, 2008). For *bithorax*'s part, instead of the mutation being applied as a genetic tool alone, how that mutation changes the fly's anatomy was regarded as potentially interesting *biologically*, as well being genetically useful.

After gaining his PhD at CalTech, where Dr. Lewis stayed ever after, Ed initiated a series of studies involving "complex loci." Those are chromosome sites at which several genetic variants were found to be clustered closely at and near a given chromosomal site. Typically, the various variants cause phenotypic changes that are related, e.g., all having to do with eye morphology. By digging into such loci, and the trio or more genotypic variants associated with each, Lewis was once again dealing with (I say again) *genetic* genetics: here meaning how various combinations of genetic changes at a given locus were apparently acting and interacting in – indeed – complex ways. Such Lewisian analyses seemed to consider the phenotypes – external anatomical changes caused by the heritable variants – as means to an end, allowing elucidations of the genetic interactions as such. Lewis himself recounted these elements of his career in the written version of his Nobel Prize Lecture [See Lewis (1995), if you are willing to grapple with the many genetic complexities he describes.]

Professor Lewis spent a fair proportion of his career investigating one of these complex loci, and his studies took on increasing degrees of *biological* significance (Lewis, 1995): how genotypic changes point to ways that genes materially influence animal *development*. This brings us back to the original mutant noticed by Bridges: *bithorax* (Lindsley and Grell, 1968c). Lewis established several additional genetic variants at the relevant autosomal locus, which became known as the BITHORAX COMPLEX (BX-C). A given BX-C-based variant – along with combinations of

mutations – caused changes in the fly's external anatomy that were regarded as increasingly interesting, phenotypically. Without burdening readers with hard genotypic specificity, suffice it say that one of these combinations led to development of the famed "4-winged" fly (depicted, on behalf of Lewis, by Lakhotia, 1995). This highly anomalous animal had a pair of the usual wings, extending from the middle part of the fly's thorax, *and* an additional pair extending from the immediately posterior portion of this "major body region." Thus, this BX-C combination caused the highly-evolved dipteran insect in question (*Drosophila melanogaster*) to take on attributes like that of a more primitive insect, such as the normally 4-winged dragonfly. Another BX-C genotype caused legs to extend off an anterior segment of the fly's abdomen, à la primitive invertebrates that possess legs extending from nearly all body segments, e.g., a centipede or millipede. Such phenotypic changes are arguably more interesting than many others known in *D. melanogaster*, such as those involving altered pigmentation, e.g., the already introduced *yellow* mutant.

Now some of the mutations mapping to (within) the chromosomal locus in question (BX-C) have such severe effects on development as to kill the animal during a given pre-adult stage. These *lethal* BX-C genotypes can be exemplified in two ways: (1) *Ultrabithorax* (*Ubx*), whereby *Ubx/Ubx* kills developing *Drosophila*. (2) A deletion of the entire BX-C, which once again is a recessively lethal genotype. The latter type of genotypic change, a.k.a. *Deficiency* (*Df*), in general has lethal consequences, with the proviso that the *Df* in question removes an appreciable number of "chromosomal bands." To re-introduce the cyto-genetic matter: These intra-chromosomal entities, which will come heavily into play in a chrono-genetic context later, were described in magnificent detail by observing "giant chromosomes" contained within salivary gland cells of *D. melanogaster* larvae (e.g., Bridges, 1935). This genome – again at the level of the relevant larval cells – is composed of approximately 5,200 chromosomal bands. Each of the long chromosomal "arms" (one for the X, two each for each of the two large autosomes) harbors ca. 1000 such bands. As for deletions, their existence and attributes can be assessed by microscopic observations of larvae carrying *Df/+* genotypes. Yet if only one or two faint bands got removed by a particular *Df,* that genotypic alteration tends to be nearly impossible to resolve microscopically. A very short deletion of this type might be a "homozygous-viable" genotype, difficult to know if the *Df/+* or *Df/Df* type is "cytologically" unresolvable. Empirical fact: A deletion that removes a readily observable number of salivary-chromosomal bands (practically meaning a quartet, up to a dozen or more) invariably removes at least one v*ital gene*, such that those *Df/Df* genotypes are indeed lethal. One of many such examples: the cytologically demonstrable *Df* of BX-C (when homozygous) kills *D. melano-*

gaster at an early developmental stage. Such animals – to a first approximation – exhibit embryonic *segments* that appear to all alike among them (Lewis, 1995). It is as if this higher insect has been *transformed* to take on qualities of a most primitive invertebrate, viz. an annelid. This category of BX-C-based transformation is analogous to those described above, whereby viable genotypes change adult-segment qualities in the direction of evolutionary primitiveness. Implication (of a Nobel Prize winning variety): Genes of the BX-C, in their normal forms, evolved to *suppress the latent capacities* of *Drosophila* to develop in ways that more primitive invertebrates do. Yet BX-C genes turned out to be significant *way beyond* the invertebrate arena, as will be mentioned later.

The matter of lethal genotypes brings us back to reconsider multiply inverted *balancer* chromosomes (within this article, as a reminder, abbreviated *Bal*). X-chromosomal *Bal*'s usually do not carry recessive-lethal marker mutations, allowing X-*Bal*/Y males to live. *Bal/lethal-variant* females – expressing the effect of a non-lethal dominant marker mutation – are also alive, if the lethal factor carried "under" the *Bal* is recessive, à la Muller's discovery, alluded to above, of lethal genotypes newly induced by ionizing radiation. How about balancer chromosomes generated on behalf of the *D. melanogaster* autosomes? Consider the long "third chromosome," which harbors the BX-C. This developmentally (and evolutionarily) important genotype has long been maintained in a *Bal*(*3*)/*Df*-BX-C "stock." For this, the balancer (as for all such multiply-inverted autosomes in *D. melanogaster*) was deliberately constructed to contain a dominant marker, whereby *Dom*/+ causes an conveniently recognizable anatomical change at the level of live flies. Such a stock is true-breeding, whereby only *Bal*(*3*)/*Df*-BX-C live flies are produced down the generations because the dominant mutation was deliberately chosen to cause, as well, *recessive lethality*. This "strain maintenance" situation allows developing animals homozygous for *Df*-BX-C to be "pulled out," whenever one wishes, for observations, etc. And the deletion genotype can never be lost in terms of routine stock preservation, because any live fly manifesting effects of the balancer's *Dom* marker must be *heterozygous* with *Df*-BX-C; this is because the multiple inversions disallow meiotic recombination, which otherwise would lead to *Dom*-with-*Df*-BX-C or "+ +" recombinant genotypes.

An insert, with a bit more about inverted chromosomes in *Drosophila*: I find it interesting that an eventual Nobel Prize winner – George Beadle, who came to study biochemical genetics in *Neurospora* (reviewed by Dronamraju, 1991) – was a geneticist studying fruit flies in his earlier days. For example, Beadle analyzed the properties and cellular behavior of *Drosophila* inversions, working with Sturtevant (e.g., Beadle and Sturtevant, 1935; historically summarized by Hawley and Ganetzky, 2016).

The Prize just referred to (1958) was co-awarded to the biochemical geneticist Edward Tatum, who began his career by studying the genetics of bacteria with Joshua Lederberg (e.g., Tatum and Lederberg, 1947). In fact, this late 50s Prize was awarded to Lederberg, along with Beadle and Tatum. It should go without saying that deconvoluting prokaryotic genetics – including genetically & molecularly characterized "vectors" that can be introduced into bacterial cells – turned out to be quintessential for essentially the entire enterprise that can be described as "genetic engineering" (DNA cloning and molecular manipulations of such macromolecules).

I should pause to mention that Beadle's and Tatum's exploitation of *Neurospora* genetics (e.g., Tatum and Beadle, 1942) was *not* designed to find out all that much about fungal genotypes alone (genetic genetics). By later analogy, *N. crassa* was magnificently utilized, from another *biological* perspective, to analyze bona fide circadian rhythms in that fungus. This microbial enterprise was pioneered and sustained by Jerry Feldman, Jay Dunlap, Jennifer Loros, and colleagues, who made a highly-successful genetic plus molecular assault on "what is the clock?" in this supposedly lower eukaryote (summarized, during the rather early days of these inquiries, by Dunlap, 1999).

With regard to a *higher* eukaryote, the principal *genetically* based power of inversion application as set-up to be possible by the likes of Beadle and Sturtevant has to do with – you guessed it – *recombination-eliminating balancer* chromosomes. I provided a primer about such chromosome aberrations of the multiply inverted variety and about other types of homozygous-lethal genotypes, for reasons extending beyond a tip of the hat pointed toward the estimable Professor Lewis. As one of the most knowledgeable and proficient drosophilists, ever, he was well aware of the power of balancer chromosomes, crucially applied in his case to maintain strains of developmentally interesting lethal factors. Now many other drosophilists, including bio-geneticists ever emerging as the 1970s unfolded, were also astute enough to be aware of (a) pertinent cytogenetic tools, e.g., *Bal*'s, or "the attached-X," which will re-surface yet again below, and (b) how lethal mutations can cause interesting biological changes as well as pointing to putatively interesting genes, in terms of what such factors are doing on behalf of wild-type development. Two such investigators, who began to work together during the 1970s, were Christiane (Janni) Nüsslein-Volhard and Eric Wiechaus. They initiated their developmental-genetic investigations by treating "normal" males from some wild-type stock or the other with a powerfully acting *chemical mutagen* (abbreviated EMS). Its properties and utility had been rattling round the fly world as of mid/late 1960s or so, and EMS has been applied to generate from-scratch a variety of interesting, novel mutants (see

below.) Lewis himself published a brief research note (Lewis and Bacher, 1968), which described EMS mutagenesis and how conveniently it can be effected by merely feeding flies on sugar water laced with the mutagen (thus no need to do something like inject the substance into treated, starting flies).

So Nüsslein-Volhard and Wieschaus, operating as third or fourth generation "mutagenizers" (late '70s, vis-à-vis a perceivable starting point for systematic developmental genetics approximately one decade earlier) proceeded roughly like this: treated the aforenoted starting flies and mated them to *Bal*-bearing females contained in a *Bal* + *Dom1/Dom2* stock, again of the true-breading variety, because each such *Dom* – here designated generically as containing 2 different dominant markers – was and is a recessive-lethal mutation, to repeat a previously introduced point. Next-generation flies – either *Bal/mutagenized autosome* (*mut*) females or *Dom2/mut* males – were backcrossed to *Bal* + *Dom1/Dom2* flies. Now any offspring of a *Dom2/mut* male that does not display the marker effect of *Dom2* must carry *mut*, because there is no recombination within meioses of males in *D. melanogaster* and thus no need for the *Dom2* marker to be contained within a multiply inverted autosome. Result: Nüsslein-Volhard's and Wiechaus's *mut* chromosomes were contained in next-generation offspring of *both* sexes (e.g., *Bal* + *Dom1/mut* females and *Dom2/mut* males). Question: Will any offspring in the *subsequent* (ultimate) generation *not* express effects of either *Dom* marker? If not, then *mut/mut* was a non-lethal genotype. Yet many of these ultimate matings, for the many "lines" established by this pair of developmental geneticists, each line originating from an individual *Bal* + *Dom1/mut* female or *Dom2/mut* male, displayed only *Dom* males and females, signifying that the *mut* in question carried at least one newly induced lethal mutation. So I hope the reader can discern how powerful are applications of balancer chromosomes: Observing the *Dom* marker *phenotypes*, as outlined briefly above, unequivocally reveals to the investigator the animal's *genotype*. Reality check: Nüsslein-Volhard and Wieschaus infused additional genetic "tricks," which made the genetic side of their screening even more proficiently performable (summarized by Wieschaus, 1995). As usual, these bio-geneticists tapped into the rich array of extant genetic variants and the "extra" opportunities afforded by them, beyond usage of standard *Dom* markers, for example.

The developmental(bio)-geneticists in question observed what amounted to dying embryos, as to *which* if any might manifest anatomical anomalies that could suggest the mutationally defined gene at hand to be involved in "pattern formation:" the insects formative body plan (e.g., nature of embryonic segments, segment identities). Thus, Nüsslein-Volhard and Wieschaus discovered mutants and genes – a small but appreci-

able subset of those pointed to by their newly induced mutations – that could mutate to lethality for reasons more bio-interesting than in cases where a normally appearing mutant might just grind to a developmental halt or begin to generically to disintegrate.

Here is an ostensibly stray aside (but not really), related to the outcomes of Nüsslein-Volhard's and Wieschaus's mutant hunting. The matter now at hand is known as "genetic saturation", which will also rear its handsome head later. Here, a not-always-appreciated element of these investigators' findings involved *numbers* of newly induced mutants. This quantitative matter was summarized, during the decade subsequent to the pertinent Prize winning paper (1980), by Nüsslein-Volhard (1995) and Wieschaus (1995). An editorial note: After this pair of biologists published their announcement report (1980), in a vanity journal not conducive to full disclosures (*re* page limitations), they followed up with "journal of record" reports (cited by Nüsslein-Volhard, 1995) that laid out *all* aspects of their successful hunting, including things like "just how many mutations" corresponded to a given genetic locus hit by the mutagen. Nowadays, not all out-of-breath "banner" papers put forth full accounts, of what was done and found; and usefully dense follow-ups tend minimally to appear in the later literature. In any case, the mutant hunters at hand documented a huge number of developmental-lethal mutations in *Drosophila*, only a subset of which caused apparent abnormalities of embryonic pattern formation. Yet that relatively small proportion of "extra interesting genes" was defined by appreciable more independently induced mutations than genetic-loci defined, even though some of the latter were pointed to by only "one hit" each (summarized by Wieschaus, 1995). Therefore, it was as if these developmental geneticists could have been approaching, but not-yet "at", a situation of genetic *saturation*. This scenario would have been altogether different if every new mutation turned out to define a previously *un*-identified gene. Certain of the *chrono*-genetic sub-enterprises revealed a similar state of affairs, as we shall see, related to number games played in context of # mutations vis-à-vis # genes.

A fair number of the developmental genes under consideration within this portion of the essay implied to the larger "developmental genetic community" that such factors could be molecularly operating on behalf of *regulating other genes*, ultimately those immediately underpinning the relevant anatomical structures. Indeed – and owing to the fact that certain mutations in fruit flies and in many other forms not only cause ostensibly interesting abnormalities, they also (I say again) identify genetic loci – when several of the genes "pointed to" by the Nüsslein-Volhard/Wieschaus mutations were identified based on where they were located chromosomally, a large proportion of the encoded products – determined post-DNA cloning – seemed as if their would be *transcription factors*

(summarized by Wieschaus, 1995). Again, these outcomes were as gingerly predicted (e.g., Lawrence, 1992). As well, those types of gene products come out of Lewis's (originally Bridges's) BX-C. More broadly, a rather large percentage of "developmental genes" operating in all kinds of animals on behalf of embryonic pattern formation appeared – via first-blush "clone and sequence" data – to encode proteins with DNA-binding capacities. Nucleotide sequencing of the genes' coding regions divulged on-paper proteins, several of which harbor "motifs" known to promote physical associations between such polypeptides and DNA sequences surmised to possess gene-regulatory functions.

The ever-expanding significance of the purely bio-genetic discoveries put forth by the 1995 Prize winners was based in part on molecular geneticists, working in a variety of "non-insect" systems, identifying "cognates" of BX-C genes along with several of those pointed to by the Nüsslein-Volhard/Wieschaus mutations, thereby describing molecularly interpretable products to emanate from way more than the "fly genes" alone (reviewed, from a neuro-perspective, by Bellen et al., 2010). It bears mentioning that the "evo-devo" extravaganza – studying development from the perspective of homologous genes acting analogously among separate species – was pioneered by developmentally investigating drosophilists. They prayerfully wondered whether DNA sequences cloned from "homeotic" genes, such as within the BX-C, might be applicable to identify molecular relatives in other species of animal; several such prayers were answered (beginning with McGinnis et al., 1984a, b, for example; reviewed by Gehring et al., 2009).

How about the *chrono*-genetic enterprise? To ramp up toward recounting that currently important sub-story, it must be first noted that bio-genetics in *Drosophila* began to leak out a little into the literature several years before the Prize-wining reports of Lewis (1978) plus Nüsslein-Volhard and Wieschaus (1980). How systematic bio-genetics started can be exemplified by the *developmental*-genetic accomplishments of David Suzuki and co-workers. Back before he became a generic science publicizer (popularizer?) in his native Canada, he began his bio-career at a small level, doing a bit of *Drosophila* genetics at Amherst College in Amherst, Massachusetts, under the aforementioned Phil Ives (à la Hall, who was mentored by Ives later on). Suzuki went on to earn a PhD, studying aand investigating under a hard-core fruit-fly geneticist, leading the former on his own to perform early-career studies at the faculty level in Western Canada in that restricted investigative area. But as of the mid/late 1960s, Suzuki slipped sideways into performance of bio-genetic studies: He generated anew and identified developmental mutants that were *temperature-sensitive* (TS), e.g., as caused by heat-sensitive lethal mutations (reviewed by Suzuki et al., 1976).

Remarkably one of the "classic" *bithorax* mutants (Lindsley and Grell, 1968d) was found retrospectively to be a TS developmental mutant, e.g., Villee, 1945; Kaufman et al., 1973). Beginning in the early 1970s, Suzuki's group began also to induce and isolate *adult-functional* mutants. For this, these investigators exploited the famed attached-X type of chromosome aberration, as follows: Starting flies (genetically normal XY males) were treated with the powerful mutagen EMS (as introduced already) then crossed to attached-X/Y females. Individual male offspring who had inherited a treated X from their father were "backcrossed" to the same attached-X type, to created a series of "putant" lines (putative mutants). One type of adult-mutant hunting effected by the Suzuki group entailed ferreting out "patho-physiological" mutants, viz. post-natal flies that were paralyzed at high temperature but OK at lower ones (e.g., Suzuki et al., 1971; Poodry et al., 1973).

One might call the Suzukian enterprise just outline an aspect of "behavior genetics," although it is difficult to pit mutationally-caused TS paralysis against a normal phenotype (elementary well-being at relatively low temperatures). This issue, if it is one, introduces the matter of *bona fide* behavioral genetics, whereby measurable phenotypes of the normal variety can be quantitatively compared with mutationally caused abnormalities or anomalies. In this respect, a key behavioral genetic pioneer was Seymour Benzer, who searched for novel behavioral sports in a dedicated manner: induction from scratch via application of the same chemical mutagen under consideration here, followed by systematic screening for heritable variants manifesting adult defects (Benzer, 1967). What Benzer had opted to do career-wise was leave the microbial genetic field in which he had toiled, notably during the 1950s, to become a higher-eukaryotic biogeneticist. After treating starting flies with EMS and mating them to – you guessed it – Muller's attached-X, *y f*, the remainder of Benzer's genetic tactic was identical to that already described, Suzuki-wise. Ultimately, groups of males from a given putant line – each carrying the same originally mutagenized X – were tested for responses to light; this led to several sports that manifested subnormal or anomalous "phototaxis" (Benzer, 1967).

Shortly after the time of the report just cited, a student in Benzer's CalTech lab, Ronald (Ron) Konopka, carried out the precise same genetic tactic. In this case, two generations from the start, the lines were screened for males that might exhibit abnormalities of *daily rhythmicity*. Three such putant lines were identified, out of some 2000 tries (Konopka and Benzer, 1971). Usefully, it seemed, the *female* progeny in these late-60s/early-70s operations served as *internal controls* for the mutant hunting. By-eye observations of intra-line offspring were doable even at low resolution, because the *yellow* body-color phenotype is readily observable without recourse to a microscope.

Konopka's original trio of mutants, *each* involving mutagen "hits" at *one* X-chromosomal locus that got named *period* (*per*), arguably displayed interesting rhythm abnormalities (Konopka and Benzer, 1971): no rhythmicity, 19 hour cycle durations in constant darkness, or 29 h cycles in that condition (the latter two phenotypes being *way* off the ca. 24 h norm, for this species of insect and for animals in general). "Interesting" mutant phenotypes or not – your choice – this search for novel *circadian rhythm* mutants (referring to the altered cycle durations, or lack of rhythmicity, in a constant environmental condition) was clearly successful. It would not have been, had Konopka been "0 for 2000." Importantly, I think this mutant hunting was not *hyper*-successful, as it could have been perceived to be if the numerator had been something like 200 or 300. That kind of outcome could have prompted the following types of questions: *Which* among an avalanche of new mutants would one plan to pursue? *And* could such a large number (hundreds instead of a tractable trio) imply that a great many of separate genes feed somehow into rhythm-regulating processes, many or most of these genic factors causing such effects indirectly? Instead, the *one* gene defined by the seminal *period* mutations allowed Ron and his boss gingerly to surmise that the factor pointed to by the trio in questions is a "clock gene": hence the title of Konopka's and Benzer's seminal paper. The assumed product of such a gene would operate, again hypothetically, somewhere near the "core" of *Drosophila's* circadian clock.

In this regard – perhaps unwittingly allowing for eventual identification of his *period* gene at the molecular level – Konopka applied his *period* mutations to determine the gene locus's location at very high intra-chromosomal resolution. This nuts and bolts operation (genetic bookkeeping) was accomplished in conjunction with proving that all three of Ron's mutations mapped to one X-chromosomal site (Konopka and Benzer, 1971; Smith and Konopka, 1981; augmented by elements of Young and Judd, 1978). What Konopka, who was a fine chronobiologist *and* geneticist, accomplished accordingly was to "meiotically map" his *period* mutations, via application of Sturtevantian principles and practices (cf. Sturtevant, 1913). As well, Konopka applied various chromosome aberrations – notably deletions – that had to involve intra-X-chromosomal sites near *per's* map position, to refine the gene's location (Konopka and Benzer, 1971; Smith and Konopka, 1981). This chrono-investigator was fortunate that his *per* gene happened to be located near the classic *white* (*w*) one, mutational identification and mapping of which led to generating many other types of "near-*w*" variants (e.g., Judd et al., 1972; Young and Judd, 1978).

These genetic practices became the norm, of a sort, when subsequent rhythm-interested drosophilists went to school on the Konopka approach. Such successors of Konopka mutagenized starting flies, as

usual with the workhorse chemical referred to already, or by what is known as "transposon mobilization"; then they worked through mating schemes strongly analogous to those effected by Nüsslein-Volhard and Wieschaus (making, you guessed it, crucial application of dominantly marked balancer autosomes). But in this case – three generations after the mutagenesis-based starting points – the investigators who set out to identify novel rhythm mutants with autosomal etiologies looked for "lines" in which *mut/mut* genotypes, as explained above, segregating out of the final-stage matings allowed for adult viability and daily-rhythm testings (behaviorally).

It follows that the post-Konopka chrono-mutant hunters exploiting *Drosophila*-based opportunities were hoping mutationally to define rhythm-related genes beyond the X chromosome (cf. Konopka and Benzer, 1971; Konopka et al., 1991). This "worked out," as summarized in the relatively early days of post-Konopka mutant hunting by Hall (2003). Two features of these outcomes: (1) At least a couple dozen novel rhythm-altering mutations were induced and identified, *but* they defined only about a half dozen novel genes; it follows that "repeat hits" kept being effected at previously identified loci, as if still further mutations with clear effects on daily rhythmicity were rather unlikely to expand the overall genetic horizon toward oblivion, e.g., three dozen mutations defining that many genes, implying that "mutation #37 or so" would point to yet another factor that would have warranted some sort of ever-broadening analysis; (2) As just implied, the genetic loci determined for the new rhythm mutations were homed-in-upon by genetic and *Df*-based mappings, so that these genes could be identified and physically isolated at the DNA level (Hall, 2003).

Once again, these investigative processes exemplify the "power of forward genetics" starting with mutants then genetic map positions and subsequent "positional cloning" (DNA-based identification of interesting genes based on their chromosomal locations). Yet it is fair to say that the chrono-molecular-genetic investigators coming up with this array of mutants and molecular clones could hardly ever predict what kinds of protein products would be encoded by a given rhythm-related genes; thus a different scenario than in the case of developmental-cum-molecular genetics (e.g., Lawrence, 1992; Wieschaus, 1995). As we shall see, "just what" a given chrono-molecular "is doing" on behalf of cellular and molecular processes – harking back to the initially mysterious PERIOD (PER) protein – had to be figured out by down-to-earth empiricism (histologically and biochemically based lab work).

I exaggerate a bit, for there were additional rhythm-related factors initially identified in their *normal* forms, by application of various "purely molecular" tactics (summarized, again, by Hall, 2003). For example, a

gene inferred to encode a neuropeptide in *Drosophila*, eventually named "pigment dispersing factor" (albeit not operating on behalf of any pigment dispersal), was identified and isolated at the DNA level as a molecularly normal factor. This led to establishment of the *pdf* gene's chromosomal location, by effecting a cyto-molecular procedure requiring no genetic variant to be in hand (Park and Hall, 1998). These findings allowed for the possibility of *subsequent* identification of a *pdf*-locus mutation, whose effects on behavioral rhythmicity could be assessed and were (Renn et al., 1999). This kind of strategy is known as "reverse genetics:" start with a gene in its normal form, whose significance cannot necessarily be inferred by the encoded product's attribute "on paper," viz. "clone and sequence;" but then come across or deliberately generate mutations within the relevant loci and ask about their potential effects on the bio-process of interest.

I should pause further to exemplify what reverse genetic starting points have meant in the rhythm arena. One of the new "clock genes" found by the post-Konopka mutant hunting referred to above was named *Clock* (*Clk*). Why? This was because positional cloning of the *Clk*-identified locus in *D. melanogaster*, followed by DNA sequencing of the gene's coding subset (Allada et al., 1998), came up with an on-paper protein quite similar to that emanating from the *Clk* gene in mouse, which had hopefully been named *Clock*, even though the original variant solely signified "rhythm mutant," as opposed to a circadian-pacemaker variant, necessarily. After the murine factor originated by way of a chemically induced mutation (Vitaterna et al., 1994), à la Konopka, the responsible variant was fine-level mapped; this allowed for molecular cloning in mouse of *Clk* DNA (Antoch et al., 1997; King et al., 1997). The CLK protein's nature (on paper) suggested that it could have "something to do with" PER protein (cf. Allada et al., 1998), as if CLK were indeed operating on behalf of the circadian clock in mammals.

Findings of this sort made various investigators wonder whether might there be *per*-like genes in such animals. For several years, after *period* was cloned in *Drosophila*, molecular searches for mammalian *per*'s came up dry; until 1997, when two separate research groups identified molecular relatives of fruit-fly *per* in human and mouse (Sun et al., 1997; Tei et al., 1997). This suggested reverse-genetic possibilities, whereby the separate *per*-like genes in mouse (n=3 per mammalian species) were reverse-genetically mutated (e.g., Bae et al., 2001; reviewed in a broad context by Bućan and Abel, 2002).

How about going in the other direction interspecifically? Well, cloning of mouse *Clk* made certain chrono-drosophilists imagine that a molecular relative of mammalian *Clk* might be identified and isolable in *Drosophila*. Yes, as it turned out. This led to manipulations of fly-*Clk* DNA in its nor-

mal form, leading to inferences as to how CLK in flies functions as part of the insect's clockworks (e.g., Darlington et al., 1998). But pure reverse genetics as such was not necessary here, because fly-*Clk* was co-identified initially by an arrhythmia-inducing mutation (staring with EMS mutagenesis, as noted), which proceeded to *forward*-genetic elucidation of the gene and its product (Allada et al., 1998). This strategy had to include overt rhythm significance for *Clk* gene action in *Drosophila* (as in mouse), because the starting point was a rhythm-defective mutant: behaviorally arrhythmic *and* with the *Clk* mutation causing *per* product levels in *Drosophila* to be very low (Allada et al., 1998).

What was just summarized points, once again, to matters revolving round "genetic satutation" of the situation revolving round rhythm-regulation in animals. First, let it be noted the *Jerk* mutant (as fly-*Clock* was provisionally named pre-cloning) could have involved "anything" in terms of encoded-product quality. Yet what that gene makes in the fly is none other than a very similar rhythm-related protein in mammals. As the post-Konopka mutant hunting matured, Young's lab mutationally identified a locus (Price et al., 1998) called *doubletime* (*dbt*). It turned out to encode a known category of kinase enzyme (Kloss et al., 1998), which in *Drosophila* targets PER protein as one of its substrates (e.g., Syed et al., 2011). Incidentally, *dbt* – essentially at the same time it was discovered via rhythm mutants – could not have been surmised to be "chrono-specific," because the gene was contemporaneously identified via mutations at the self-same locus that cause embryonic lethality (Zillian et al., 1999). Thus *dbt* – *un*like *per* and the Young lab's famed *timeless* (*tim*) gene (originally Sehgal et al., 1991, 1994) – is developmentally vital. Therefore, in turn, the DBT enzyme must target substrates not limited to the inessential PER protein. Mutant fruit-flies with a ruined *period* gene, or lacking it altogether, are alive and "seem fine" until one tests them for daily rhythms, as will be certified later. So the case of *doubletime* caused that gene to take on expanded biological significance, even though the overall case of clock genes in flies and mammals caused the story *not* to get bloated beyond a relatively small number of key "players."

More about mammals, to drive home the point just claimed. Within a non-murine mammalian species, a dramatic rhythm-affecting mutation was run across: a spontaneous sport called *tau* which, when homozygous, causes *hamsters* to manifest ca. *20-hour* cycle durations in constant darkness (Ralph and Menaker, 1988). Even though forward-genetic possibilities are minimal for this type of mammal, Joe Takahashi's research group managed to home in upon the relevant genetic locus, in order to clone *tau* DNA from hamster (Lowrey et al., 2000) then reveal that the encoded product is none other than a close molecular relative of *Drosophila*'s DBT kinase (cf. Kloss et al., 1998). Once again, TAU could have been anything,

barely predictable pre-cloning; but the outcome was to *re*-identify an already appreciated clock-functioning factor.

These molecular-genetic examples of saturation-signifying claims are analogous to "more rhythm-affecting mutations than genes pointed to." Here, the matter of *Clk* (née *Jerk*) in *Drosophila*, followed by the case of *tau* in hamster, could well have identified previously unknown (even unanticipated categories of) players materially contributing to animal clockworks. But both such cases got squeezed down to clock factors already on the "animal-rhythm table."

Another intra-fly example, whereby yours truly realizes he keeps getting ahead of the overall sweep of chrono-molecular history: The Konopka-like, but autosomally based, screening for new rhythm mutants resulted in afore-described *Clk* and a separate gene (*cycle*, a.k.a. *cyc*) whose category of protein-product co-functions with CLK (Rutila et al., 1998; cf. Allada et al., 1998). Two *additional* rhythm-altering mutations fell out of this screening, beyond those that which eventually were revealed to generate two independently induced mutations at *each* of the *Clk* and *cyc* loci.. The additional two – for a total of six – co-mapped with one another, at an autosomal locus well separated from those of *Clk* or *cyc* (Suri et al., 2000). Now the pair of "extra" mutants could have recognized a brand-new rhythm-related factor; instead the (non-*Clk*, non-*cyc*) mutations at hand turned out merely to re-identify *doubletime* (Suri et al., 2000), just as *tim* was hit by further mutageneses carried out in Young's research group (e.g., Rothenfluh et al., 2000) *and* independently within the Brandeis-based one (Rutila et al., 1996).

I have been soft-pedaling the matter of "cloning the first clock gene": yes, *period* in *Drosophila*. Owing to the 2017 Prize (Physiology or Medicine) being based in part on a trio of lab-heads whose research groups basically co-identified *per* at the molecular level, here is some requisite historical information. First, let it be known that the aforementioned matter of "genetic saturation" has to do with much more than "lots of *rhythm*-affecting mutations defining a much smaller number of genes." Thus the saturation issue was in play when reasons for Janni Nüsslein-Volhard's and Eric Wieschaus's anointment as Nobel laureates were outlined in previous passages. An earlier example from the sub-field of developmental genetics: A well-defined segment of *Drosophila*'s X chromosome – near the *white gene* as noted – was saturated by the research group of Burke Judd, in terms of loci that can mutate to lethality. Cleverly applying various genetic tricks (crucially, as usual, dependent on pre-existing chromosome aberrations), these investigators induced hundreds of novel mutations, all necessarily confined to a small, near-*w* region of the X chromosome, defined by the length of a certain X deletion. But that many mutations defined only a bit more than one dozen loci; it followed

that *that* many "vital genes" were contained within the region (Judd et al., 1972). In other words, any further induced lethal mutation would almost certainly be yet another repeat hit at a locus already mutated. But these investigators wondered whether additional genes might be harbored within the region at hand, loci that could not mutate to lethality.

Now Mike Young (at least) was aware that Konopka had mapped his *per* gene to right smack in the middle of the intra-X segment in question (Judd et al., 1972; cf. Konopka and Benzer, 1971). A crucial concern here: All of Konopka's *per* mutants had to be alive, based on his chrono-screening tactic (Konopka and Benzer, 1971); yet this gene *could* have been a vital one, capable of mutating to lethality. If that were so, a set of allelic lethals from the "saturation screen" – lethal mutations independently mapped to be at least very near *per* – would fail to "complement" certain *per* mutations: e.g., arrhythmia-inducing per^{o} "over" given such lethal would cause locomotor arrhythmicity, just as Konopka had found *Df-per/* per^{o} to cause. Yet Young found that all the relevant lethals complemented per^{o}: None of the pertinent heterozygous types was arrhythmic, just as was known for the strongly rhythmic attributes of per^{o}/+ or *Df-per*/+ types (Young and Judd, 1978; cf. Konopka and Benzer, 1971). Thus, Judd et al. (1972) had not really "saturated the region" in terms of identifying *all Drosophila* genes contained therein, only for the developmentally vital ones. It followed that the *period* gene in this insect cannot mutate to lethality – only, it seemed in terms of most severe mutational effects, causing biological arrhythmicity.

An obvious implication of the foregoing micro-history is that Dr. Young's interest in the *period* gene harked back to his formative years, when he was a graduate student in Judd's lab. When this investigator expanded his horizons on behalf of bio-*molecular* genetics, he began to study a classic "developmental gene" in *Drosophila* called *Notch* (*N*). Don't ask, but register that this factor had long been regarded as potentially interesting. And fly *N* is related to strongly analogous factors in other animals, of course (reviewed by Bellen et al., 2010), just as are several of the Prize-winning "Lewis/ Nüsslein-Volhard/Wieschaus" genes. Well, the *N* locus is on the X chromosome of *D. melanogaster*, not all that far from *w* and *per*. Thus Young's own research group – once he started his lab in New York City after his grad student days and a "molecular post doc" in California – aimed to "clone *Notch*." He did so (e.g., Kidd et al., 1983, 1986) and realized that this afforded the possibility of effecting a "molecular chromosomal jump" from the *Notch* locus to the *period* one. This, too, worked out thanks to clever application of the relevant molecular clones *and* of chromosome aberrations whose lesions crucially "touched" both of the pertinent intra-chromosomal loci (Bargiello and Young, 1984).

Contemporaneously, the neighboring Hall and Rosbash Labs: (within Waltham, Massachusetts) opted to "try and clone *per*" in competition with Young and co-workers. Why at Brandeis University? This motivation harked back to Hall, working with a postdoc C.P. (Bambos) Kyriacou in the late 1970s, stumbling upon a short-term behavioral rhythm in *Drosophila* connected with normal male courtship behavior (Kyriacou and Hall, 1980). Those two investigators were aware that the only extant rhythm mutants in this species were Konopka's *period* ones. Therefore Hall, who had long been in touch with his former labmate Konopka, asked the latter if he would send a trio of stock containers, representing the famed trio of *per* mutants. Konopka so transmitted, along with a snail-mailed note saying words to the effect of "that sounds like a nice experiment." Hall wondered why Ron would say such a thing: How could one expect mutations, which by definition affect daily rhythmicities, also to cause alterations of the one-minute courtship rhythm in question? Yet each of those three mutations *did* so affect (Kyriacou and Hall, 1980). Disclaimer: Other courtship-involved investigators are skeptical of that study and related ones that followed, as initially reported in the very early '80s. I will not burden you with this matter, but see the review of Hall and Kyriacou (1990); also Alt et al. (1998); Stern (2014); Stern et al., (2017); Kyriacou et al. (2017, 2019).

Because various elements of *Drosophila* courtship are species-specific, Kyriacou and Hall asked whether the "one-minute" rhythm displayed by *D. melanogaster* males would be the same, different, or non-existent in other species. They found and reported about five years later that males of *D. simulans* (here abbreviated *sim*) generate the relevant "courtship-song" rhythm with a cycle duration of only about 40 seconds, compared with about 60 sec for "*mel*" males (Kyriacou and Hall, 1986). As was kind of customary for species-diverging characters, these reproductive-behavioral investigators generated interspecific hybrids between *mel* and *sim*. Those two *Drosophila* types are close evolutionary relatives, long known to generate hybrid progeny when males of one species are coaxed to mate with females of the other. Yet, it was also known that hybrid males with a *mel* X (over a *sim* Y) are developmentally dead; whereas the sexually reciprocal type (*sim* X/*mel* Y) is alive and behaves OK or better. In this regard, Kyriacou and Hall were aware of – dare I say again? – the lore of *Drosophila*. Thus they applied a mutation in *D. simulans* known as *Lethal hybrid rescue*; contained in a *sim* male parent, it mated to a standard *mel* female, leading to viable *mel* X/*sim* Y sons. The reciprocal mating effected by these investigators was standard *sim* males x attached-X *mel* females; their sons are *sim* X/*mel* Y. Yet again (!) the "power of the attached-X," applied here to control for "maternal effects" on hybrid progeny properties, viz. species-specific egg cytoplasm influences

thereon, putatively *not* operating in this case, for both female-parent types were *D. melanogaster*. Behavioral results: The reciprocally hybrid male types put forth courtship-song rhythms matching the type of X chromosome coming in from the dual-species parents (Kyriacou and Hall, 1986). It followed that the etiology of the species difference in rhythm singing mapped to only one chromosome, not the typical outcome inferable from the behavior of hybrid animals. So, could it be that said etiology was harbored within but *one genetic locus* on that chromosome? It was (and is) "the X," where the *period* gene is located.

Well, this question was in-principle answerable, as of the early 1980s: (A) Could the *period* gene be cloned from *D. melanogaster*? Yes, as exemplified above. This would allow for quick cloning of *per* from *D. simulans*, which would have to harbor a strongly homologous *period* gene. Now, a pair of *Drosophila* biologists-cum-molecular geneticists had burst forth contemporaneously with the wherewithal to generate routinely "DNA transformed" fruit flies (Spradling and Rubin, 1982; Rubin and Spradling, 1982). The potential for revolutionizing bio-molecular-genetic studies of this insect was realized by an endless number of DNA-mediated *transgenic* types designed and generated by drosophilists, thanks to proficient transgenesis doability established by Allan Spradling and Gerry Rubin, cited above. What *kinds of DNA engineerings* could be effected – followed by introduction into *Drosophila* and insertions of the introduced "constructs" into the flies' chromosomes – seemed limited only by investigators' imaginations. Here is but one example thereby, inserted here as something of a conceit. We at Brandeis, working with the research group of Steve Kay elsewhere, wondered whether "molecular cycling" of *period* gene products, dealt with historically below, might be monitorable at the level of *individual live flies*. Thus we fused a regulatory region cloned from the *per* locus to a DNA sequence (*luc*) encoding fire-fly luciferase; and when that transgenic type was fed the relevant enzyme substrate, "glow rhythms" could indeed be measured all day, and during the next and the next (Brandes et al., 1996). This ploy led, for instance, to establishment of "molecular-rhythm parameters" that (in contrast) can be mushily apprehendable via sacrificing and extracting-from large numbers of flies "per time point." This "*per-luc*" transgenic-type was exploited in several ways subsequently. For example, *Drosophila* carrying pre-mutagenized chromosomes *and* that transgene were screened for novel rhythm mutants, not based on behavioral phenotypes; instead upon mutational alterations of normal LUC-reported cycling (Stanewsky et al., 1998). This led to identification of a new mutant named *cry*, along with forward-genetic identification of the corresponding DNA. Not to burden readers with hardly any details: *Drosophila* CRY functions on an "input pathway" involving light-induced daily resets of the fly's circadian clock (e.g., Hall, 2000).

This enterprise within the enterprise exemplifies how certain fruit fly chrono-geneticists came to study not only core clock functions (via *period* and a handful of additional genes), but also the important matter of environmental *inputs to the clock*. Circadian clocks, in general, are not very accurate; but they are easily reset, daily, and notably by photic stimuli. We and various other research groups also hoped to tackle the aforementioned matter of *outputs* "from the clock" (e.g. Peng et al., 2003; cf. Park and Hall, 1998; Renn et al., 1999), realizing that a clock at its core does nothing for the organism, unless key elements of central pacemaker function "feed forward" into the regulation of revealed rhythmicity.

Back to the matter of courtship-song rhythmicity. Spradling's and Rubin's transgenically based breakthrough made us at Brandeis realize one more thing that became doable: Against a backdrop of the interspecific song-rhythm difference summarized earlier, and mapping the etiology of this species-specificity to the X chromosome, we could molecularly transfer per^{+} DNA from *D. simulans* into *D. melanogaster*, whereby that latter type by itself would be genetically arrhythmic (per^{o}) for both locomotor activity (Konopka and Benzer, 1971) and male singing behavior (Kyriacou and Hall, 1980). Specific question: Would such a single-gene interspecific transfer bring with it regulation of courtship-song rhythmicity like that of the donor species (viz. ca. 40-sec cycle duration)? Many years later – however surprisingly, as just implied, or controversial (as referred to earlier) – the answer was "yes" (Wheeler et al., 1991).

Meanwhile, however, we had to get our hands on *per* DNA cloned from *D. melanogaster*. Taking a different tack from that of Young's lab (outlined above), I first realized that we at Brandeis could potentially get a running start with regard to "*per* region" DNA. How? Well this former Brandeisian – who routinely tracked the *Drosophila* literature, no matter how putatively obscure – remembered a then-recent paper, emanating in part from a university that happens to be located within the Prize awarding country: University of Lund, southern Sweden; not that important. What *was* important was that a research group headed there by Jan-Erik Edström had initiated a novel tactic for "positional cloning" by scraping DNA out of a well-defined region of the *D. melanogaster* genome (!). For this, those investigators looked at giant salivary-gland chromosomes (aforementioned) under the microscope and applied a fine-tipped glass needle to an intra-X segment near the *white* gene, thus also near the *period* one (Scalenghe et al., 1981). This subset of the genome was chosen because it had long been so well characterized as to mutationally defined and cytogenetically mapped loci, as already summarized above in various contexts. Scalenghe and co-workers may or may not have been aware that they could have "scraped out" *per* DNA among molecular clones connecting with nearby loci. In any case, when Hall was at a conference co-attended by

one of the Edström lab collaborators (Vince, né Vincenzo, Pirrotta, cf. Scalenghe et al., 1981), the former asked the latter if a follow-up from the original report had identified any "*white*-region" clones corresponding to X subregion "3B1-2."

I burden you with that level of detail in context of (i) there being 102 major regions among the *D. melanogaster* chromosomes, going back to Bridge etc.'s original cytogenetic analyses; each such region is conventionally subdivided into about a half-dozen lettered sub-regions, which in turn are designated by further "sub-numbers" (each of which corresponds to an individualizable salivary-gland chromosomal band, amounting to a total of ca. 50 bands per major numbered region); (ii) Konopka himself, via his aforementioned fine-mapping endeavor, had localized his *period* gene "down to" 1 or 2 intra-X bands (within region 3), or perhaps to a tiny subsegment located between, indeed, bands 3B 1 & 2 (Smith and Konopka, 1981). Furthermore, Ron and his then co-worker had mated two deletion-bearing types to one another, creating *Df-1/Df-2* females (Smith and Konopka, 191), against a backdrop of each such *Df* (here generically designated) "uncovering" the arrhythmia-inducing effects of per^{o}; and one edge of *Df-1* (call it the righthand one) had been pre-designated to "just overlap" the relevant edge (lefthand) of *Df-2*; it was as if this two-deletion heterozygous type *removed* the *period* gene altogether (confirmed to be so in subsequent molecular analyses by Bargiello and Young, 1984; and Reddy et al., 1984). Relevant inferences of the pre-molecular variety were buttressed by the fact that the *Df-1/Df-2* females were found to be alive and arrhythmic (Smith and Konopka, 1981), same as for the behavior of per^{o}/per^{o} females. This cytogenetic ploy once again indicated that the *period* gene is not a vital one.

Against this background, and with apologies for including a rather genetically dense insert, the "DNA-scraper" collaborator whom Hall queried (Pirrotta) said he would check as to whether any of his and co-workers' X region-3 clones might have been found apparently to correspond with aforenoted 3B1-2 (owing to such molecular cytogeneticists having annealed in-situ their various scraped-out clones, of a labeled variety, to salivary-gland chromosomes). That cytogeneticist soon sent a pair of DNA clones to Brandeis, surmising that they could be, or be very near, the molecular starting-point for cloning *per* that we were hoping to acquire. This wish was realized, via various aspects of subsequent labwork performed in the collaborating groups of Hall plus Rosbash (Reddy et al., 1984). As already noted, Young's competing group reported similar, ultimately molecularly converging, findings, about *period* gene molecular genetics (Bargiello and Young, 1984; summarized by Young et al., 1985). Some ostensibly "banner" features of these findings, reported during a late stage of 1984, were "transgenic rescues" of per^{o}, effected by transform-

ing-in putative *per*$^{+}$ DNA (Zehring et al., 1984; Bargiello et al., 1984). It was these early-days findings that made us at Brandeis realize that doing essentially the same, but later, introduction of *per*$^{+}$ DNA from *D. simulans*, had a chance of answering the question about "interspecific genetic etiology" for a behavioral variation, cavalierly posed in previous passages.

Before such an investigative prayer could be put forth (Wheeler et al., 1991), however, various nuts and bolts characterizations of the molecularly identified and transgenically confirmed *period* gene DNA were performed after 1984 and during a fair proportion of the mid/late 1980s. For instance, coding DNA within the *per* locus was sequenced (reviewed by Hall, 2003). But these descriptive findings led, for a while, to no meaningful insights as to how the on-paper *protein* encoded by this ostensible clock gene might function within cells and tissues.

Here is a final fillip to this early history, recounted on behalf of divulging how chronogenetics, then molecular-neurobiological genetics, was – dare I say – pioneered as a (then) cottage sub-industry. It was operating back then as a small part of the burgeoning industrial-level research that the "bio-genetic" system came to be, based conspicuously on *Drosophila* research. Speaking of low-level stuff, the Brandeis groups' "*per* cloning" goal was not really met at first, because Reddy et al. (1984) identified three separate RNA's to emanate from the tiny intra-X region defined by Konopka's two-deletion ploy. Which of that trio was *per* per se (maybe more than one transcription unit)? It occurred to us to track expression of three candidate genes by extracting RNA's at different timepoints during a given daytime, then nighttime. Might one such transcript type manifest systematic fluctuations in its concentration? If so, *that* RNA might be transcribed from *per*. It would have been registered as a gene that can *mutate* to alter daily *biological* rhythms, correlated with the *normal* form of this gene making its product to define a daily *molecular* rhythm. The answer to this preliminary inquiry was wrong, x 3 or so: The incorrect third of the gene trio at hand was reported to generate a daily-oscillating transcript type, whereas what turned out to be *per* RNA was said *not* to fluctuate via whole fly RNA extracts (Reddy et al., 1984; similarly reported in a summary article by Young et al., 1985). This "wrong-RNA" matter was corrected later (Lorenz et al., 1989).

More meaningful than asking at first-blush about *per* RNA oscillations were later demonstrations of PER protein cycling, via timed immuno-histochemical assessments of final product levels in "adult head" specimens (Siwicki et al., 1988; Zerr et al., 1990). Such assessments of temporally controlled expression of the gene were performed contemporaneously with re-examination of the "cycling RNA?" matter by Hardin et al. (1990), based on "head only" extractions of RNAs at a series of timepoints across 24-hour Earth-day cycles.

An investigative fillip, which refers to one of the overall goals that sustained the chrono-genetic enterprise: Siwicki (1988), along with Liu et al. (1988), took the first meaningful step toward describing *where* a clock gene is expressed *in the fly's nervous system*, potentially pointing toward the neural substrates of rhythmic behavior. Additional early-days findings in this arena were made by Brigitte Frisch, John Ewer, and Maki Kaneko (reviewed by Kaneko, 1998). Later, this *neuro*-genetic sub-enterprise expanded into studies made in various other research groups (including that headed by Michael Rosbash, e.g., Stoleru et al., 2004).

I should leave things there, assuming that Professors Rosbash and Young will better recount how *subsequent* investigations during the 1990s and 21st century decades have elucidated "what is PER protein, and what are co-acting or interacting clock gene products, doing to form and operate a circadian clock?" The single-letter adjective italicized shortly above alludes to the fact that figuring out a fair amount of "what's the clock in *Drosophila*?" turned out to be of fine heuristic value for doing so analogously in studies of many other life forms. At a minimum, daily oscillations of clock proteins – in terms of their concentrations, qualitative attributes, or both – became the Zeitgeist of many molecular-chrono-genetic analyses, performed on behalf of investigative systems ranging from microbes to mammals. The relevant investigative extravaganza has been nicely summarized – as these studies came to the fore and, at least for animals, quasi-converged – by Dunlap (1999); Reppert and Weaver (2000); Young and Kay (2001); Chang and Reppert (2001); Kondo, 2007); and by Cohen and Golden (2015).

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This author (and anointee) finds it impossible to acknowledge all the co-workers, labmates, colleagues, and other associates who contributed mightily to studying *Drosophila* rhythms and the clocks underlying them – most valued as these many companions were. There really had to be a large overall crew to perform and sustain theses studies, because they were multi-faceted: genetics, molecular biology, neurobiology, and behavior. Suffice it to say here, with regard to my rhythm-related associates, that I gratefully acknowledge associations with the other pair of "labhead awardees:" Michael Rosbash and Mike Young *and* how that pair of professors fostered vast numbers of experimentally proficient – as well as interesting and enjoyable – interactions among supervisees working within the three research groups in question. Again, such students, postdoctorals, and other researching labmates – distributed between two groups in the Boston area and one located in New York City – have been too numerous to acknowledge individually. One exception to this, specifi-

cally with regard to the "Hall lab:" I can never forget, nor fail to appreciate retrospectively, the first professional-level associate (graduate student) within my laboratory at Brandeis University in Waltham, Massachusetts. That was now-Professor Ralph J. Greenspan (University of California at San Diego). Ralph showed the way with respect to exploiting *Drosophila* genetics in order to make that "system" a genuinely deep neuro-genetic one: hard-core cyto-genetics devoted to elucidating neurochemical, neuro-anatomical, neuro-physiological, and behavioral phenomena, forming something of a symphony of studies that Ralph made speak vigorously to one another. It was (now) Dr. Greenspan who, however unwittingly during the mid/late 1970s, showed the way whereby initially neuro- and molecular-neuro-genetic investigations of *Drosophila*'s "rhythm system" started us down a path along which *mechanistic* analyses of the flies' internal "clockworks" came to the fore in the late 1980s and beyond.

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