The Direction of Evolution in the *Drosophila melanogaster* Species Subgroup Based on Functional Analysis of the Crystal Cells

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ABSTRACT   The crystal cells in the hemolymph of *Drosophila melanogaster* contain paracrystalline inclusions and phenol oxidase. Melanization of this type of blood cell can be induced by treatment of larvae with hot water. By using this functional criterion to establish cell homology we compared the shape of the inclusions in the crystal cells of the *D. melanogaster* species subgroup and a number of distantly related species of *Drosophila*. The crystal cell inclusions in four of the sibling species *(simulans, erecta, mauritiana, yakuba)* are the same as those found in *D. melanogaster*. In the fifth species, *D. teissieri*, the crystal cell inclusions resemble those found in the hemocytes of the other larval drosophilids that were examined rather than those of *D. melanogaster*. On the basis of these observations and the phylogenetic relationships deduced from analysis of chromosomal inversions by Lemeunier and Ashburner ('76), we propose that *D. teissieri* is closer to the ancestral form or is the ancestral species of the *melanogaster* species subgroup of *Drosophila*. This is the first instance in which a hemocyte type has been used to determine the direction of evolution within a subgroup of insect species.

The larval hemolymph of *Drosophila melanogaster* contains two classes of hemocytes, plasmatocytes and crystal cells (Rizki, '57a). Plasmatocytes differentiate into podocyte and lamellocyte variants but the crystal cells, comprising 5–10% of the larval hemocyte population, remain uniquely distinguishable by their prominent paracrystalline inclusions. Such cytoplasmic structures have not, to our knowledge, been reported in the blood cells of other insects or even other species of *Drosophila*. Yet the function ascribed to these cells, storage of the components involved in hemolymph phenol oxidase activity (Rizki and Rizki, '59), is a common attribute of insect hemolymph.

Studies on a recently isolated mutant gene, *Black cells (Bc, 2–80.6±)*, agree with the localization of hemolymph phenol oxidase activity to the crystal cells (Rizki and Rizki, '79a; Rizki et al., submitted for publication). Blood cells with paracrystalline inclusions are absent in *Bc* larvae that contain instead melanized cells in the hemolymph and lymph glands, suggesting that the blackened cells are a mutant form of the crystal cells. When the cells in this mutant are melanized, the phenol oxidase within them is destroyed; hence *Bc* larvae lack phenol oxidase activity. Furthermore, melanization of the crystal cells in *Bc⁺* larvae can be induced by hot water treatment, and the distribution of heat-induced black cells in these larvae mimics the distribution of black cells in *Bc* mutant larvae. From this series of observations it is clear that two methods can be used to identify crystal cells in *D. melanogaster*: structurally, on the basis of their paracrystalline inclusions; and functionally, by hot water treatment to blacken them. Utilizing these criteria we examined the hemocytes of a number of sibling species of *D. melanogaster* and some other members of the family Drosophilidae. For the latter group, no effort was made to be exhaustive; we used species that were available in our laboratory. The five sibling species of *D. melanogaster* used are those studied by Lemeunier and Ashburner ('76). The present report summarizes these findings on *Drosophila* hemocytes and suggests that analysis of hemocyte types may be useful in delineating phylogenetic relationships in *Drosophila*.

MATERIALS AND METHODS

The sibling species used for this study were: *D. melanogaster* (Ore-R wild type), *D. erecta* (154.1), *D. simulans* (C135.20), *D. yakuba* (115), *D. teissieri* (128.2), and *D. mauritiana*. The other species examined were: *D. pseudoobscura*, *D. virilis*, *D. novamexicana*, *D. willistoni*, *D. nebulosa*, *D. buskii*, and *Zaprionus vittiger*.

All larvae were grown on cream of wheat medium with live yeast at 18°C except *Zaprionus* larvae, which were grown on corn meal–agar medium and *D. buskii* larvae, which were grown on medium containing flax seed. Mid-third instar larvae were washed with 1.2% NaClO and rinsed well with distilled H₂O to remove extraneous food particles from the body surface. Larvae were opened by tearing the body wall, taking care not to rupture internal organs, and allowing the hemolymph to flow into a small drop of buffered formaldehyde (2.3% in phosphate buffer at pH 7.2). After 4 minutes dilute Giemsa stain was added to the hemolymph sample, and the stained cells were examined. At least five camera lucida drawings of crystal cells were made for each species to assure that variations in shape and size of the inclusions were recorded.

To establish the functional identity of the cells with inclusions, larvae were immersed in hot water at 70°C. When blackening of hemocytes became apparent, the treated larvae were examined either directly under transmitted light (compound microscope: 12.5 × 16 or 12.5 × 25 magnification), or the larvae were opened in Carnoy fixative and tissues with attached darkened cells were processed and examined. The cytoplasmic inclusions are still visible in the cells that are only lightly tanned, so the cells were not permitted to blacken fully. The inclusions in the melanizing cells and in the stained preparations were compared.

RESULTS

The cytoplasmic inclusions in the crystal cells of *D. melanogaster* have a strikingly regular shape. The two longer sides of the structures appear parallel with ends that are either rectangular or conical. Since photographs of *D. melanogaster* crystal cells have been published previously (Rizki and Rizki, '59), none are included here. However, the paracrystalline inclusions in four of the sibling species, *D. simulans*, *D. mauritiana*, *D. yakuba*, and *D. erecta* are indistinguishable

from those found in *D. melanogaster* crystal, cells, and representative crystal cells from these four species are included (Fig. 1). There are variations in size of the paracrystalline inclusions, and the number of inclusions per cell also differs among crystal cells taken from the same hemolymph sample. Cells similar to the four cells in this plate, each taken from a different sibling species, can all be found in a single sample taken from any one of the sibling species, including *D. melanogaster*. Treatment with hot water blackened the cells with crystals in the four sibling species.

Figure 2 contains photographs of crystal cells from *D. teissieri*, another sibling species of *D. melanogaster*. The inclusions in these cells lack the general rectangular appearance found in the crystal cells of the other five species of the *melanogaster* subgroup of *Drosophila*. They appear chunky and globular. The inclusions are quantitatively variable but none of the inclusions resemble those found in the other sibling species. That these cells are functionally similar to the crystal cells of the other *melanogaster* sibling species can be demonstrated by immersion of the larvae in hot water, for they respond to this treatment precisely as do the crystal cells.

In all the larvae from outside the *melanogaster* species subgroup the hemocytes with inclusions resembled those found in *D. teissieri* rather than those found in *D. melanogaster* (Fig. 3). There were size and quantitative variations, but none similar to the *melanogaster* type were found. For each species the functional relationship to the *melanogaster* crystal cell was established by hot water treatment.

DISCUSSION

This report is the first attempt to use a functional criterion for classification of a specific hemocyte type in the genus *Drosophila*. Classification of insect hemocytes relies primarily on morphological characteristics, so categorization of cells to a common class among different insects is possible only when there are distinctly comparable structural traits. To draw analogies in the absence of such similarity or in the absence of information on the functions of a given cell type is unwise, since hemocytes display morphological variations in response to physiological changes in the hemolymph; they also show variations during their own stages of development (Rizki, '78). *D. melanogaster* with its many mutant strains has been exceptionally useful for studying hemocyte interrelation-
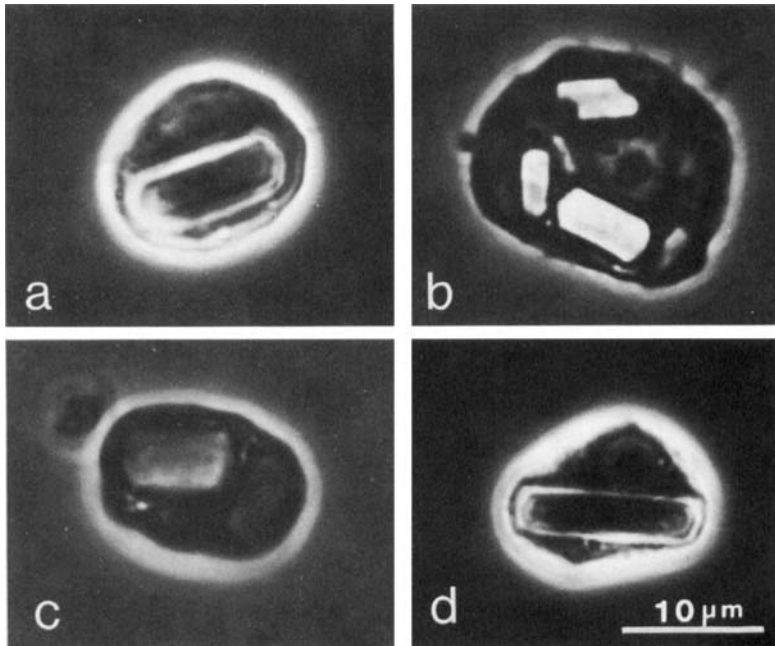
Fig. 1.   Morphology of the paracrystalline inclusions in the crystal cells of: a, *D. simulans;* b, *D. mauritiana;* c, *D. yakuba;* d, *D. erecta.*
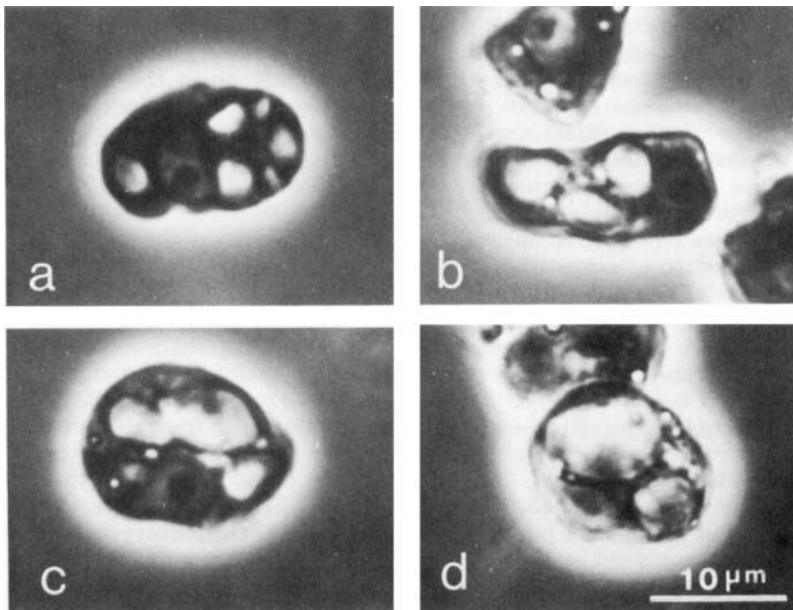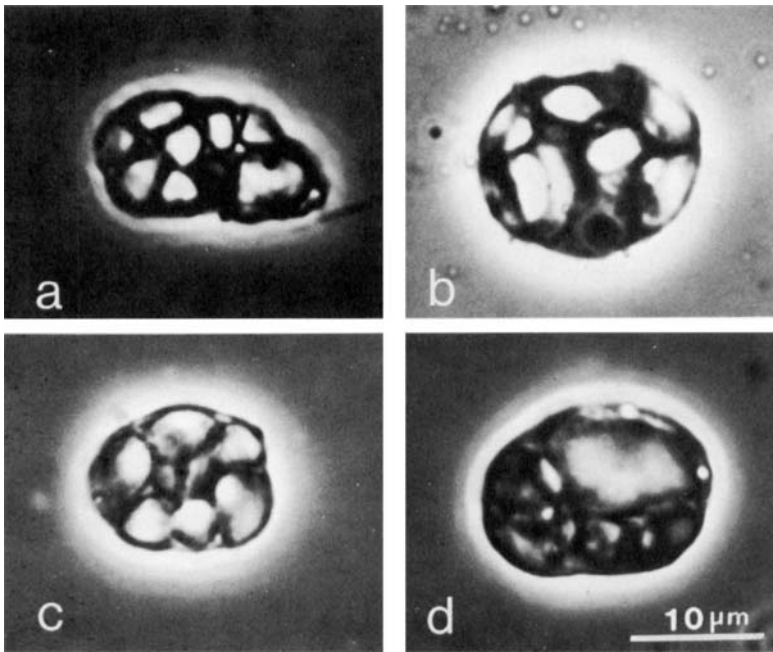

Fig. 2.   Crystal cells of *D. teissieri.*

Fig. 3.   Crystal cells of: a, *D. willistoni;* b, *D. pseudoobscura;* c, *D. novamexicana;* d, *Zaprionus vittiger.*

ships: melanotic tumor strains provided the material for studying the plasmatocytes and their morphological variants (Rizki, '57b; Rizki and Rizki, '79b); the *Bc* mutant confirmed the crystal cell as the source of hemolymph phenol oxidase activity and lead to a simple technique for releasing melanization in the crystal cells of *Bc+* larvae (Rizki and Rizki, '79a). With the latter method hemocytes that are not otherwise readily comparable by morphological criteria can be grouped into one category, and we are now able to unravel the enigma in the literature concerning crystal cells in drosophilids. Yeager's ('45) terminology was adopted for classification of the blood cells in *D. willistoni* (Rizki, '53) with the exception that one category of cells was designated crystalloid cells. These hemocytes contain highly refractile, birefringent rodlets and are found in young larvae. In subsequent studies on *D. melanogaster* (Rizki, '57a) the term crystal cell was adopted for the cell type that is the subject of the present report. Crystal cells are found throughout larval life in *D. melanogaster* and are morphologically distinct from crystalloid cells. On the basis of our published work, Nappi ('70) concluded that "crystalloid cells of *D. willistoni* and the crystal cell of *D. melanogaster* are unquestionably

morphologically similar" although Rizki and Rizki ('59) did not "analogize the crystalloid cells of *D. willistoni*" with the crystal cells in *D. melanogaster.* From the present study it is clear that the crystal cells in *D. melanogaster* are analogous to the cells in *D. willistoni* described as spheroidocytes (Rizki, '53); there is as yet no information suggesting a relationship, if any, between the crystalloid cells of *D. willistoni* and the crystal cells of *D. melanogaster.*

The fragility of the crystal cells is a distinctive feature of this hemocyte type in *D. melanogaster* (Rizki, '57a). When the body wall is opened or hemolymph is removed from the hemocoel, the inclusions within these cells begin to disintegrate and the cells often rupture. We proposed that effective separation of phenol oxidase from its substrate is achieved within the crystal cell by storage of the latter in the paracrystalline inclusions (Rizki and Rizki, '59). Disturbances that influence the integrity of the paracrystalline inclusions will allow contact of substrate with enzyme, thus resulting in melanization. Since the crystal cell inclusions are unusually sensitive to experimental manipulations, we cannot ignore the possibility that the morphological difference observed between the melanogaster and

non-melanogaster cell types is due to factors in the cells or hemolymph that influence the stability of the inclusions, rather than a difference in the structural components of the inclusions themselves. Hopefully, biochemical and/or ultrastructural examinations of these cell types will answer this question. In the meantime, the distinction between these two groups is apparent by utilizing the fixation methods employed in the present study.

A brief summary of the species included in the *melanogaster* subgroup of *Drosophila* is presented by Lemeunier and Ashburner ('76). These authors analyzed the banding patterns of the salivary gland chromosomes of the six sibling species, and on the basis of the overlapping inversions established phylogenetic relationships among the species. Their scheme includes bidirectional arrows for, as stressed by these authors, chromosomal inversion phylogenies are seldom useful for determining the progression of evolution from primitive to more recent species. Additional distinguishing features must be sought, and these might then be combined with the information from inversion phylogenies to delineate lines of descent. Among the morphological traits generally useful for distinguishing sibling species are the structural features of the male genitalia. Interestingly, structural similarity of male genitalia does not necessarily indicate genetic closeness, for the male genitalia of the sibling species *D. pseudoobscura* and *D. persimilis* are distinguishable with difficulty (Rizki, '51) whereas those of *D. simulans* and *D. melanogaster* are quite distinct (Sturtevant, '29). Indeed, the male genitalia of the six sibling species of the *melanogaster* subgroup are sufficiently different to allow recognition of these species (Bock and Wheeler, '72; Tsacas and David, '74; Tsacas and Lachaise, '74), but there do not appear to be differences and similarities in these organs that would be useful for analyzing the direction of evolution. A recent analysis of polypyrimidine stretches in drosophilids including sibling species in the *melanogaster* subgroup shows a similarity of sequences in *melanogaster, mauritiana,* and *simulans* DNA, but *erecta, yakuba,* and *teissieri* do not have polypyrimidine sequences that will hybridize with *D. melanogaster* polypyrimidines (Cseko et al., '79). Therefore, evolutionary directions for this subgroup cannot be assessed by these methods. We believe that the unique morphology of the inclusions in the crystal cells can be used for this purpose. Five of the six sibling species share a

similar morphology of these structures whereas the remaining species, *D. teissieri,* differs from these and resembles the other *Drosophila* species that we examined.

We stress that the sampling of species outside the *melanogaster* subgroup examined in this study is very limited, and we do not imply that the melanogaster-type crystal cell occurs only in this subgroup. An extensive review of the drosophilids and their phylogenetic interrelationships is presented by Throckmorton ('75). Aside from the *melanogaster* subgroup, we examined the hemocytes of *willistoni, nebulosa,* and *pseudoobscura,* representing at least two distantly related species groups of *Sophophorans,* two species from the *virilis* group, a species of the genus *Zaprionus,* and *D. buskii* in the subgenus *Dorsilopha.* The inclusions in the hemocytes of all these species are like those of *D. teissieri.* If we accept that the widely occurring characteristic is the ancestral trait, then *D. teissieri* is closer to the ancestral form or is the ancestral species of the *melanogaster* species subgroup. This suggestion is combined with the scheme of Lemeunier and Ashburner ('76) in Figure 4. Such an evolutionary pattern supports the conclusion of Tsacas and Lachaise ('74) and Throckmorton ('75) that the *melanogaster* spe-
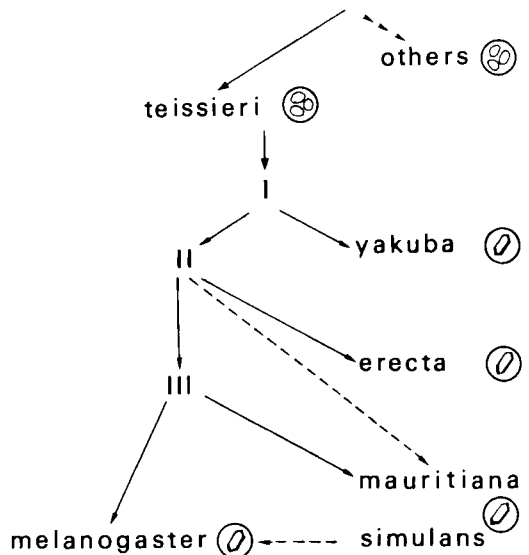

Fig. 4. The proposed direction of evolution in the *D. melanogaster* species subgroup deduced from analysis of morphology of the inclusions in the crystal cells. This scheme is based on the phylogeny of inversions presented by Lemeunier and Ashburner ('76); I, II, and III are hypothetical inversion steps and the dotted lines represent an alternate scheme proposed by these authors.

cies subgroup arose in Africa since *D. teissieri* is endemic to Africa (Tsacas, '71). Furthermore, *D. yakuba* and *D. erecta* are also African endemic species; *D. mauritiana* is known from the island of Mauritius (Tsacas and David, '74).

Mating preference has been suggested as a means for determining phylogenetic relationships among *Drosophila* species by assuming that females of an ancestral species discriminate against males of a derived species (Kaneshiro, '76). The success of reciprocal hybrid matings between *mauritiana, simulans,* and *melanogaster* was measured by Watanabe and Kawanishi ('79). These authors, however, suggest that mating preference is exerted by the females of the newly derived species and on the basis of this assumption propose the evolutionary scheme from *melanogaster* → *simulans* → *mauritiana*. If Kaneshiro's ('76) assumption is accepted, then the same data predict the direction of evolution as *mauritiana* → *melanogaster*. Crystal cell morphology together with inversion phylogeny suggests that *D. melanogaster* is the more recently derived species of this group. In line with Throckmorton's ('75) reasoning on the wide spread distribution of *D. melanogaster* and its association with man, it is interesting to consider that this association was first established in Africa.

ACKNOWLEDGMENTS

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