Genetic fixation of phenotypic response of an ultrastructural character in the anal papillae of *Drosophila melanogaster*

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The size of the apical folds of the plasmalemma in the cells of anal papillae of *Drosophila melanogaster* larvae is dependent on the osmotic pressure of the external medium. Increasing salt concentration of the external medium causes a decreasing size of the apical folds. This reaction could be genetically changed by selection on stretched anal papillae in pupae. This deviant character was correlated with a decreasing size of the apical folds, which even completely disappeared when extreme expression of the stretched character is obtained.

INTRODUCTION

Phenotypic modification by environmental factors is a widespread phenomenon in nature. These phenotypic reactions on changing environmental conditions are often considered to be adaptive. It is intriguing that these “ecophenotypes” resemble “ecotypes”, local populations with genetically different phenotypes, permanently living under one of these environmental conditions.

In the past, the origin of such adaptations was explained by Lamarckian processes. Nowadays it is seen as a consequence of natural selection on genetic variation already present in the population before the change in the environment.

The difference between genetic determination and phenotypic modification is not completely clear-cut, because phenotypic modification has a hereditary basis. It indicates that shifts are possible from acquired to inherited characters by selection on genetic variability of the “reaction norm”. This process was called “genetic assimilation” by Waddington (1953).

Evidence for genetic assimilation was obtained in several model experiments with artificial selection on morphological characters of *Drosophila* (review Waddington, 1961). Waddington (1959) also carried out an experiment with natural selection by salt media. The character which showed “genetic assimilation” in this experiment, was a measurement thought to represent the size of the anal papillae.

Anal papillae of *Drosophila* were first described by Gloor and Chen (1950) as two ventral plates on both sides of the anus (fig. 1(a,b)). The cells of the anal organ form a single layer of differentiated epidermis cells, which are distinctly larger than normal epidermis cells (fig. 1(c)). The ultrastructure of the cells is characterised by enlargements of the plasmalemma, which are associated with many mitochondria (fig. 1(d)). The plasmamembrane on the basal surface of the cells invaginates to form an anastomosing network. The plasmamembrane on the cuticular surface of the epithelial cells is arranged into folds. In the region of the anal organ, no epicuticle can be seen. All characters point to a specialised function to facilitate ion transport (Eichelberg *et al.*, 1972).

Several studies suggest that the intake of inorganic ions can be regulated by variation in the size of the total organ. These studies were done with *Aedes* and *Culex* (Wigglesworth, 1933), *Chironomus* (Strenzke and Neumann, 1960), *Drosophila* (te Velde *et al.*, in press) and several other species (Komnick, 1977).

The intake of ions can also be regulated by variation in the surface area of the plasmamembrane on the cuticular surface of the papillae cells. Examples of this second type of variation came from experiments with the anal papillae of *Aedes aegypti* (Sohal and Copeland, 1966) and *Drosophila* (Chen and Brugger, 1973; this study). Similar phenomena were demonstrated by the transport epithelium in the ventral tube of some

Figure 1 Anatomical position and details of the anal papillae.

(a) black stained (AgCl) papillae at the ventral side of the larva. (b) the two anal papillae in detail (scanning electron microscopy). (c) cross-section (light microscopy). (d) electron microscope picture of a cell. an. (anus); a.p. (anal papillae cells); ap.f. (apical folds of the plasmamembrane); bas.f. (infoldings of the basal membrane); cut. (cuticle); ep. (epidermis); mit. (mitochondria); pr. precipitate of AgCl).

Collembola species (Eisenbeis and Wichard, 1977).

Environmental and genetic variation in the size of the apical folds of the plasmalemma in anal papillae of *Drosophila* was subject of this study.

MATERIALS AND METHODS

Strains and media

The selection lines described in this paper were derived from a cage population established in 1972 with a large number of flies caught in Dahomey (now Benin, West Africa). It was maintained at 25°, the food was according to Mittler and Bennet (1962), composed of sugar, agar and dead yeast. In 1975 a cage population was started on a medium with sodium chloride which was gradually increased to 2 per cent. In 1977 approximately 20 per cent of the pupae showed a deviant character

which was only occasionally found in the base population, when pupae were grown in a medium containing 2 per cent NaCl.

In these pupae the remnants of the anal papillae are large, because the papillae remained stretched during puparium formation. In normal larvae the papillae are retracted during puparium formation, leaving a narrow ridge at both sides of the inter-papillar zone (fig. 2(a,b)).

Figure 2 Illustration of the anal papillae of the R- and S-strain on 2 per cent NaCl-medium. (a) retracted (R) anal papillae in a pupa (the dark lines; between these lines an inactive zone can be seen). (b) stretched (S) papillae in a pupa. (c) the apical border in the cells of the R-strain with a deeply folded plasmalemma, covered by a normal cuticle. (d) extreme expression of the apical border in the cells of the S-strain with complete disappearance of the folds of the plasmalemma and a changed structure of the cuticle.

On this population artificial selection was performed for and against this deviant character. After 20 generations of divergent selection, each line contained more than 95 per cent individuals showing the desired character, i.e., stretched (S), respectively retracted (R) papillae.

Flies were allowed to lay eggs during one night in 125 ml bottles which were placed upside down on watch glasses covered with agar (2 per cent solution) with a piece of yeast. The eggs were washed from the agar on an egg sieve. They were put on media with different salinities with the aid of brushes and grown at 25°C. Third stage larvae (one day before pupation) were used for the experiments. The media used were: (1) normal

medium, (2) this medium with 2 per cent or 4 per cent NaCl and (3) diluted medium (0.25 of all ingredients, washed agar and demineralised water). One experiment with the R-strain was done by maintaining the larvae for 1 hour in 10 per cent NaCl-solution, following the method of Chen and Brugger (1973).

Light microscopy

Larvae were put into a 1 per cent AgNO_3 -solution for 3 minutes, which gives a black precipitate of AgCl on the anal papillae (fig. 1(a)). At the posterior and anterior end, a small piece was cut off. They were fixed in Bouin's fluid, dehydrated via alcohol and amylacetate and embedded in paraffin. Transverse sections were cut (5–7 μ thick), transferred to water, stained with haemalum-eosin according to Mayer (Romeis, 1968), dehydrated and embedded in DPX.

Electron microscopy

The posterior end of the larvae was cut off just before the anal papillae, allowing fixative to penetrate both sides of the tissue. Tissues were fixed at room temperature for 2 hours in 6 per cent glutaraldehyde, buffered with 0.1 M phosphate buffer plus 0.6 per cent MgCl_2 . During the first 20 minutes the fixation was carried out in vacuum to remove remnants of air from the tissue. After 1 hour in buffer, the tissue was post fixed in buffered 1 per cent OsO_4 for 1 hour (refreshment after 30 minutes) and 1.5 per cent potassiumferrocyanid (De Bruyn, 1980) during 15 minutes. Fixation was followed by dehydration in a graded series of acetone finally added with 5 per cent D.M.P. Infiltration of the embedding medium was carried out as follows: over night in a 1:1 araldyte/acetone mixture, 8 hours (one refreshment) a 2:1 mixture, 24 hours in a 3:1 mixture (one refreshment) and 24 hours in pure araldyte. Sections of 1 μ were cut up to the anal papillae. Thin sections were cut on a Reichert Om U2 ultra microtome of the area surrounding the anus. They were stained 5 minutes with 1 per cent uranyl acetate and with lead citrate. The electron microscopes used were: Philips EM 201 and Philips EM 200.

In addition to this standard treatment other methods were tried to get better results for larvae grown at different salinities, viz., as different percentages of glutaraldehyde, a longer osmium-fixation time (with refreshment) and changing osmolality of the fixative.

Scanning electron microscopy

Larvae were fixed in 3 per cent glutaraldehyde, buffered in 0.1 M phosphatebuffer postfixed in buffered 1 per cent OsO_4 and dehydrated in a graded series of ethanol. They were dried following the "critical point drying method" with the aid of liquid CO_2 . Larvae were fixed with tape on an object-table and sputtered with a thin layer of gold (Edwards sputter coater). The microscope used was a Cambridge stereoscan 600 M.

RESULTS

A preliminary electron microscopy study with the selection lines, indicated that the apical folds of the plasmalemma had disappeared in the line with stretched papillae in the pupae. The cuticle was much thinner and the regular structure was replaced by a flaky structure (fig. 2). The frequency of the stretched character after selection was approximately 98 per cent. An environmental stimulus of 2 per cent NaCl was necessary for expression of the character in the pupae. On normal medium the normal cuticle reappeared, but not the apical folds. In the R-line, with retracted papillae in the pupae, both on normal and 2 per cent NaCl containing medium, a regular apical folded pattern of the plasmamembrane is present. In the pupae the anal papillae show the normal wild type pattern on both media.

Later a more detailed study was carried out with a strain which showed the S-character for approximately 80 per cent. It was very difficult to find a suitable fixation method for the anal papillae of larvae reared at different salinities. (This problem is not unknown for electron microscope work on anal papillae; see Meredith and Phillips, 1973.)

Results of bad fixation are revealed by swollen or bursted mitochondria, holes in the cell content and an inferior structure of the cell organella. The problems with fixation indicate, that the cell content of the anal papillae had a different osmotic pressure at different environmental salinities. The folds on diluted media were very thin, while on salt media they were thick and swollen (fig. 4, R-1 compared to R-4).

Results of this study are presented in figs 3 and 4. Fig. 3 shows four pictures of the extremes: the very elongated apical folds of the R-line in diluted medium (a); the normal wild type like folds of the R-line on normal medium (b); strong reduction of the folds of the R-line exposed to a high salt stimulus (c); complete disappearance of the apical fold of the S-line on normal medium (d).

Figure 3 Variation of the apical folds in cells of the anal papillae. (a) R-strain on diluted medium. (b) R-strain on normal medium. (c) R-strain treated with a 10 per cent NaCl-solution. (d) S-strain on normal medium with complete absence of the apical folds.

Fig. 4 shows a summary of the results:

R-1 dilute medium. In these cells we can see a drastic increase of the size of the apical folds; they penetrate deeply into the cell. The density of mitochondria is also increased and they are also situated between the apical folds.

R-2 normal medium. These cells show the pattern known from wild type. Mitochondria are spread through the whole cell (see also fig. 1(d)); at the apical region they are situated just at the border of the folds and sometimes between the folds.

R-3 2 per cent NaCl. Compared with the situation on normal medium the size of the apical folds is slightly decreased. Further, no clear differences were found.

R-4 4 per cent NaCl. The length of the apical folds has decreased compared with the 2 per cent larvae. The folds are oriented regularly, hardly branched, swollen (compared with the 0 per cent larvae) and very close together. (Probably this approximates more the situation *in vivo* since at lower salt contents the folds are hypo-osmotic with regard to the fixation-liquid and therefore shrank.)

Figure 4 Variation of the apical folds in cells of the anal papillae; R: R-strain, S: S-strain. R-1 diluted medium. R-2 normal medium. R-3 2 per cent NaCl. R-4 4 per cent NaCl. R-5 treated for 1 hour with 10 per cent NaCl. S-1 diluted medium. S-2 to S-5 normal medium.

R-5 10 per cent NaCl treatment. The apical folds are shortened very much. Density and size of mitochondria have decreased.

S-1 dilute medium. The apical folds show a tendency to increase but the flexibility of the character

is reduced compared with the R-line. Many mitochondria can be found also between the apical folds.

S-2 to S-6 normal medium. A very large variation with regard to the length of the apical folds was found from slightly less the size of the R-line on the same medium to complete absence (S-5). In cells with very reduced apical folds a high increase of mitochondria could be observed, which, however, was not consistent.

Fig. 5 shows three classes of expression of the stretched character in the pupae. It strongly suggests a correlation between the percentage of S-papillae in a population and the rate of expression of the character.

Selection for extreme expression, in which from each generation pupae with expression c (fig. 5) were used as parents for the next generation, was very successful within 10 generations (te Velde, 1985, in preparation). The anal plates in the pupae with extreme expression appear as very transparent, swollen areas (fig. 5). The cuticle of the anal plates of such pupae is very transparent, when looking from the inner side of the empty pupal case. This is probably a consequence of the complete absence of the apical folds, since it can be seen on fig. 2 that this is correlated with a very thin cuticle.

Figure 5 Top. Expression of the stretched character of the anal papillae in pupae in three classes. From (a) to (c): increasing expression of the character. In (c) only a very thin cuticle covers the anal papillae. Bottom. Abscissa: percentage of the S-character in the four populations. Ordinate: the frequency of the expression classes, arranged according to increasing expression of the character.

DISCUSSION

Anal papillae have the characteristics of typical transport epithelia (Berridge and Oschman, 1972), also called "chloride epithelia". These cells are situated at different sites in aquatic insects and have an osmoregulatory function. Anal organs are characteristic for *Diptera* (Komnick, 1977). They are able to absorb inorganic ions against a great concentration gradient (Koch, 1938; Wigglesworth, 1938).

Some investigators suggest an excretory function in hyperosmotic environments. Meredith and Phillips (1973) did not find ultrastructural differences in the anal papillae of the salt water mosquito *Aedes campestris* compared with fresh water species. Moreover, they did not find major differences when *A. campestris* was maintained in hyperosmotic (normal) and hypo-osmotic (diluted) external media. They also showed that the capacity of absorbing ions in diluted media was not lost.

Reversal of ion transport in fish gills is accompanied by marked changes in ultrastructure of the chloride cells. Meredith and Phillips (1973) suggest, that other transport activities may be involved in reversal of ion transport in the anal papillae of *A. campestris*. They do not have much positive evidence for this hypothesis, however. The smaller anal papillae of *A. campestris* compared with the fresh water species *A. aegypti* (Meredith and Phillips, 1973) and the very small anal papillae of another salt water species *A. detritus* (Beadle, 1939) do not fit their hypothesis.

The present evidence suggests, that the anal papillae have an osmoregulatory function in hypo-osmotic environments only (te Velde *et al.*, 1985, in preparation). This is supported by morphological changes. In hypo-osmotic medium the folds of the apical plasmamembrane increase in size and penetrate more deeply into the cell (Chen and Brugger, 1973; this study). In addition, the size of the total organ increases (Wigglesworth, 1933; Komnick, 1977; te Velde *et al.*, in press).

In hyper-osmotic medium the opposite occurs, both with the apical folds (Sohal and Copeland, 1966; Chen and Brigger, 1973; this study) and the size of the anal papillae (e.g., Wigglesworth, 1933; Komnick, 1977; te Velde *et al.*, in press). These data suggest that in hypo-osmotic media the uptake of inorganic ions is improved and that in hyperosmotic media the rate of leakage is decreased. Perhaps such a decrease can also occur by changes at the molecular level which then could explain the situation in *A. campestris*.

In the experiments with *Aedes aegypti* (Sohal and Copeland, 1966) much smaller differences in salt content caused changes in the apical folds. Apparently these anal papillae are more sensitive to different salinities than those of *Drosophila*. This is probably related to the broader range of salt concentrations in which *Drosophila* can live.

The examples of developmental plasticity demonstrated in the anal papillae illustrate adaptability, the feed-back type of environmental modification in which an environmental stimulus causes a morphological change, which adapts the organism specifically to that stimulus (Warburton, 1956). Waddington (1959) showed that such a reaction could be genetically fixed. However, in his experiment with salt adaption he did not measure the real anal papillae but the "papillae-like" inactive zone between these organs (te Velde *et al.*, in press). Our experiments show, that another character of the anal papillae which shows an adaptive response, *i.e.*, the ultrastructure of its cells, can be fixed by selection. The selection applied was an indirect one. We selected for a threshold-like morphological character "stretched" (S) or "retracted" (R) anal papillae in pupae and found that this was correlated with ultrastructural changes in the cells of the anal papillae of larvae. A salt stimulus (2 per cent NaCl) was necessary for penetrance of the stretched papillae character. On normal medium the S-selection-line showed the retracted character again.

In the S-strain we found complete disappearance of the apical folds of the plasmamembrane in some individuals, even at the normal medium. On diluted media the ability to enlarge the folds was highly reduced. This was accompanied with reduced osmoregulatory capacity on that medium, while the osmotic value of the haemolymph in media with high salt content did not rise as much as in the R-strain. This was correlated with an approximately six times higher survival rate on medium containing 6 per cent NaCl (te Velde, *et al.*, 1985, 1988, in preparation). This agrees with the hypothesis, that in hyperosmotic media the disappearance of the folded zone diminishes the leakage of ions into the haemolymph.

The disappearance of the apical folds appeared to be a gradual change, either by environmental or genetical causes. So we found a continuous changing character in larvae underlying a threshold character in pupae.

Part of this continuous variation appeared also to be expressed in the S-character of the pupae. It seems that expression of this character was increased when the apical folds decreased (fig. 5, fig. 6). Complete disappearance of this zone was accompanied by a changed, very thin cuticle at the site of the anal papillae (fig. 2(d)) which can be seen as a transparent site in empty pupal cases with highest expression of the stretched character.

The difference between the R- and S-strains revealed that the "stretched" character and the

Figure 6 Diagram of the relation between the expression of the apical folds of the plasmalemma in the anal organ cells and the character in the pupae. (1) decrease of the apical border is necessary to stretch the papillae in the pupae. (2) a 2 per cent NaCl-stimulus is necessary for the expression of the S-character. (3) at highest expression of the S-character the cuticle is very thin and its structure is flaky.

difference in survival has a polygenic determination (te Velde *et al.*, 1985, in press). This shows the importance of the folding of the apical plasmalemma for the salt adaptation and strongly suggests that the individual phenotypic reaction of the plasmalemma folding on different salt concentrations is really an adaptive reaction.

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