# Adaptation of *Drosophila melanogaster* to high and low osmolarity promotes evolutionary change in the phenotypic plasticity of the larval anal organs

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**Supplementary information:** Movie S1: Fstarch_on_Mstarch.avi, Movie S2: Fcontr_on_Mstrch.avi, Movie S3: Fsalt_on_Msalt.avi — three videos based on the corresponding microtomography reconstruction of larval AO structure: https://drive.google.com/drive/folders/1r368iv8hiwWnx9JR55-UlluVBTuSMs7p?usp=drive_link.

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## Abstract

The anal organs (AO) of *Drosophila* larvae provide a textbook example of phenotypic developmental plasticity as they expand in low osmolarity conditions and shrink when larvae are reared on a high-salt medium. Waddington (1953) experimentally showed that these plastic changes might undergo "genetic assimilation": the adaptive plastic change in the size of AO under high salinity conditions became hereditary after a few generations of rearing on a high-salt medium. Later attempts to replicate this finding produced ambiguous results. To clarify the question, we analyzed plastic osmolarity-related changes in the size of AO in three *D. melanogaster* lines adapted to low, intermediate, and high osmolarity during a long-term evolutionary experiment. We failed to replicate the genetic assimilation. However, we find that the reaction norm became wider in the low osmolarity adapted line. Additionally, we studied the fine structure of AO in fly lines using dying, SEM, and microtomography and revealed phenotypic plasticity of nanoscale pits on the surface of AO as well as confirmed the reduction of the epithelial cells of AO in flies adapted to higher osmolarity.

**Keywords:** anal papillae, osmotolerance, fly, adaptation, phenotypic plasticity, genetic compensation, genetic assimilation.

## Introduction

### Phenotypic plasticity and genetic assimilation

Phenotypic plasticity (PP) is the ability of individual genotypes to produce different phenotypes when exposed to different environmental conditions (Pigliucci, Murren, and Schlichting, 2006). The ability for such plastic changes in the phenotype in response to certain stimuli is determined genetically and epigenetically and can evolve under selection (Nijhout, 2003; Iordansky, 2009; Pfennig et al., 2010; Markov and Ivnitsky, 2016; Nishikawa and Kinjo, 2018). PP can influence the course of evolution in several ways (Kamshilov, 1972; Shishkin, 1988; Nijhout, 2003; Ehrenreich and Pfennig, 2016; Markov and Ivnitsky, 2016), including through the mechanism that Conrad Waddington (Waddington, 1953; 1959) called genetic assimilation (GA). In this evolutionary scenario, a new feature first appears as a plastic change (modification) due to the changes in environmental conditions. Over time, if the changed conditions persist long enough and the new trait is adaptive, then alleles that stabilize this modification (i. e., increase the likelihood of its implementation in ontogeny) may spread in the gene pool (Walworth et al., 2016; Wood et al., 2023). As a result, the dependence of that

trait on specific environmental cues may weaken or even disappear. The trait appears in any conditions, that is, becomes "innate" rather than "acquired". GA can occur due to stabilizing selection or mutational degradation of the mechanisms responsible for choosing developmental trajectories depending on the environment (Markov and Ivnitsky, 2016). Waddington demonstrated the feasibility of the GA scenario in classical experiments on *Drosophila* (Waddington, 1942; 1953; 1956). He showed that the changes in the wing venation caused by heat shock can become genetically encoded and that major developmental deviations, such as an additional pair of wings (*bithorax* phenotype), can undergo GA. However, both examples were illustrations of non-adaptive PP, in contrast to the changes in the anal organs size in *Drosophila melanogaster* larvae, which Waddington also showed to be susceptible to GA.

## Anal organs of *Drosophila* larvae

Many Diptera larvae, including *Drosophila*, have special organs on the posterior-most segments of the body. They are variously referred to as "anal papillae", "anal pads", "perianal pad", "anal plates", "perianal plate", "silver-reducing organs", or "ventral hypodermal organs" (Stoffolano, 1970; Krivosheina, 2005). We use the term "anal organs" (AO). Two AO in *D. melanogaster* larvae are located ventrally, to the right and left of the anus. They usually look like slightly swollen, transversely oriented oval pads, separated by the so-called inactive region surrounding the anus (Gloor and Chen, 1950).

AO, along with the Malpighian tubule system, serve for osmoregulation. Gloor and Chen first demonstrated the osmoregulatory function of AO in *Drosophila* larvae (Gloor and Chen, 1950). They found that AO darkened when the larvae were immersed in an $AgNO_3$ solution and then illuminated with ultraviolet light. $AgNO_3$ was absorbed precisely in the AO zone, and under the ultraviolet light, it decomposed into atomic silver, giving the inky color.

The AO of *D. melanogaster* larvae is a great example of a morphological structure subject to adaptive PP. The change in the AO size depends on the osmotic potential of the medium: the size of the AO decreases with the increasing salt content of the medium and increases with its decrease. Numerous studies have confirmed the AO of *Drosophila* and other Diptera larvae serve only for the transport of ions from the environment to the hemolymph at low salt content and not for excretion at high salt content (Wigglesworth, 1933; Koch, 1938; Ramsay, 1953; Bradley, 1987; Jarial, 1987; Donini and O'Donnell, 2005; Durham and Grodowitz, 2012). Therefore, it is beneficial for larvae to reduce the surface area that absorbs ions in the environment with high salinity and, on the contrary, to increase this area when ions are in deficit.

For instance, the AO of *Aedes (Stegomyia) argente* mosquito larvae shrank when they were transferred from a diluted Ringer's solution, corresponding to a hypotonic medium, to a concentrated solution. It is clear that this response helps organisms avoid osmotic shock (Wigglesworth, 1933). Moreover, the larvae of *Aedesa egypti* and *Culex pipiens* formed atypically large AO if they were grown in a medium with low osmotic potential. Surely, this adaptation helped organisms absorb more ions in the conditions of their shortage (Wigglesworth, 1938).

Also, it was shown that changes in salinity may cause some modifications in the fine structure of AO. The cellular structure of this organ in dipteran larvae has been studied in representatives of different Cyclorrhapha fly families: Ephydridae, Drosophilidae, and Muscidae (Gloor and Chen, 1950; Copeland, 1964; Stoffolano, 1970; Meredith and Phillips, 1973; Mullins and Cochran, 1974; Yakovleva et al., 2023). AO consists of typical transport epithelial cells with a polar structure and numerous infoldings of the apical membrane adjacent to the cuticle, which lies above. This cuticle is significantly thinner than the rest of the cuticle covering the body and contains small (about 100 nm in diameter) pits in the exocuticle layer (here and after referred to as the nanoscale pits) (Wipfler et al., 2013).

It was shown that with a sharp increase in the environmental salinity, the AO of *Aedes* mosquito larvae not only became shorter, but their epithelial cells degenerated: the number of infoldings of the basolateral and apical membranes decreased until they disappeared entirely, the membranes became thicker, and the number of mitochondria decreased (Martini-Hamburg, 1922; Edwards and Harrison, 1983). The same changes in the *Aedes* mosquito larvae were observed under high salinity conditions in larvae adapted to moderate salinity (Wigglesworth, 1933; Sohal and Copeland, 1966). Jarial showed experimentally that when *D. melanogaster* larvae were growing on the medium with a high osmotic potential (medium with 7 % NaCl), AO epithelial cells degenerated considerably: membrane infoldings and invaginations were absent, the number of mitochondria was reduced, and cell nuclei were disrupted (Jarial, 1987).

In his classic article on "genetic assimilation of acquired characters" Waddington experimentally demonstrated the phenomenon of GA using the example of AO in *Drosophila* larvae (Waddington, 1959). According to Waddington, plastic changes in the AO size that occurred in larvae living in a salty environment were partially stabilized (subjected to partial GA) after two dozen generations of life in such an environment. More specifically, naïve (non-adapted, then control) larvae grown on salty medium had a larger AO than the larvae grown on the standard medium, and then after 20 generations of adaptation to salty medium, the adapted lines had a

larger AO than the control lines grown on both the standard and salty medium. Overall, the main result was that the adapted lines had a larger AO than the control ones at any environmental salinity (Waddington, 1959).

Waddington's results contradicted other research devoted to the relationship between the AO size of dipteran larvae and the environmental salinity, which shows that AO actually decreases in size with an increase in salinity (Miyoshi, 1961; Te Velde, Molthoff, and Scharloo, 1988). Waddington's results were explained in the 1980s. Apparently, Waddington measured not the size of the AO but the size of the inactive area between them, and not in larvae but in early pupae, in which the AO shrank and became almost indistinguishable, and the inactive area, on the contrary, protruded and looked like a pair of pads, the size of which correlated negatively with the size of the larval AO (Te Velde, Molthoff, and Scharloo, 1988).

### Statement of the problem

Our goal was to reproduce Waddington's classic result confirming the "GA of an acquired adaptive character", using our laboratory lines of *D. melanogaster* reared on three media with contrastingly different osmotic potential (high, intermediate, and low) for eight years (approximately 160 generations). Thus, our fruit flies had much more time for GA than in the experiments of Waddington (about 20 generations), Miyoshi (25 generations), and Te Velde (no more than five years). Otherwise, the setting of the experiment was similar to Waddington's original experiment.

Firstly, we aimed to elucidate the nature of the PP of AO size in *Drosophila* larvae depending on the environmental salinity to make sure that AO decrease in size with an increase in salinity and vice versa. To this end, we compared the AO size in fly lines adapted to high osmolarity grown on the three media — with high, intermediate, and low osmolarity. And we did the same with fly lines adapted to normal and low osmolarity.

Secondly, we planned to determine whether the direction and magnitude of the reaction norm evolved during the flies' long-term adaptation to the media with low and high osmolarity. To do this, we compared the AO size in fly lines adapted to high, intermediate, and low osmolarity grown on the media with high osmolarity. Next, we made the same comparison for those three lines grown on the medium with normal osmolarity and the same on the medium with low osmolarity.

Next, we checked whether there were stable differences in the AO size between the lines that correspond to the concept of GA (for example, the AO in the lines adapted to salt might be smaller than in the control lines grown under the same conditions). It would help us to conclusively determine whether this experimental system confirms Waddington's idea of GA.

Thirdly, we examined other plastic changes in the phenotype determined by the osmolarity of the environment, which could also become innate during the adaptation to environments with different osmolarity, that is, undergo GA.

## Materials and methods

### Fly lines

Third-instar larvae of *D. melanogaster* from three laboratory outbred lines were studied:

1. Control line Fcontr reared on a standard rich laboratory food medium (Mcontr) with normal osmotic potential for *Drosophila* larvae (60 g of inactivated yeast, 35 g of semolina, 50 g of sugar, 45 g of crushed raisins, 8 g of agar, 2 g of propionic acids per 1 liter of medium).
2. Fsalt line reared on a salty medium (Msalt) with extremely high osmotic potential (Mcontr medium supplemented with 7 % NaCl).
3. Fstarch line reared on a depleted starch-based medium (Mstarch) with reduced osmotic potential (60 g inactivated yeast, 30 g starch, 8 g agar, 2 g propionic acid per 1 liter of medium).

The fly line reared on the medium with 4 % NaCl was also used to study the presence of nanoscale pits on the AO cuticle.

All lines originated from the outbred population derived from 30 wild individuals of *D. melanogaster* obtained in September 2014 in the southwest of Moscow. Standing population sizes were approximately a thousand adult flies in the Fcontr line, several hundred in the Fsalt line, and about one hundred in the Fstarch line (Belkina, Naimark, Gorshkova, and Markov, 2018). At the beginning of the study, all lines were reared on their media for about eight years (approximately 160 generations). Lines were reared in cages ($16.5 \times 16.5 \times 25$ cm$^3$) and had tens to hundreds of individuals in the population continuously. All lines were reared with natural daylight at room temperature with standard humidity. The article by Belkina and coauthors (Belkina, Naimark, Gorshkova, and Markov, 2018) gives a detailed description of the lines.

Previously, we showed that our laboratory lines reared on the Msalt and Mstarch media became better adapted to their media compared to a control line reared on Mcontr (Yakovleva, Naimark, and Markov, 2016; Gorshkova et al., 2019).

### Larvae preparation for the AO measurement

To analyze PP and evolutionary changes in the AO size, the larvae of each line (Fstarch, Fcontr, Fsalt) were grown on each of the three media (Mstarch, Mcontr, Msalt).

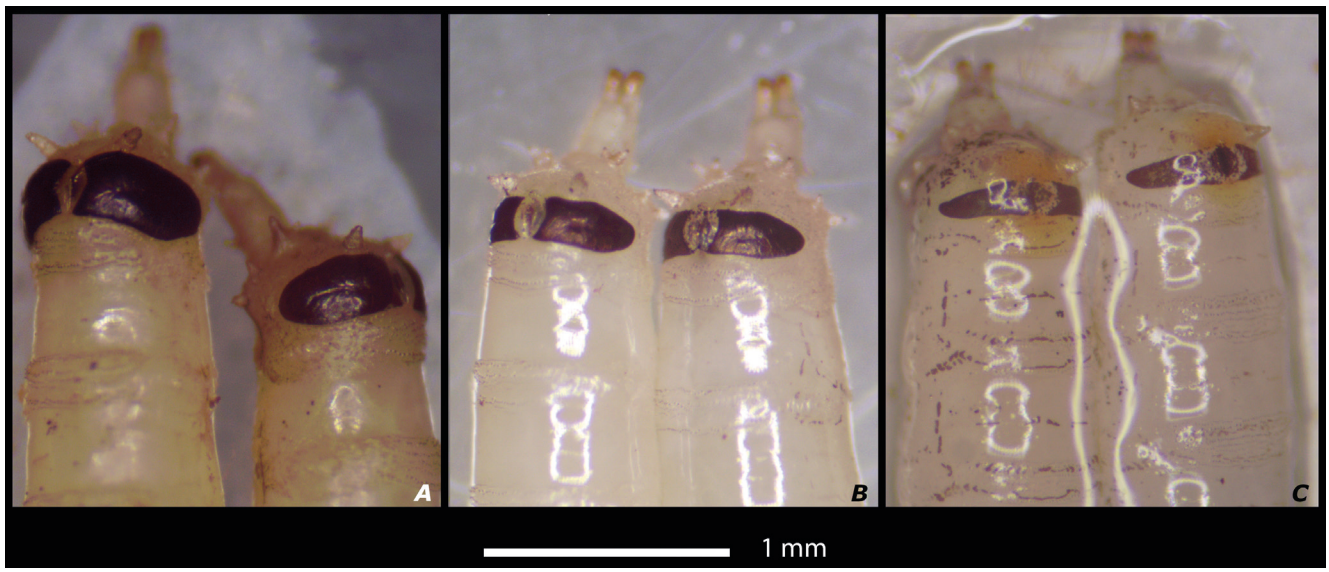


**Fig. 1.** An example of silver nitrate-stained AO in Fsalt line grown on Mstarch (A), Mcontr (B), or Msalt (C) media.

In total, nine combinations were used: Fstarch larvae grown on Mstarch; Fstarch larvae grown on Mcontr, etc. Larvae from the Fstarch line, not adapted to a medium with a high salt concentration (7 %), hardly develop in such a stressful medium (occasionally, individual larvae survive). That is why, to measure the AO area, we used Fstarch larvae grown on the medium with 4 % NaCl which was also a stressful high-salinity environment.

To obtain eggs, 4 to 10 test tubes with the appropriate medium were placed for 1 to 3 days in the population cages of the fly lines. The number of tubes and tube placement period depended on the number of adult flies in the population cages so that the density of larvae developing in the test tubes would be comparable. Then, adult flies were removed from the tubes, the tubes were removed from the boxes, plugged with cotton plugs, and incubated at room temperature for 4 to 6 days until the third-instar larvae appeared. After accumulating the required number of the third-instar larvae in test tubes, 50–80 individuals were removed and rinsed in fresh water for further staining.

The anal organs were stained according to the method described by Gloor and Chen (Gloor and Chen, 1950). Washed *D. melanogaster* larvae were placed in hot water (80–90 °C) to relax and kill them. Cooled larvae were immersed for 2 to 30 minutes in a 1 % $AgNO_3$ solution (2–3 minutes for the Fcontr and Fstarch lines grown on Mcontr and Mstarch; 15 minutes for the Fcontr and Fstarch lines grown on Msalt and for the Fsalt line grown on Mcontr and Mstarch; and 30 minutes for the Fsalt line grown on Msalt medium). Differences in solution immersion time were determined empirically depending on the AO staining intensity in different fly lines to achieve comparable staining. Next, the larvae were placed under ultraviolet light for 20–30 min until their AO stained inky, then they were washed in distilled water to prevent further excessive staining.

To characterize the size of the AO, the surface area of the right or left half of the symmetrical organ was measured. For comparability, it was normalized to the length of the larval abdomen since the head capsule and the thoracic segments of the larvae were often retracted. The abdomen length and the AO surface area were measured on the images (example in Fig. 1) using the ImageJ program.

The images were obtained using a Micromed MS-5-ZOOM LED stereoscopic microscope (Russia) and a ToupCam UHCCD05100KPA eyepiece microscope camera (China) using the ToupView software (Windows).

## Larvae preparation for SEM and microtomography

For scanning electron microscopy, the treatment was carried out in polypropylene 25 mL tubes with flat-top screw caps. Third-instar live larvae were washed from medium particles in fresh water, then kept in a 5 % KOH solution for 3 minutes, and then rinsed again. After that, the larvae were placed in hot water (80–90 °C). Cooled larvae were fixed in ethanol series (30 % — 50 % — 70 % — 95.6 %), then subsequently dehydrated in an ascending ethanol series: 95.6 % ethanol, 95.6 % ethanol and 100 % acetone (1 : 1), 100 % acetone was changed two times (30 min per step).

Dehydrated larvae were dried at the critical point on a Hitachi Critical Point Dryer HCP-2 (Hitachi, Japan). Dried specimens were glued to aluminum stubs

**Table 1. Length of abdomen and relative size of *Drosophila* anal organs in all variants of the experiment**

| Line / medium | Sample size | Length of abdomen, mm, mean ± standard deviation | Relative AO size, sq. mm/mm, mean ± standard deviation |
|---|---|---|---|
| Fstarch / Mstarch | 59 | 3.4586 ± 0.4945 | 0.0385 ± 0.0095 |
| Fcontr / Mstarch | 50 | 3.6093 ± 0.2818 | 0.0287 ± 0.0036 |
| Fsalt / Mstarch | 81 | 3.8104 ± 0.3461 | 0.0277 ± 0.0056 |
| Fstarch / Mcontr | 50 | 3.5792 ± 0.2061 | 0.0195 ± 0.0025 |
| Fcontr / Mcontr | 50 | 3.4635 ± 0.2353 | 0.0138 ± 0.0019 |
| Fsalt / Mcontr | 66 | 3.8367 ± 0.2292 | 0.0169 ± 0.0019 |
| Fstarch / Msalt | 50 | 3.1082 ± 0.1746 | 0.0073 ± 0.0010 |
| Fcontr / Msalt | 49 | 3.3433 ± 0.2209 | 0.0080 ± 0.0016 |
| Fsalt / Msalt | 50 | 3.5824 ± 0.2586 | 0.0085 ± 0.0017 |

and coated with an alloy of gold and copper (Eiko IB-3, Eiko, Japan). The images were obtained using scanning electron microscopes Jeol JSM-7000 (Jeol, Japan) using JCM 7000 software — JEOL and TESCAN VEGA-II XMU (Brno, Czech Republic) using TESCAN Essence software. The images were processed in the GIMP program.

Sample preparation of *D. melanogaster* larvae for 3D microtomography reconstruction was carried out similarly. The tomograms of the larvae of the Fstarch, Fcontr, and Fsalt flies grown on their media were made on Neoscan N80 tomograph (Belgium) at the Borisyak Paleontological Institute of the Russian Academy of Sciences. The CTvox Version 3.3 program was used for image processing and video editing.

### Statistical analysis

The larval abdomen length and the relative AO size were examined with a two-way analysis of variance (ANOVA), considering the factors "line", "medium", and their interaction. Dependent variables were log-transformed to meet the assumptions of this method. The best model was selected using the Akaike information criterion (AIC). The relative contribution of individual factors was estimated using partial $\eta^2$ (eta squared), which shows the fraction of variance of the dependent variable, remaining after considering other variables explained by this independent variable. Multiple comparisons were made with Tukey's HSD test. The calculations were carried out using the R software (R Core Team, 2021). Designations in tables with results: SS — the sum of squares (the total variation between the group means and the overall mean); df — the degrees of freedom for the independent variable; MS — mean of the sum of squares (SS / df); F — test statistic from the F-test; p — p-value for the F-statistic.

## Results

Table 1 contains the measurements of the abdomen length of third-instar larvae of *D. melanogaster* and their surface area of AO adjusted for the abdomen length, this is what we call relative AO size.

### Relative size of AO

Our experiments confirmed that the AO size in *Drosophila* larvae was subject to a pronounced PP depending on the osmotic potential of the environment: AO were much larger in larvae grown on a medium with low osmotic potential than in larvae grown on a medium with high osmotic potential (Table 1, Fig. 2).

Statistical analysis showed that the relative AO size strongly depended on the medium which the flies were reared on and, to a lesser extent, on the line they belonged to and on the interaction of these factors (Table 2). On the Mstarch, the relative AO size was maximum, and on the Msalt, it was minimum, and their distributions practically did not overlap (Fig. 3). As for the lines of flies, the control line Fcontr had the smallest relative AO size, and Fstarch had the largest one. Noteworthy: although the relative AO size decreased on Msalt, the AO of Fsalt flies did not become smaller on average. At the same time, the interaction of factors was also significant: the Fstarch larvae grown on their own Mstarch medium had the largest relative AO size out of all nine variants ($p < 0.001$ in all cases), but on Msalt, the relative AO size in Fstarch larvae

**Table 2. Influence of line and medium on relative AO size**

| Factor | SS | df | MS | F | p | Partial $\eta^2$ |
|---|---|---|---|---|---|---|
| line | 2.89 | 2 | 1.45 | 48.6 | < 0.001 | 0.15 |
| medium | 153.63 | 2 | 76.81 | 2579.8 | < 0.001 | 0.90 |
| line × medium | 5.10 | 4 | 1.27 | 42.8 | < 0.001 | 0.24 |

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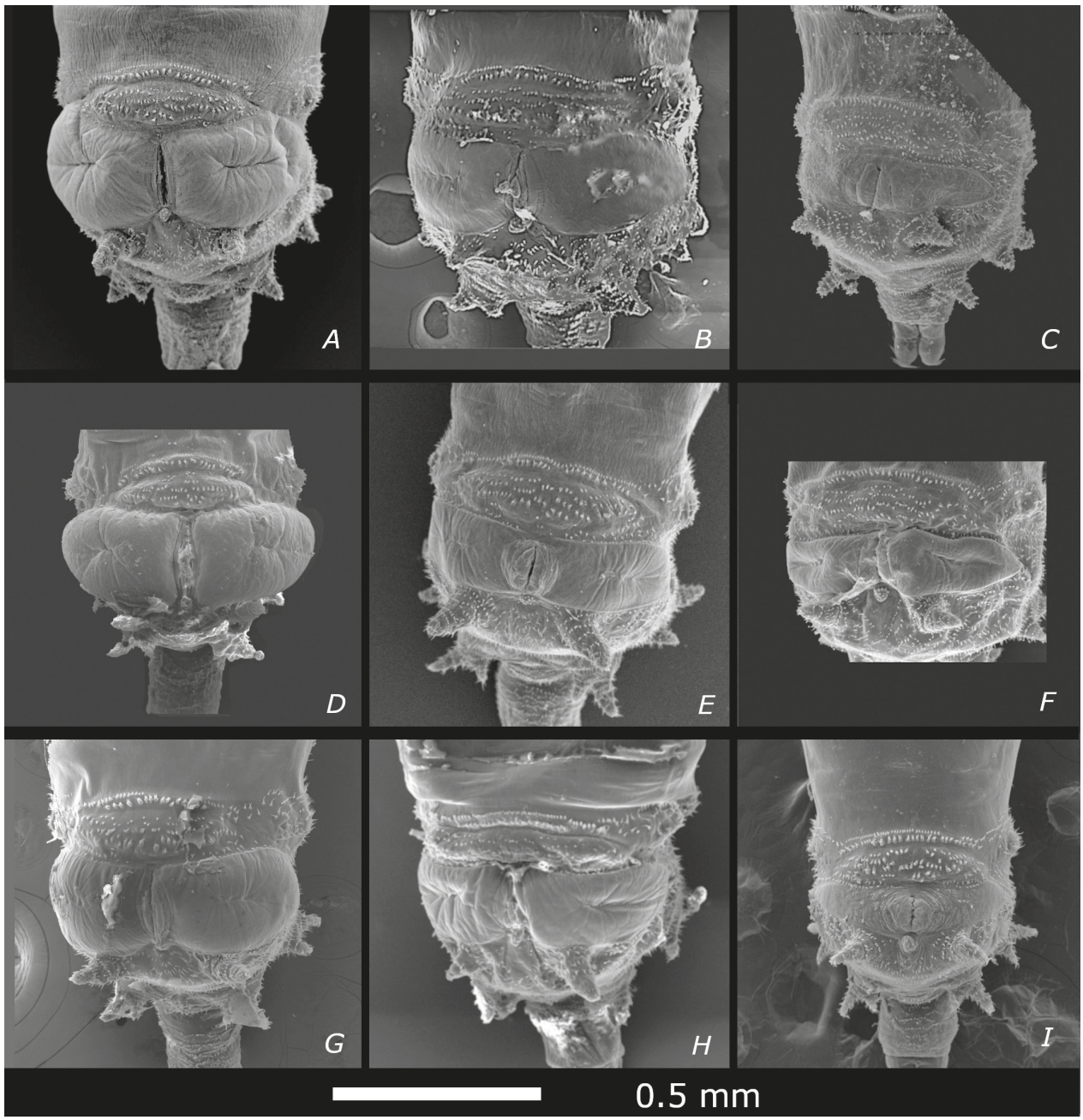


**Fig. 2.** AO of *D. melanogaster* from starch (A–C), control (D–F), and salt (G–I) lines grown on media with different osmolarity. A — Fstarch on Mstarch, B — Fstarch on Mcontr, C — Fstarch on Msalt (4 % NaCl), D — Fcontr on Mstarch, E — Fcontr on Mcontr, F — Fcontr on Msalt (4 %), G — Fsalt on Mstarch, H — Fsalt on Mcontr, I — Fsalt on Msalt (7 %).

was minimal with $p < 0.001$ in all cases, except the nonsignificant ($p = 0.373$) difference between Fstarch and Fcontr on the Msalt (Fig. 3). However, the difference between Fstarch and Fcontr was significant ($p = 0.024$) with the less conservative Fisher LSD post-hoc test.

## Length of the abdomen

Length of the abdomen depended on the fly line, medium, and the interaction of these factors. The influence of the line was stronger than the influence of the medium, as indicated by the larger partial $\eta^2$ (Table 1, 3).

The Fsalt larvae, adapted to the salty medium (Msalt), grew longer on all three media than the larvae of two other lines. At the same time, on the Msalt,

**Table 3. Influence of line and medium on the length of the abdominal segments of fly larvae**

| Factor | SS | df | MS | F | p | Partial $\eta^2$ |
|---|---|---|---|---|---|---|
| line | 1.01 | 2 | 0.51 | 77.0 | < 0.001 | 0.22 |
| medium | 0.71 | 2 | 0.36 | 54.1 | < 0.001 | 0.16 |
| line × medium | 0.18 | 4 | 0.04 | 6.8 | < 0.001 | 0.05 |

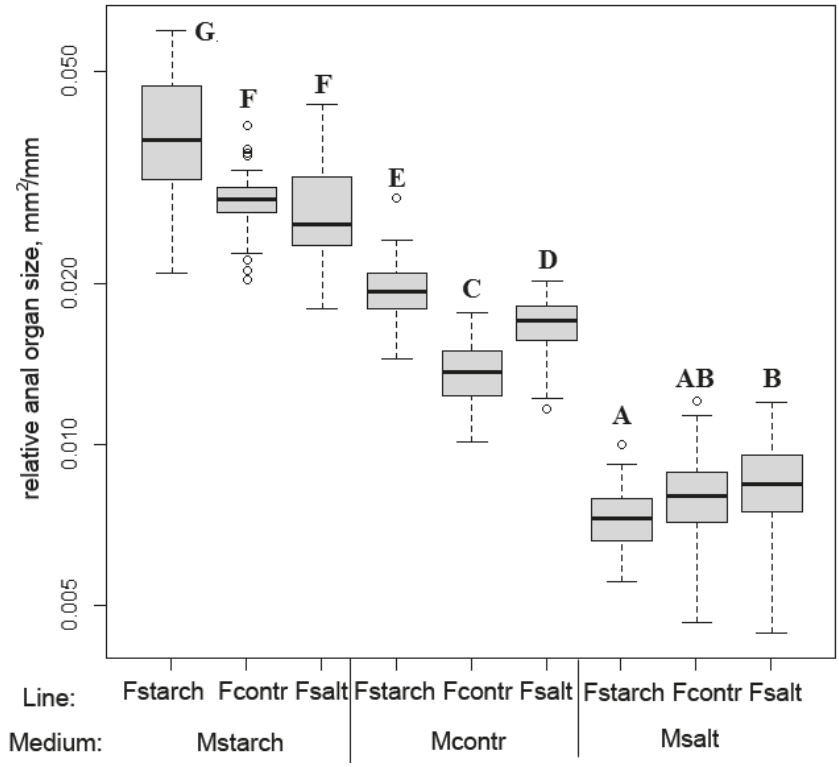


**Fig. 3.** Relative AO size of fly larvae in Fstarch, Fcontr, and Fsalt lines grown on media with different osmolarity. The absence of common letters indicates statistically significant differences on Tukey's HSD test. The box on the diagram denotes interquartile range (IQR: from Q1 to Q3), bold line shows the median (Q2), vertical bars are assessed as min{min value; Q1 – 1.5IQR} and max{max value; Q3 + 1.5IQR} respectively, and the circles represent outliers.

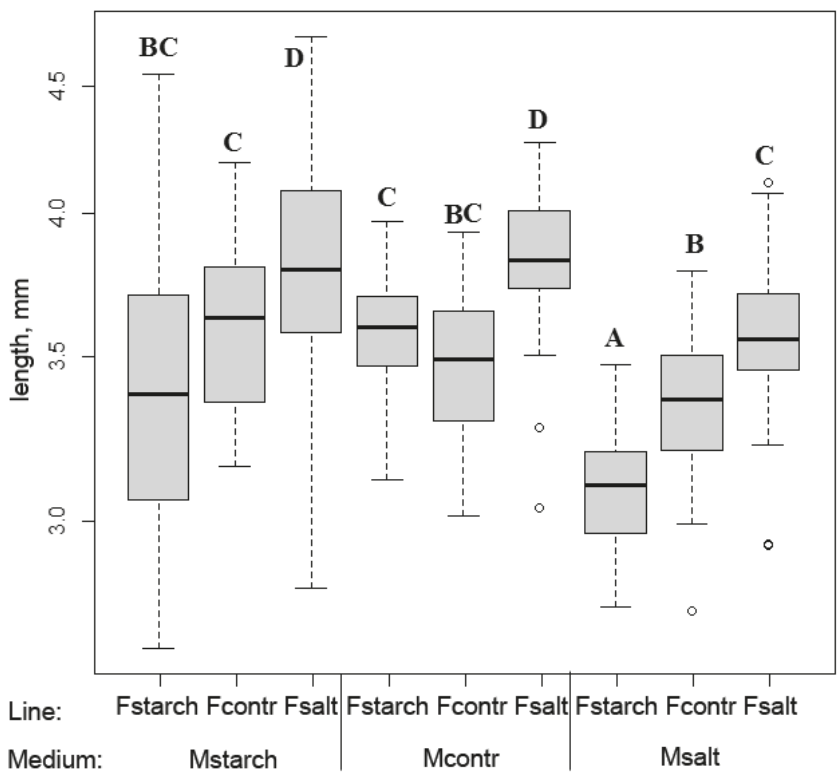


**Fig. 4.** The length of the larval abdomen in Fstarch, Fcontr, and Fsalt lines grown on media with different osmolarity. The absence of common letters indicates statistically significant differences on Tukey's HSD test. The elements on diagram represent the same as in Fig. 3.

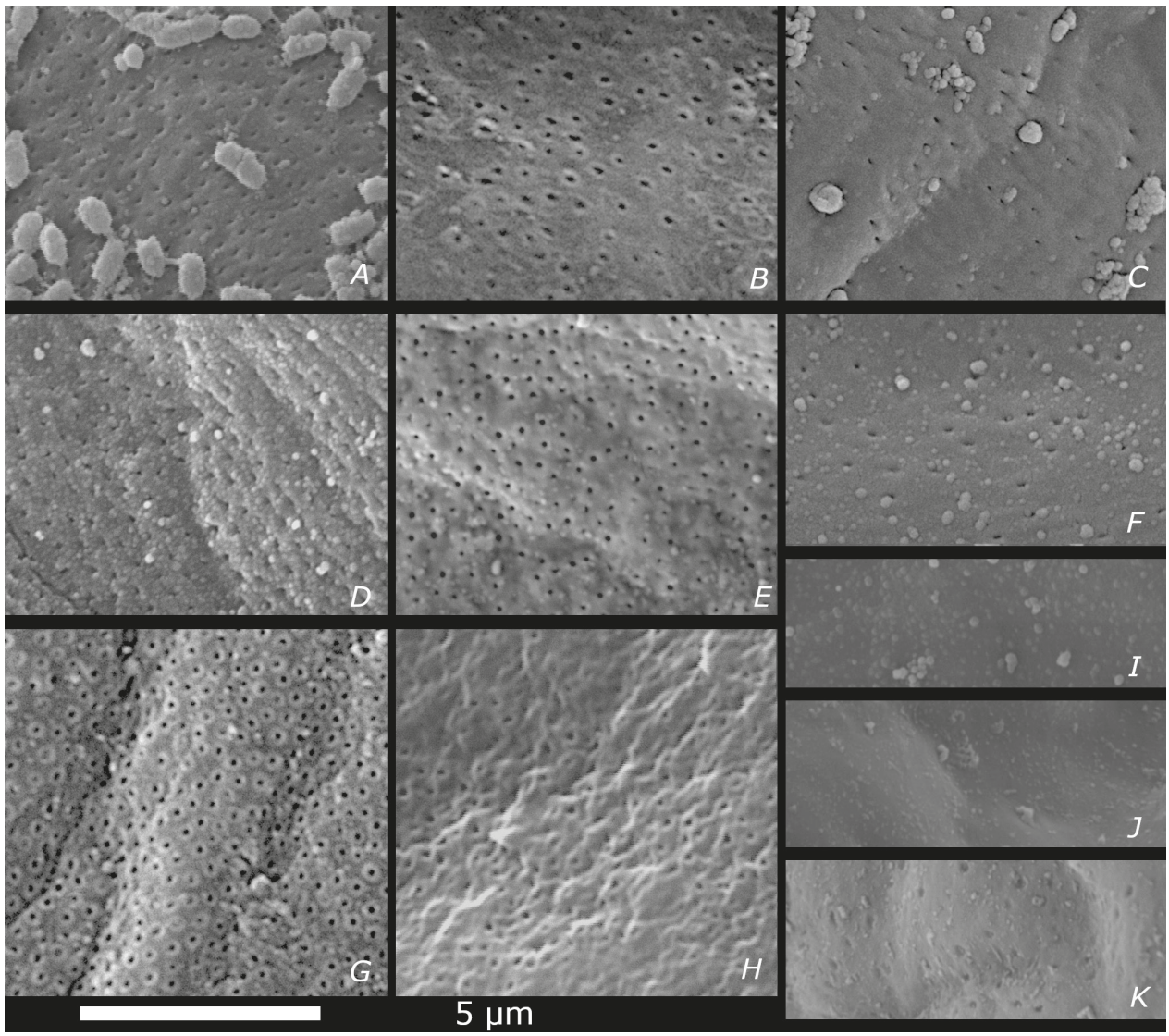


**Fig. 5.** Nanoscale pits on the cuticle of larval AO in starch (A–C), control (D–F), and salt (G–K) lines grown on media with different osmolarity. A — Fstarch on Mstarch, B — Fstarch on Mcontr, C — Fstarch on Msalt (4 % NaCl), D — Fcontr on Mstarch, E — Fcontr on Mcontr, F — Fcontr on Msalt (4 %), G — Fsalt (7 %) on Mstarch, H — Fsalt (7 %) on Mcontr, I — Fsalt (7 %) on Msalt (7 %), J — Fsalt (4 %) on Msalt (4 %) without nanoscale pits, K — Fsalt (4 %) on Msalt (4 %) area with rare shallow nanoscale pits.

all larvae grew shorter than on the standard and starch (Mcontr and Mstarch) media. Differences in length between Fcontr and Fstarch larvae were significant only on Msalt (Fstarch larvae were smaller). On the Mstarch, the larval length variability was significantly higher than on the Msalt and Mcontr (Fig. 4).

## Nanoscale pits on the AO cuticle

Using SEM, we found that nanoscale pits were present on the AO cuticle of *Drosophila* larvae in almost all variants of the experiment. The only exception was larvae adapted to the medium with 7 % NaCl, grown on a medium with the same NaCl content (Fig. 5I).

To clarify whether only the fly larvae adapted to very high salinity demonstrated that PP, we studied larvae from another fly line that was reared for eight years on 4 % NaCl medium. Carefully examining the surface of the AO cuticle of seven individuals reared on their 'native' medium (with 4 % NaCl), we failed to detect regular nanoscale pits (Fig. 5J). Only in one larva from this line, we found a small area where something similar to such pits was observed (Fig. 5K). However, their number and depth were less than in the Fsalt line grown on media without salt. This probably indicated that the ability not to form nanoscale pits on the medium with high osmolarity developed gradually in the process of adaptation, starting with a decrease in the number and depth of nanoscale pits. On the other hand, nanoscale pits were found only in one of seven Fsalt (4 %) larvae and only in a small area of the AO; therefore, the development of nanoscale pits on the salty medium may be not typical for Fsalt flies adapted to 4 % NaCl, just as well as for Fsalt flies adapted to 7 % NaCl.

As for the other test variants, we can notice that the distance between nanoscale pits increases with the increase in salinity. This tendency is especially notable considering that the area of the AO decreases (the surface of the AO shrinks) with the increase in salinity (Fig. 2).

## Discussion

### Relative size of AO

***Phenotypic plasticity.*** Our results confirm the conclusions of previous studies on the nature of PP in *Drosophila* larvae: a medium with low osmotic potential promoted an increase in the AO size in larvae, whereas a high salt medium led to a decrease. This is most probably an example of adaptive PP, since the AO function is to transport ions from the environment into the hemolymph of the larva but not in the opposite direction. In a hypotonic environment, it is beneficial for larvae to have larger AO with well-developed epithelial cells to transport deficient ions from the environment efficiently. On the contrary, in a hypertonic environment, it may be advantageous to have reduced AO with nonfunctional epithelial cells since the AO do not take part in removing the excess ions from the body.

***Changes in the reaction norm.*** The differences between the fly lines shown in Figs 2 and 3 reflect the changes that occurred during adaptation to hypotonic (Fstarch line) and hypertonic (Fsalt line) conditions. These changes do not fit the definition of GA. In the Fsalt line, there is no hereditary decrease of AO size on all media; moreover, the relative AO size in Fsalt is not less than in the control Fcontr line on all the media. These results contradict the results of Waddington (Waddington, 1953) and agree with the results of Te Velde (Te Velde, Molthoff, and Scharloo, 1988). Also, in the Fsalt line, the AO size does not differ significantly from the AO size in the Fcontr flies on both Msalt and Mstarch; therefore, the reaction norm has not narrowed during the flies' adaptation to the medium with high osmolarity.

On the Mstarch, low osmolarity-adapted Fstarch larvae have significantly larger AO than two other lines grown on the same medium. This probably represents an adaptive change. However, it was achieved not by the genetically encoded adaptive plastic change (that is, not according to the GA scenario) but by expanding the reaction norm. During the adaptation to the hypotonic medium Mstarch, not an "unconditional" trait developed (propensity to form larger AO under all conditions), but a "conditional" one — a propensity to develop larger AO only on hypotonic (Mstarch) and standard (Mcontr) media. On the contrary, under hypertonic conditions, the AO in Fstarch larvae are smaller than in Fsalt (the difference is statistically significant) and somewhat smaller than in Fcontr (although the difference reaches statistical significance only with the less conservative post-hoc test). This result is consistent with the idea that the Fstarch flies adapted to the hypotonic medium Mstarch by expanding the reaction norm (the magnitude of PP increased). In other words, the sensitivity of the trait (AO size) to the environmental factor (osmotic potential) increased. It is possible that the permeability of the AO cuticle increased in Fstarch larvae during their adaptation to hypotonic conditions, which could further complicate their development in hypertonic conditions. Probably, that is why the AO of these larvae contracted more strongly under hypertonic conditions than in the other fly lines.

### Other changes in the phenotype

***Length of the larvae.*** Our results (Tables 1 and 3, Fig. 4) show that in the Fsalt line during adaptation to a salty medium the growth dynamics have changed: the larvae of this fly line grew larger on all three media. At the same time, since the larvae of all three lines grew smaller on Msalt than on the other two media, the salty medium itself slows down the growth. Thus, the change in the Fsalt line is directed oppositely to the direction of the PP. It can be hypothesized that the plastic decrease of the larval length on the salty medium is an example of a non-adaptive PP since body size is a universal indicator of health and fitness in many animals, and large larvae generally develop into larger and stronger adults. The non-adaptive PP, expressed in this case as growth retardation on a salty medium, could enforce "compensatory" selection for enhanced growth. The Fsalt flies were probably selected for their ability to withstand unfavorable plastic change: the phenomenon known as selection against the environmental gradient. In this case, the change in the Fsalt lines can be considered as an example of genetic compensation, a phenomenon opposite to GA (Grether, 2005; Ghalambor et al., 2015; Markov and Ivnitsky, 2016).

***AO tomograms.*** Based on the obtained tomograms, we can conclude that with an increase in the osmotic potential of the medium, the layer of X-ray dense epithelial cells underlying the AO cuticle became thinner (Fig. 6, highlighted in white on the right images). This could be due to the less necessary ion transport into the body in lines living on a medium with high osmotic potential. The need for ions, in this case, could be fully met by obtaining them from food.

Tomograms allow us to suggest that the AO cuticle is thinner in larvae from the Fstarch and Fcontr lines, which may facilitate the transport of ions through the AO (video based on the tomograms in the supplementary materials). On the contrary, the cuticle covering the rest of the body of Fstarch larvae is more transparent than the cuticle of

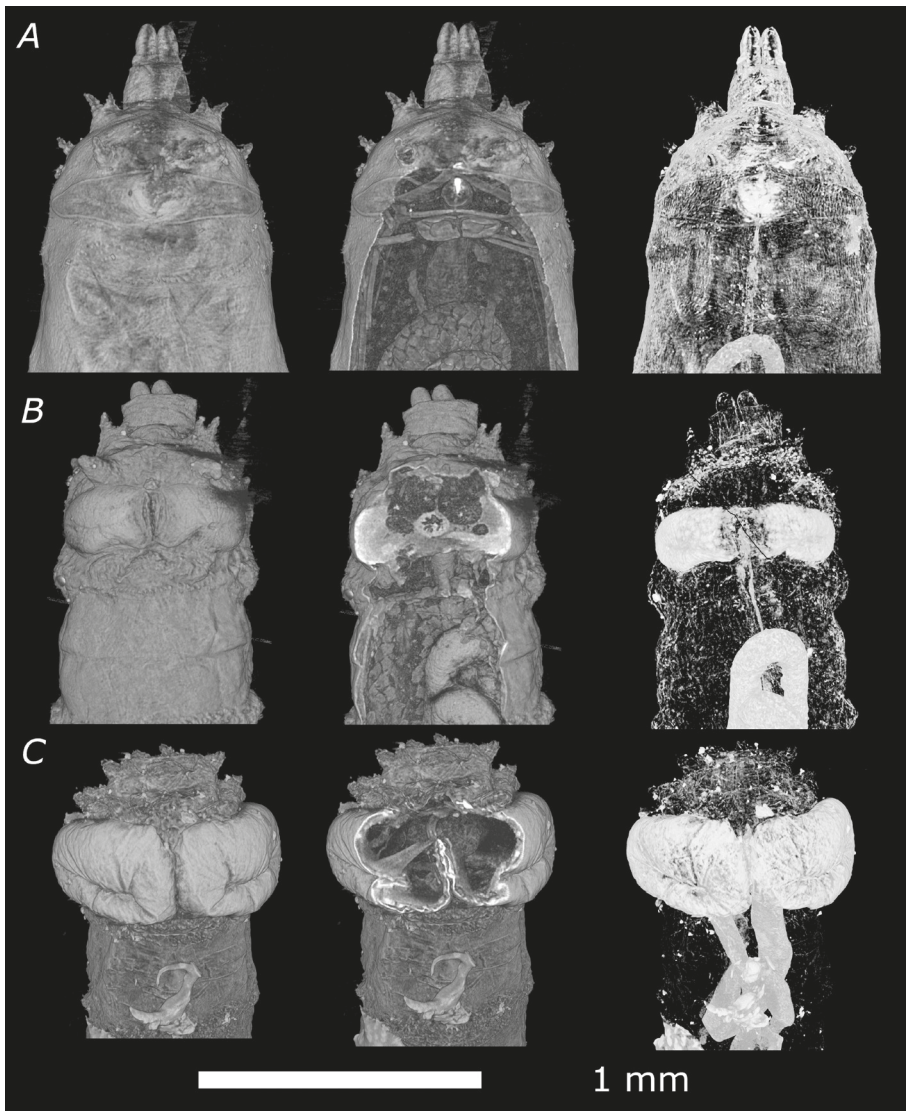


**Fig. 6.** AO of *D. melanogaster* from Fsalt (A), Fcontr (B), and Fstarch (C) lines reared on their own media. Images based on the tomograms: on the left — a general view, in the center — the frontal section of the AO, on the right — the same as on the left, but with increased contrast in density (more dense areas are white, less dense areas are transparent).

Fcontr larvae, and Fcontr larvae's cuticle, in turn, is more transparent than the cuticle of Fsalt larvae. It might be an adaptation against excessive water loss in a hypertonic environment (Fig. 6, images in the center).

***Nanoscale pits.*** The Fstarch and Fcontr fly lines, not adapted to the salty medium, retain nanoscale pits on the surface of the AO cuticle at any salinity of the medium (Fig. 5A–F), while the Fsalt line, adapted to the salty medium Msalt, evolved the ability not to develop nanoscale pits on the AO cuticle when growing on their salty medium (Fig. 5I, J). Since these structures increase the area of the epicuticle contacting the external environment and serve to intensify the absorption of ions (Chen and Brugger, 1973), it is possible that getting rid of nanoscale pits is an example of adaptation to living in a hypertonic environment. At the same time, flies of this line (Fsalt) retain the ability to form nanoscale pits when grown on a medium with lower osmotic potential, Mcontr, and Mstarch (Fig. 5G, H). Therefore, the reduction of these structures in the Fsalt line is not an example of a genetically encoded unconditional phenotypic change but rather an expansion of the reaction norm during the adaptation to a medium with high osmolarity. Moreover, we showed this for the flies adapted to the medium with 4 % NaCl (Fig. 5J) as well as for those adapted to the medium with 7 % NaCl (Fig. 5I).

The differences in the AO staining efficiency between the lines are consistent with what was said above. To obtain the same degree of staining in flies of all lines, the time of immersion in silver nitrate varies 15-fold: for Fsalt grown on Msalt, it is about 30 minutes, while for Fstarch grown on Mstarch, it is only about 2 minutes. This fact indicates the differences in the degree of the AO cuticle permeability, which is higher in flies adapted

to a hypotonic environment (Mstarch) and lower in flies adapted to a hypertonic environment (Msalt). The differences in the AO permeability may be partially due to the tendency to form or not to form nanoscale pits on the AO cuticle surface, to the differences in the cuticle thickness, and to the changes in the thickness of the epithelial cell layer, which is thicker in the Fstarch line and thinner in the Fsalt line, as indicated by the tomograms (Fig. 6, right images, video in supplementary materials). Another reason might be the changes in the epithelial cells' structure, for example, the reduction of apical membrane infoldings in the larvae reared on salty food, as previously demonstrated by Jarial (Jarial, 1987).

## Conclusions

Our study showed that the experimental system used by Waddington in his classic paper to demonstrate the possibility of genetic assimilation of adaptive plastic change (adaptive modification) was probably unsuitable for this purpose. In our experiment, the genetic assimilation of adaptive plastic changes did not occur during the adaptation of *D. melanogaster* to environments with different osmotic potentials. Instead, other evolutionary scenarios were involved, namely:

1. Reaction norm expansion — enhancement of the plastic response of the AO size in the Fstarch line adapted to the hypotonic environment to changes in the osmotic potential of the environment; ability not to form nanoscale pits on the surface of the AO cuticle in a hypertonic environment in the Fsalt line adapted to this environment. The latter may be one way to reduce the AO cuticle's permeability.
2. Genetic compensation — an increase in body length in Fsalt larvae adapted to a hypertonic environment that slows down the growth.

## Limitations

In fact, the identified phenotypic changes may have a genetic or epigenetic basis. Further research may clarify whether the observed phenotypic changes could potentially be ascribed to epigenetic rather than genetic effects. The populations will be subjected to a common environment before taking phenotypic measurements. This step will reduce the influence of transgenerational effects, which can endure for 2–3 generations in *Drosophila* (Shannon and Weaver, 1949; Kawecki et al., 2012; Markov et al., 2016).

For a long time, we studied 3–4 replicates in Fstarch, Fcontr, and Fsalt (2, 4, 7 % NaCl) lines, and our observations suggest that phenotypic plasticity in different replicates of the same line is similar. However, here we present comprehensive measurements only for one replicate of Fstarch and Fcontr lines and for two Fsalt lines (4 and 7 %). The large sizes of the studied populations suggest that the potential effects of drift on the obtained results are probably small.

Approximately thirty flies were collected from nature for the origin of the evolutionary experiment, so the gene pool could be described as having one rarest allele and 59 others. While the initial population was reared, the number of flies increased to one thousand (Belkina, Naimark, Gorshkova, and Markov, 2018). According to the Hardy — Weinberg law, there were 33 rare alleles and 1967 others in that population. At this point, other lines were created. Standing population sizes were approximately several hundred in the Fsalt line and about one hundred in the Fstarch line (Belkina, Naimark, Gorshkova, and Markov, 2018). We can analyze the probability of rare allele loss using a chi-squared test.

For Fsalt, at least for 200 individuals (400 alleles), the p-value is 0.0097, and Yates's correction for continuity gives $p = 0.0187$; both are less than the alpha level of 0.05. Hence, the null hypothesis, that there is no statistically significant difference between the expected frequencies (0 %) and the observed frequencies (1.67 %), is rejected. The chance of losing rare alleles was very low; therefore, there was no founder effect.

For Fstarch, for 100 individuals (200 alleles), the p-value is 0.0672, and Yates's correction for continuity gives $p = 0.1271$; both are higher than 0.05. The null hypothesis is not rejected. In this case, we could suggest the possibility of the founder effect if the size and morphology of AO were monogenic. But such phenotypic traits as the AO's shape and area, the cuticle's smoothness or wrinkling, and the presence of nanoscale pits on it (each of which is not discrete) are determined with high probability by many genes.

According to the Wright — Fisher model and considering the 1.67 % frequency of rare alleles at the beginning, we cannot entirely exclude the presence of genetic drift. On the one hand, given the polygenic nature of the analyzed quantitative traits, the preferential fixation of one allele in the majority of replicates in the absence of selection would suggest the possible role of genetic drift in the divergence of populations based on quantitative traits with a given population size and a given number of generations. On the other hand, the effect of genetic drift should result in high variability of traits within one sample. However, within all three lines, we did not observe a high variance value of the measured traits. Therefore, the observed changes in the traits might be explained as a result of selection.

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