CHARACTERIZATION OF EGGSHELL DIVERSITY AMONG *DROSOPHILIDAE* SPECIES: SIGNALING, PATTERNING AND MORPHOGENESIS

By

ALESSIO S. RUSSOMANNO

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Dr. Nir Yakoby

and approved by

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Nir Yakoby

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Eric Klein

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Angélica L. González

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THESIS ABSTRACT

Characterization of eggshell diversity among *Drosophilidae* species: signaling, patterning and morphogenesis

by ALESSIO S. RUSSOMANNO

Thesis Director:
Dr. Nir Yakoby

Morphological diversity is exhibited throughout nature yet the differences in the developmental mechanisms which contribute to the variety of traits remain mostly unknown. *Drosophila melanogaster* oogenesis is a model system used for understanding tissue patterning and development. During oogenesis, the follicle cells, a monolayer of epithelial cells engulfing the developing oocyte, are instructed by several cell to cell signaling pathways, including the epidermal growth factor receptor (EGFR) and bone morphogenetic protein (BMP), to transform from a 2 dimensional (2D) cell-layer into the eggshell's 3D structures. The eggshell itself possesses features that are common or unique to each species, including the number of the dorsal appendages (embryo respirators), and the presence or absence of a dorsal ridge (a lumen-like structure along the dorsal side of the eggshell). It was previously shown that changes in EGFR and BMP signaling are consistent with the morphological differences observed among the eggshells of *Drosophila* species. Here, we aim to study a new morphology, the respiratory stripe, featured on the eggshell of *Scaptomyza anomala* and *Scaptomyza elmoi*. These species are close relatives to Hawaiian *Drosophila* species. This thesis includes the characterization of eggshells' morphologies, the spatiotemporal changes in EGFR and BMP signaling, and determination of some aspects of patterning changes related to the eggshells of *S. anomala* and *S. elmoi.*

ACKNOWLEDGEMENTS

I would like those who have ensured my success as a graduate student and individual in no particular order. I would like to thank my family for their help and support throughout my life. I thank Nir Yakoby for his guidance and mentorship in the success of my research and academic career. My lab colleagues for their many fruitful comments and discussions; especially Cody Stevens for keeping everything hunky dory, Nicole Revaitis for her help with imaging, and the undergrads who worked with me on this project, Marina Mikheil and Nguyen Nguyen. Also special thanks goes to Vikrant Singh, Matthew Niepielko, and Rob Marmion for their help and advice at succeeding in the world of science.

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Introduction

Morphological diversity is a phenomenon seen throughout nature. Diversity of traits can be seen amongst species from the same habitat such as Darwin's finches [1]. In this case, the diversity lies in the shape and length of the beaks due to the bird's food source. Like the beaks of the finches, the eggshells of *Drosophila* are also morphologically diverse. The differences in the eggshells lie in the number and length of the dorsal appendages, and also the presence or absence of the dorsal ridge [2-6]. The mechanisms responsible for diversity of traits among organisms are not fully understood and require further investigation.

Drosophila oogenesis

Drosophila melanogaster oogenesis, which occurs in the ovary of the female fruit fly, is a well-established model system for the study of tissue patterning and morphogenesis. Each ovary consists of 14 -16 ovarioles which contain egg chambers that develop in an assembly-like fashion. The process of oogenesis consists of 14 morphologically defined stages which takes approximately 3 days to complete [7, 8] (Fig 1A). The main compartments of the egg chamber include the germline cells, including 15 nurse cells and the oocyte, which are surrounded by a monolayer of follicle cells (Fig 1B). The nurse cells are responsible for providing the growing oocyte with nutrients, RNAs, and proteins. The oocyte will become the developing embryo upon fertilization. Within the oocyte, the initial position of the nucleus is in the posterior end. As the oocyte grows, the nucleus anchors itself asymmetrically at the dorsal anterior via microtubule formation [9]. Towards the end of oogenesis, the follicle cells fold into the 3D structures, and secrete the eggshell (Fig 1C). The structures of the

Drosophila eggshell are the micropyle which is used for sperm entry, the operculum from which the larvae will hatch, and the dorsal appendages which are used for embryo respiration (Fig. 1C). This system can be utilized to study developmental mechanisms including the signaling pathways and subsequent tissue patterning involved[3, 10].

A Cavaliere *et. al*, 2008

B Yakoby *et. al*, 2007

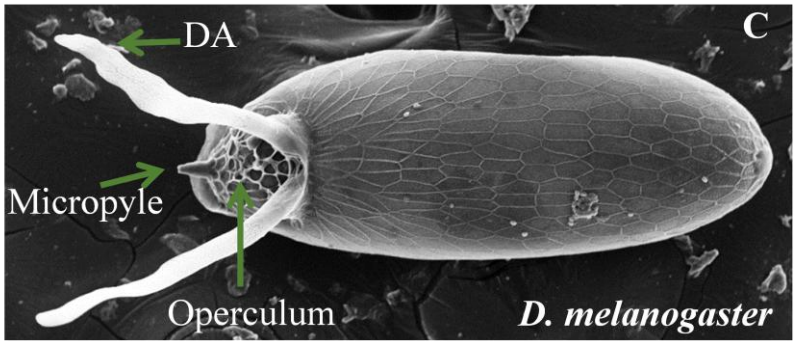


Figure 1 *Drosophila* oogenesis: (A) The fourteen stages of oogenesis (Cavaliere *et. al*, 2008). **(B)** An artificially colored egg chamber at stage 10B to denote the different compartments of the egg chamber. The blue cells represent the nurse cells (NC), the red cells represent the follicle cells surrounding the oocyte (the white large compartment). The tan dot with * denotes the nucleus. A denotes anterior, P denotes posterior, D denotes dorsal, V denotes ventral (Yakoby *et. al* 2008). **(C)** The different structures of a *D. melanogaster* eggshell, (DA) denotes dorsal appendage.

EGFR and BMP signaling pathways

The two major signaling pathways involved in the patterning of the follicle cells are EGFR and BMP [3, 11-13]. Epidermal growth factor receptor (EGFR) signaling pathway belongs to the receptor tyrosine kinase (RTK) family of growth regulators [14-

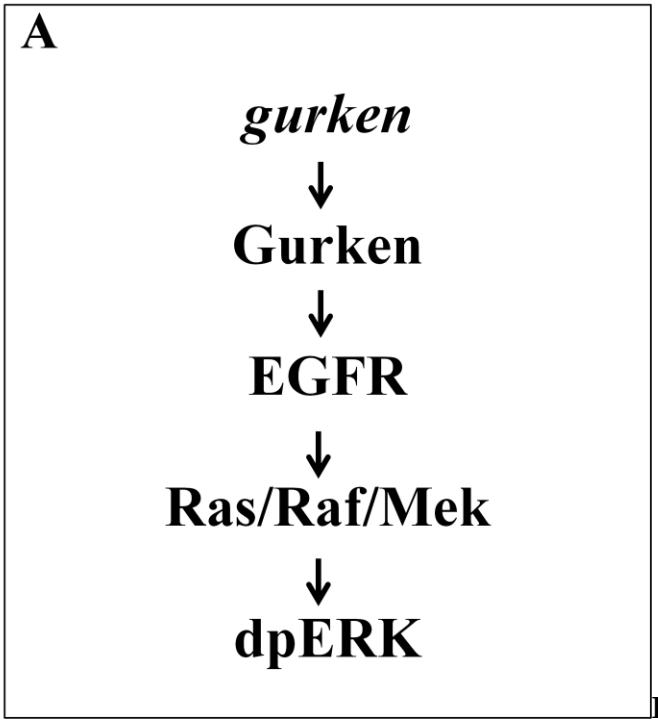
16]. This pathway is evolutionarily conserved among species and has been found to play a role in cell proliferation, differentiation, and apoptosis . Upon binding of the growth factor to the receptor, a signaling cascade known as the Ras-Raf-Mek-Erk is induced [14, 15]. Human pathologies, such as cancer, have been linked to malregulation of this pathway, consequently components of the pathway are targets of therapeutics [17, 18].

Bone morphogenetic protein (BMP) signaling pathway is another pathway involved in tissue development, apoptosis, and proliferation. BMP signaling activation occurs when a dimer molecule binds to both type I and type II receptors [19-21]. Upon ligand binding to the complex of type I and type II receptors, the type II receptor phosphorylates the type I receptor, and the later phosphorylates the intracellular signaling mediator Mothers Against DPP (MAD). Two activated MAD molecules (P-MAD) bind to Medea (MED), then this complex translocates to the nucleus to regulate gene expression. [20, 22]. Although initially thought to be only involved in bone development, it has since been found to be involved in the development of other tissues, including the digestive, muscular, and nervous tissue [19, 23, 24]. Developmental abnormalities and cancer have been linked to malregulation of this pathway [24-26]

Cell to cell signaling during oogenesis

In *Drosophila* oogenesis, the ligand for EGFR is Gurken (GRK) and the ligand for BMP is Decapentaplegic (DPP) [3, 22, 27, 28]. GRK is a TGF-α-like ligand and its mRNA is localized around the oocyte's nucleus, thus the nucleus serves as the ligand source (Fig. 2A, B). The activation gradient of EGFR in the follicle cells is initiated by the secretion of the ligand GRK from near the oocyte nucleus to the perivitelline (Fig.

2C) . Upon GRK binding to its receptor, EGFR which is uniformly expressed in the overlying follicle cells, a cascade of phosphorylation is initiated via the Ras-Raf-Mek-dpERK, which activates or deactivates transcription factors (Fig 2A, D) [13, 29]. During oogenesis, the position of the nucleus within the oocyte is dynamic. Initially, the nucleus is at a posterior position and later it is anchored to a dorsal position. Consequently, the position of GRK is dynamic [30, 31]. The dynamics of GRK's positions are critical to set both anterior/posterior and dorsal/ventral axes of the fly [28]. In addition, EGFR signaling is crucial for the correct development of the eggshells. In the absence of signaling all dorsal structures are lost [32, 33].



B, C, D Niepielko, unpublished

Figure 2 EGFR activation during oogenesis: (A) Schematic of EGFR signaling cascade **(B, C)** *gurken* mRNA and Gurken protein are localized near the oocyte nucleus **(D)** Gurken activates EGFR signaling (monitored by dpERK). Yellow arrows denote the posterior boundary of each pattern. **(B, C, D)** Images by Matthew Niepielko

The *Drosophila* BMP2/4 ligand homolog DPP is secreted from the follicle cells covering the nurse cells and the centripetally migrating follicle cells at the oocyte and nurse cells border (Fig. 3A). The activation of BMP signaling, monitored by P-MAD, in the follicle cells is dynamic. Early, the anteriorly secreted ligand is perceived by a uniformly expressed type I receptor thickveins (TKV) (Fig. 3B). Consequently, early BMP signaling is detected as an anterior band near the DPP source (Fig. 3D, E). Later, the TKV is expressed in two dorsolateral patches on either side of the dorsal midline, allowing the emanating ligand to signal away from the source, where TKV is expressed (Fig. 3C, F). [10, 12, 20-22, 34, 35]. It was previously shown that the early BMP signaling is conserved across *Drosophila* species while the late patterns are diverse and dynamic [36].

A, B, C, D, E, F Niepielko *et. al* 2012

Figure 3 BMP signaling during oogenesis: (A) The *dpp* ligand is expressed in anterior follicle cells. **(B)** Early *tkv* expression is uniform in all follicle cells. **(C)** Late *tkv* is expressed in two dorsolateral patches on either side of the dorsal midline. **(D)** Schematic representation of BMP signaling activation. **(E)** Early BMP signaling, monitored here by P-MAD (green), is restricted along the anterior. **(F)** Late BMP signaling appears as two dorsal patches on either side of the dorsal midline. In all figures, the broken yellow line denotes the anterior boundary and the white arrow denotes the dorsal midline. Figure from Niepielko *et. al* 2012.

Follicle cell patterning

Patterning of the follicle cells results is the process of non-uniform gene expression. These genes are targets of several signaling pathways including EGFR and BMP [3, 28, 30, 37, 38]. A previous screen showed that 81 such genes are dynamically expressed in 36 distinct patterns during oogenesis [39]. Two such examples of follicle cell patterning that are widely used to describe targets of EGFR and BMP signaling are Broad (BR) and Rhomboid (RHO). These genes mark two functional cellular domains that participate in the production of the dorsal appendages. BR marks the future roof (top) domain of the dorsal appendages and RHO marks the future floor (bottom) domain of the appendages [3, 40-42]. Genetic perturbations of EGFR and BMP signaling have also been shown to affect dorsal appendage formation by changing BR expression [10, 11, 21, 36, 40, 43].

Changes in cell signaling and tissue patterning are the driving forces of the diversity of eggshell structures. For example, it has been shown that the number of activated EGFR domains coincides with the number of dorsal appendages produced [2]. Another structure, known as the dorsal ridge, is also found in certain *Drosophila* species. It was found that the pattern of EGFR activation coincides with its placement, shape, and length along the dorsal most side of the eggsehll [4, 27].

Specifically, the distribution of GRK has also been shown to be critical in dorsal ridge formation [27]. It was previously shown that in *D. melanogaster*, a species that does not have a dorsal ridge on its eggshell, that the GRK protein is detected at the dorsal anterior end with a 51% of the oocyte length extension towards the posterior end. In species with a dorsal ridge, like *D. willistoni*, GRK extends 76% towards the

posterior end. The corresponding extensions are also found in the activation patterns of EGFR signaling (monitored by dpERK). Using RNAi against *D. willistoni grk*, it was found that GRK is necessary for DR formation. The importance of GRK in dorsal ridge formation was further verified with the construction of a transgenic *D. melanogaster* fly containing *D. willistoni* GRK. This fly was able to produce a dorsal ridge on its eggshell [27].

Investigation of eggshell development in another genus of *Drosophilidae*

Scaptomyza is a genus of *Drosophilidae* which evolved from Hawaiian *Drosophila* approximately 20 - 30 million years ago [44, 45]. Unlike *D. melanogaster* that feeds on yeast, these fly species have acquired a preference for plants as their food source [44, 46]. The only eggshell previously imaged was that of *S. albovittata*. It was shown that this eggshell lost its dorsal appendages in favor of two respiratory stripes on either side of the dorsal most side of their eggshells [46]. This intriguing image sparked my interest in the evolution eggshell morphology as well as the evolution of the respiratory stripe among species.

Here, I present the first comprehensive analysis of *Scaptomyza* eggshell development by analyzing the morphology, signaling pathways, and tissue pattering in the species available which are *S. anomala* and *S. elmoi*. Due to difficulties raising *S. anomala*, data for this species is included when available. The eggshells for these species are presented in figure 4. By analyzing the eggshells of these species, a unique structure known as the respiratory stripe was characterized along its dorsal side. It was found that the respiratory stripe differed in size as well as in the interpillar network located inside the structure when compared to the dorsal ridge. Reduction in BMP, as

well as the late phase of EGFR activation, were found in both *Scaptomyza* species. Also, while EGFR activation was found to occur in the future respiratory stripe domain, there were found to be differences in cell shape suggesting differences in morphogenesis.

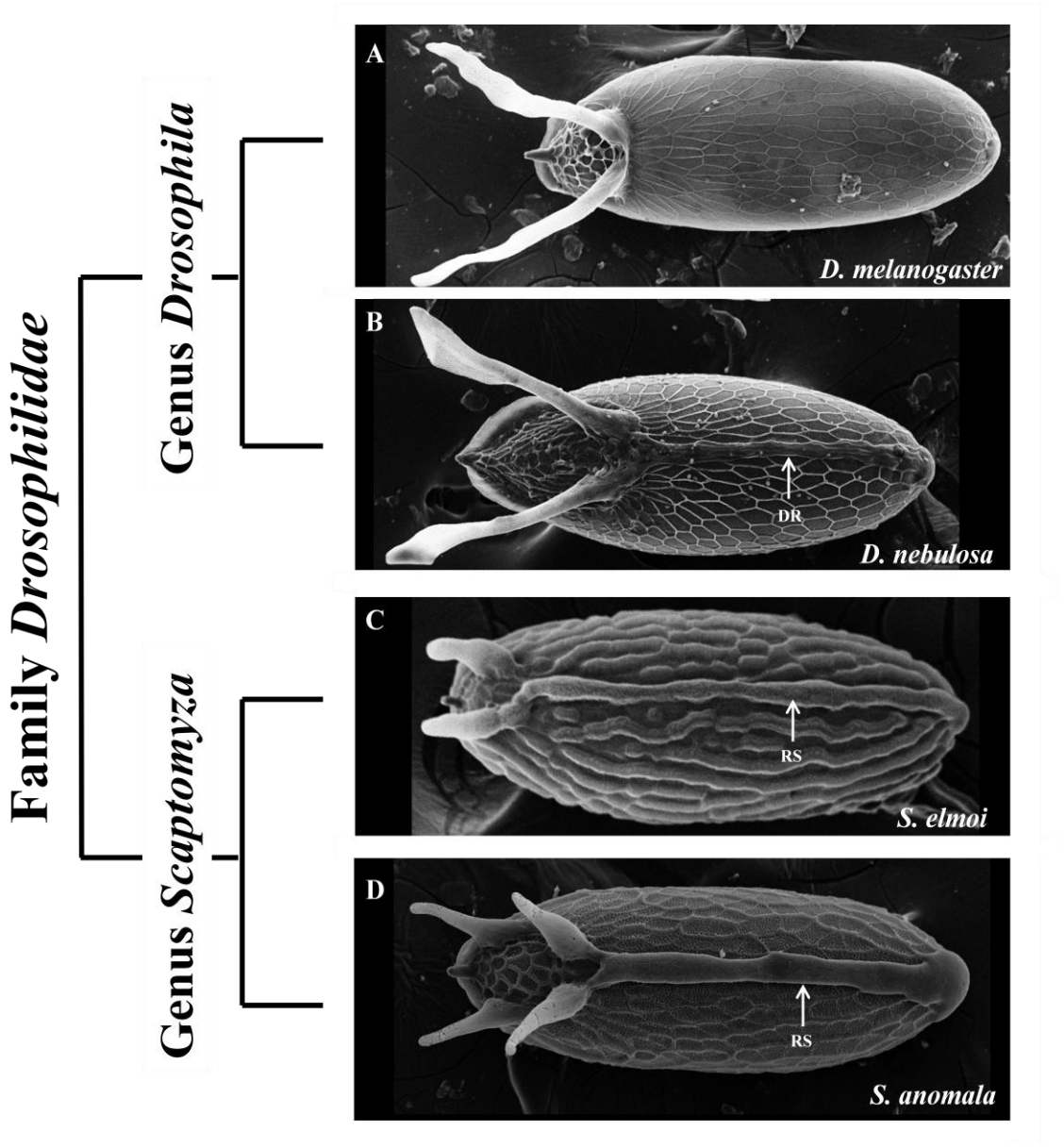


Figure 4 Eggshells of interest: (A) *D. melanogaster*, does not have a dorsal ridge on its eggshell. **(B)** *D. nebulosa*, a species with a dorsal ridge (DR) on its eggshell. **(C)** *S. elmoi*, a species with a respiratory stripe (RS) on it eggshell. **(D)** *S. anomala*, a species with a respiratory stripe (RS) and four dorsal appendages.

Materials and Methods

Flies

The following flies were used in this study: *D. melanogaster* (wild-type, OreR), *D. nebulosa*, *S. anomala*, and *S. elmoi* (UC San Diego *Drosophila* Stock Center). All flies were fed dry yeast 1-2 days prior to dissection. *S. anomala* was raised on cornmeal with a piece of mushroom placed on top of the media. *S. elmoi* was raised on cornmeal with a piece of red cabbage. *D. melanogaster* and *D. nebulosa* were raised on standard cornmeal with no supplementation. All flies were raised at room temperature (23°C).

Floatation experiments

To test possible functions of the eggshells, eggs were laid on grape plates and allowed to settle for 1 day. Eggs were then dispersed with water and poured into a clear plastic cup to monitor ability to float.

Immunohistochemistry

To study pathway activation and tissue patterning, immunohistochemistry was employed as stated here. Ovary dissection and fixation for BR, FAS-III, and P-MAD were carried out as previously described [10] except ovaries for *S. anomala* and *S. elmoi* were dissected into a mixture of 200 µL of 0.2% Triton in PBS and 180 µL Paraformadelhyde. Ovary dissection and fixation for dpERK which was carried out as previously described [47] . The primary antibodies used were anti-mouse BR-Core (1:100 DSHB), anti-mouse FAS-III (1:100 DSHB), anti-rabbit anti-phosphorylated-Smad1/5/8 (1:3500, a gift from D. Vasiliauskas, S. Morton, T. Jessell and E. Laufer),

anti-rabbit dpERK (1:100 Cell Signaling). Secondary antibodies used were 488 anti-mouse and 568 anti-rabbit (1:1000 Invitrogen).

Microscopy

To analyze results from immunohistochemistry experiments, egg chambers were imaged using Leica SP8 confocal microscope. To study the eggshell morphology, eggshells were collected on grape plates using yeast (for *Drosophila* species), cabbage (for *S. elmoi*), mushroom (for *S. anomala*). Eggshells were mounted on double sided carbon tape and coated with gold/palladium for 45 seconds. SEM images were taken using Neoscope JCM-6000 and SEM LEO 1450EP.

Colchicine treatment

To study EGFR activation in respiratory stripe formation, *S. elmoi* flies were fed colchicine mixed with yeast paste (50 µg/mL) for 48 hours on grape plates. After two days, the plate was replaced for another 24 hours and then number of eggs were counted.

Results

The respiratory stripe is morphologically different from the dorsal ridge

The eggshells of both *S. elmoi*, a species with a respiratory stripe, and *D. nebulosa*, a species with a dorsal ridge, are depicted in figure 5. The respiratory stripe in *S. elmoi* was found to contain two distinct surfaces with the middle layer being more porous in appearance (Fig. 5A, A', C, C'). The dorsal ridge in *D. nebulosa* was found to contain one distinct surface and the imprint of the follicle cells used in the dorsal ridge's formation are seen (Fig. 5B, B', D, D'). Cross sections were also obtained of the dorsal structures in both species (Fig. 5E, F). In the cross sections obtained, the respiratory stripe in *S. elmoi* is approximately three times higher than the dorsal ridge in *D. nebulosa* (n=2, mean=10.91µm and n=2, mean=3.62µm, respectively). It is also important to note that the respiratory stripe was found to have two distinct pillar matrices inside with the top portion being on average 3.66 µm (n=2) in height and the bottom on average being 7.26 µm (n=2) in height. Due to difficulty obtaining cross sections, measurements were also taken at lateral views of the eggshell and it was found that the respiratory stripe was an average 10.48 µm (n=5, ±0.62SD) in height and the dorsal ridge was an average 3.84 µm (n=6, ±0.17SD) in height.

What is also interesting in these flies is the thickened chorion located on the main body of the eggshells, most notably that of *S. elmoi* (Figs. 4C, 5A, C, E). Investigations into other insect eggshells revealed that another cabbage eating fly, *Erioischia brassicae*, describe similar longitudinal grooves along the lateral sides of the eggshell. It was found that these grooves in *E. brassicae* allowed the eggshell to float horizontally much like a boat or canoe[48]. To test the ability to float in *Drosophilidae*,

we started with *D. melanogaster* and *D. nebulosa* and found that they floated at an oblique angle with the dorsal appendage remaining above water (Fig 6A, B). We next investigated *S. elmoi* and found that its eggshell floated horizontally (Fig. 6C). All eggshells were able to float (Fig 6D).

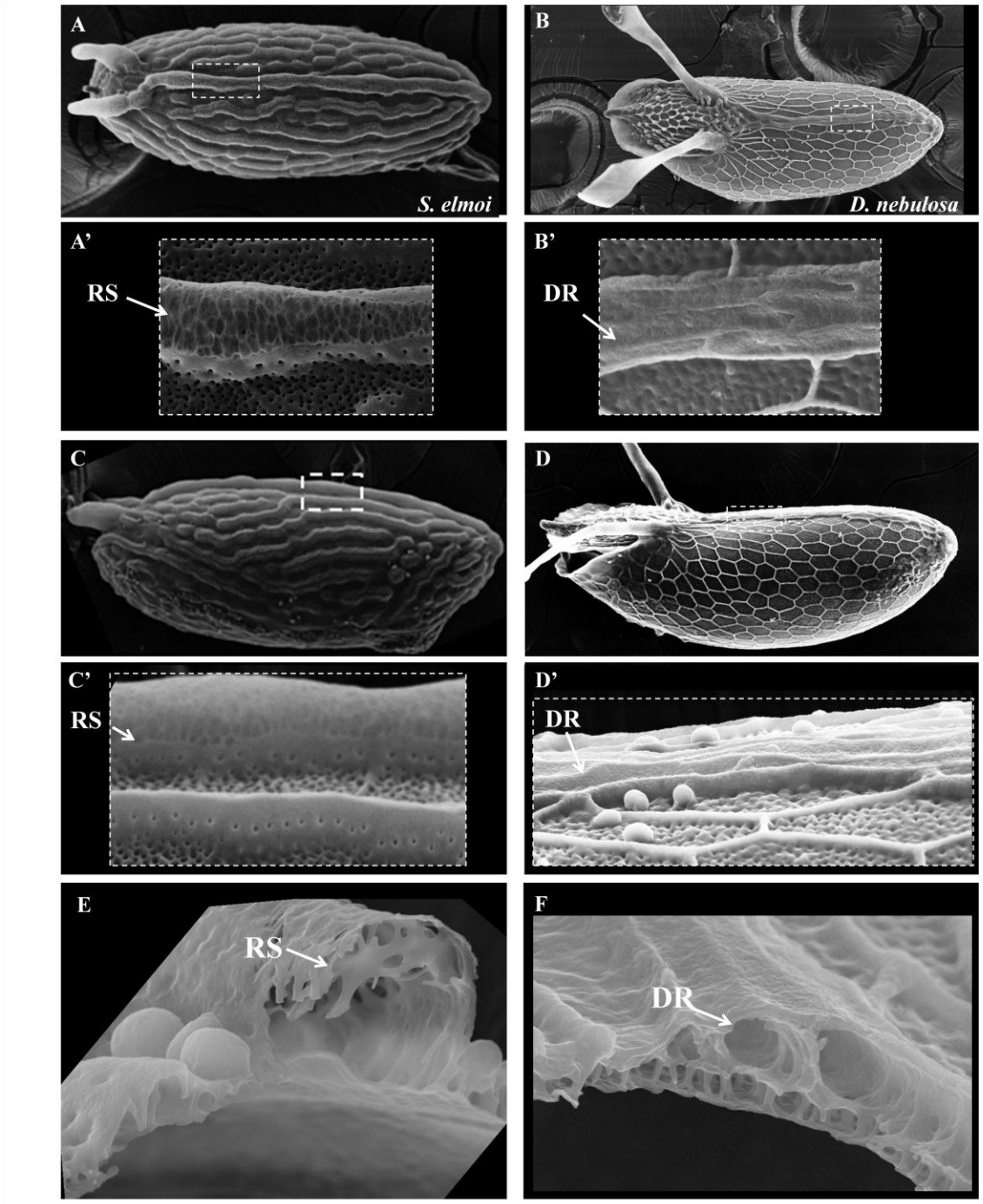


Figure 5 SEM images of eggshells: (A, A') Dorsal view of *S. elmoi* eggshell. **(B, B')** Dorsal view of *D. nebulosa* eggshell. **(C, C')** Lateral view of *S.elmoi* eggshell. **(D, D')** Lateral view of *D. nebulosa* eggshell. **(E, F)** Cross-sections of respiratory stripe (RS) and dorsal ridge (DR), respectively.

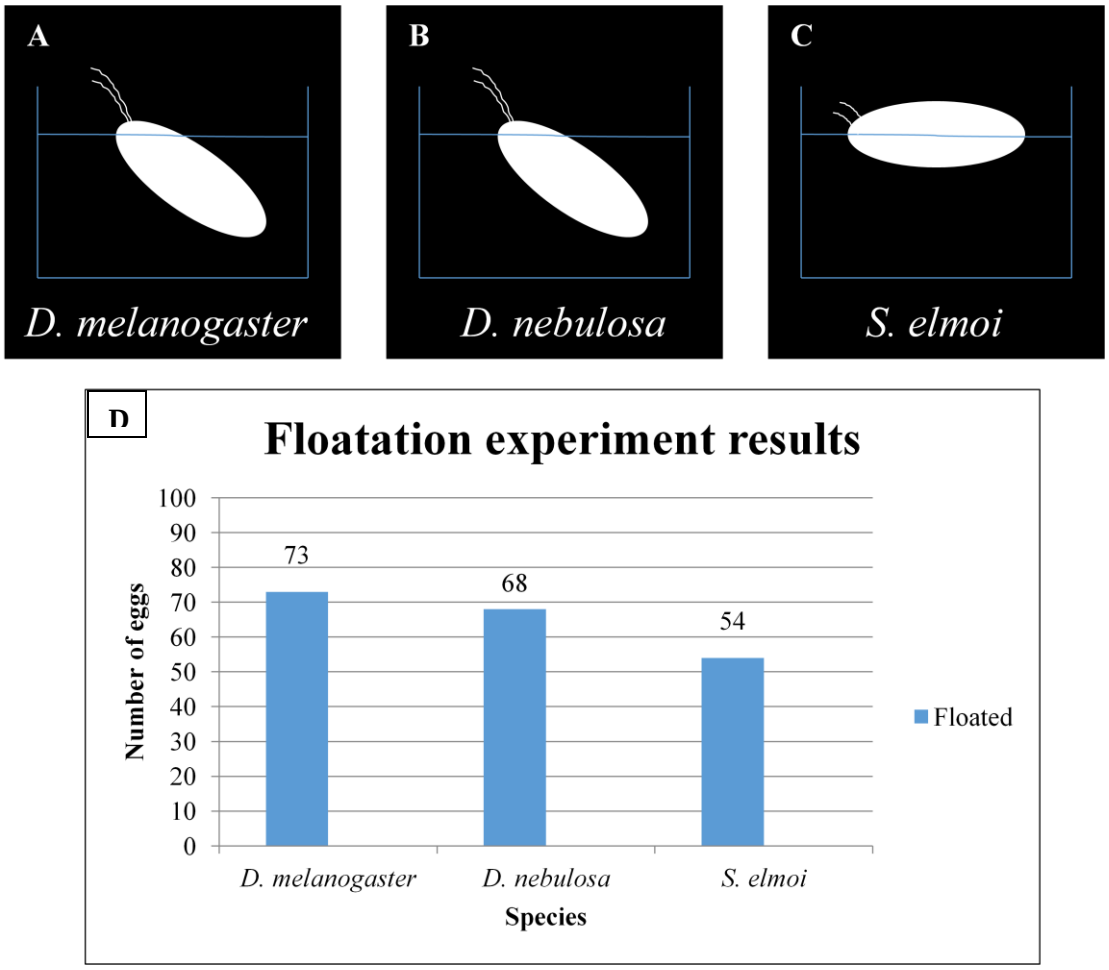


Figure 6 Floatation of the eggshell: (A, B, C) Representation of how a *D. melanogaster*, *D. nebulosa*, and *S. elmoi* eggshells floated when placed in water. **(D)** Graph showing that all eggs for each species floated (*D. melanogaster*, n=73, *D. nebulosa*, n=68, and *S. elmoi* n=54)

BMP dynamics in *Scaptomyza*

It was previously discovered that early BMP signaling dynamics are conserved across *Drosophila* species, represented by *D. nebulosa* (Fig. 7A) [36]. This is also the case in *Scaptomyza* with similar activation pattern in the follicle cells along the anterior border of the nurse cells and oocyte (Fig. 7D, G). However, as the egg chambers transition into the later stages, we found reduced activation of BMP signaling. Starting with stage 10B, a pattern of P-MAD 5-7 cells wide is seen in *D. nebulosa* (Fig. 7B).

The *Scaptomyza* species display reduced signaling when compared to *D.* nebulosa (Fig. 7B, E, H). At stage 11/12, a broad pattern of P-MAD is seen in *D. nebulosa* on either side of the dorsal midline (Fig. 7C). The signal remains in the anterior most cells in *S. elmoi* (Fig. 7F). In *S. anomala*, the signal moves 1-2 cells towards the posterior with symmetry along the dorsal midline (Fig. 7I).

A, B, C Niepielko *et. al* 2011

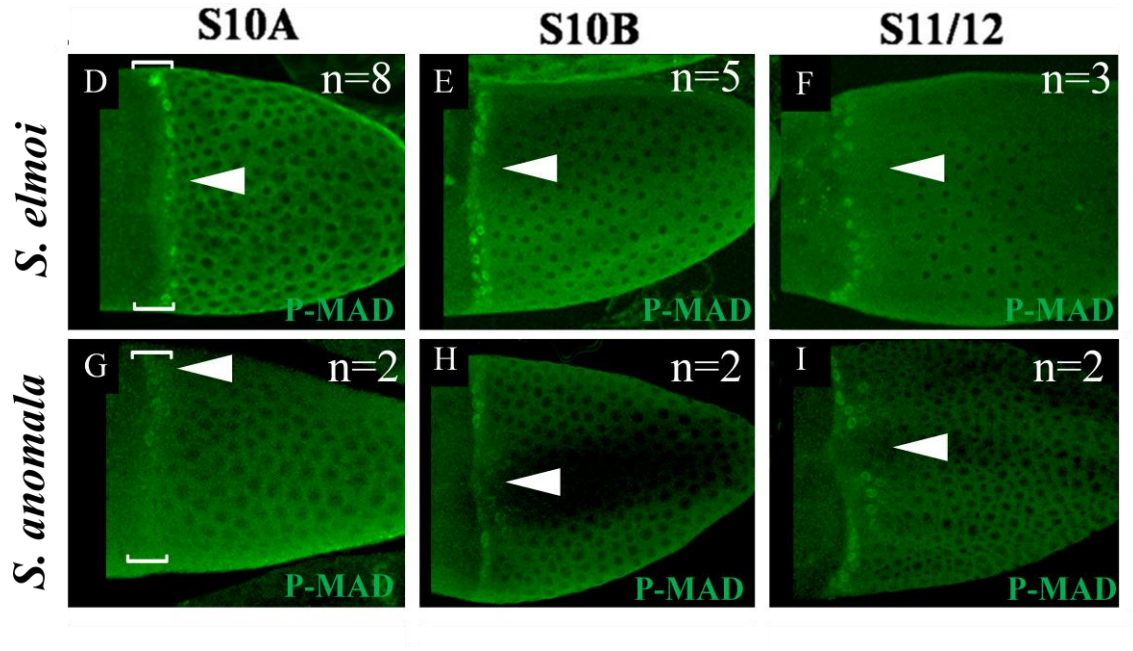


Figure 7 BMP in *Drosophila* and *Scaptomyza*: **(A, B, C)** BMP activation monitored by P-MAD (green) in *D. nebulosa* (Niepielko, *et. al* 2011). **(D, E, F)** BMP activation in *S. elmoi.* **(G, H, I)** BMP activation in *S. anomala.* Arrowhead marks the dorsal midline, brackets mark the anterior. Images A, B, C from Niepielko, *et. al* 2011

Broad expression is reduced in *Scaptomyza*

To further investigate dorsal appendage formation in *Scaptomyza,* we looked at BR which marks the future roof of the dorsal appendages in *D. melanogaster* [3, 40, 42]. BR expression was found to be reduced in *Scaptomyza* species when compared to *Drosophila* species (Figure 8). *D. nebulosa,* with two dorsal appendages, contains about 25-30 cells in each of its BR patches which is typical for species with two dorsal appendages (Fig. 8A). *S. elmoi,* a species with two shortened dorsal appendages, contains about 5–10 cells only 1-2 cells wide in the anterior-most cells (Fig. 8B). BR expression is also reduced in *S. anomala*, a species with four shortened dorsal appendages (Fig. 8C). Interestingly, as showed for other species with 4 dorsal appendages, including *D. virilis* [2], we found that BR has two patches on either side of the dorsal midline in *S. anomala* (Fig. 8C). In *D. melanogaster*, BR regulates TKV expression and consequently controls the pattern of BMP signaling (Fig. 8D).

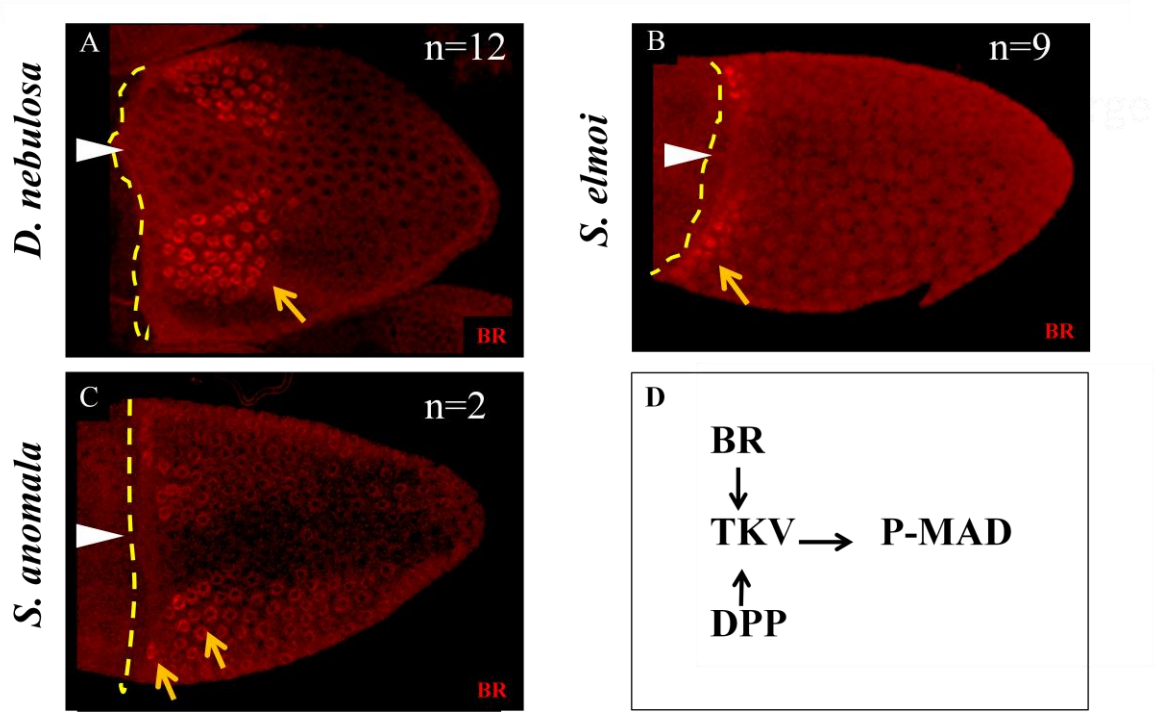


Figure 8 Broad is reduced in *Scaptomyza* species: (A) Broad (BR) expression (red) in *D. nebulosa.* **(B)** BR expression in *S. elmoi.* **(C)** BR expression in *S. anomala.* (D) Schematic describing how BR controls TKV expression and consequently the pattern of P-MAD. Yellow broken line denotes the anterior, arrowhead denotes the dorsal midline, arrow denotes BR expression.

EGFR activation reflects final eggshell morphology

Previous investigations into dorsal ridge formation have shown that EGFR activation is required for its formation [4, 27]. Here, using a similar procedure, EGFR activation was determined by performing immunohistochemistry to detect the intracellular molecule, dpERK, which is activated during the phosphorylation cascade downstream of EGFR activation (Fig. 2A, D). It was previously found that EGFR activation corresponds with the final eggshell morphology (Figure 9). In *D. melanogaster*, EGFR activation, as monitored by dpERK, can be seen extending

approximately 50% from the anterior to the posterior (Fig. 9D). In species with a dorsal ridge, it was found the activation of EGFR activation correlated to the length of the dorsal ridge on the eggshell. For example, in *D. nebulosa*, it was found that EGFR activation, as monitored by dpERK, can be seen extending all the way to the posterior (Fig. 9E) [27]. In *S. elmoi* EGFR was found to be activated along the dorsal midline from the anterior all the way to the posterior end (Fig. 9F). Due to lack of genetic tools in this species, we used colchicine to disrupt microtubule formation and thus mislocalized the nucleus, and consequently mislocalized the nuclear associated GRK, as previously described [4]. We were able to disrupt the respiratory stripe and also disrupt dorsal appendage formation and the operculum was also found to be severely reduced (Fig. 10).

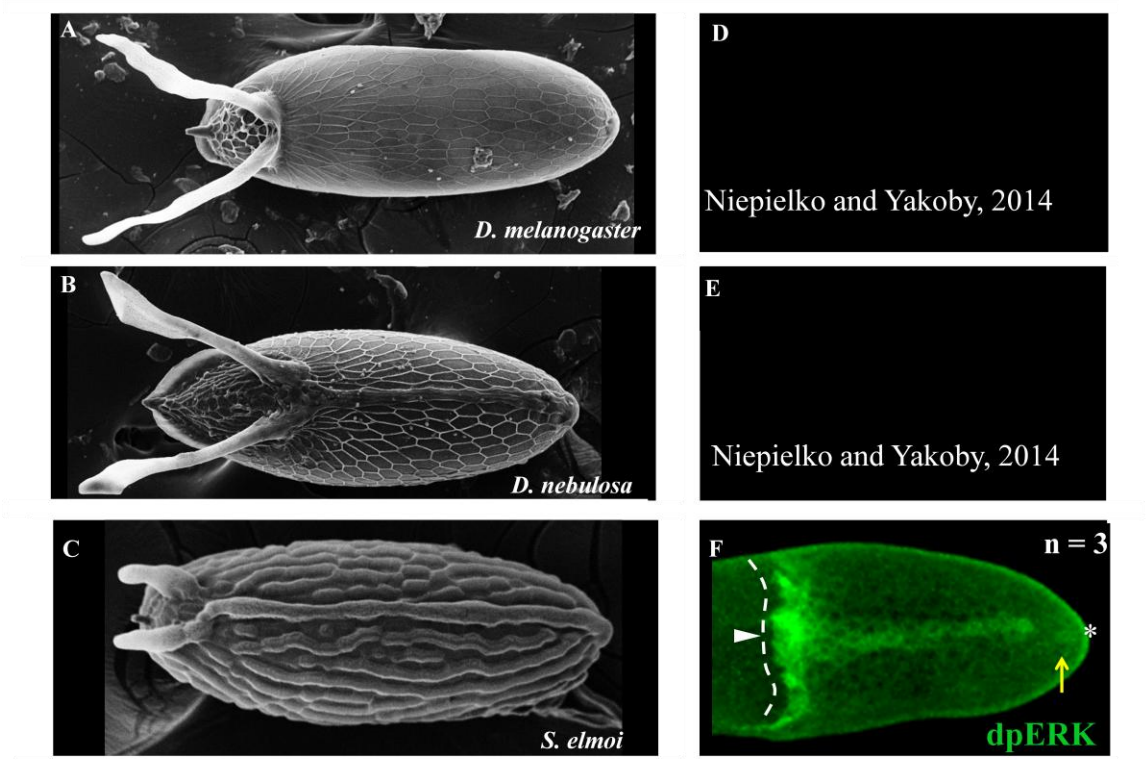


Figure 9 EGFR activation reflects final morphologies: **(A, B, C)** Eggshells of species of interest. **(D)** dpERK showing extension 50% towards the posterior in *D. melanogaster* (Niepielko and Yakoby 2014). **(E)** dpERK activation extending all the way to the posterior end in *D. nebulosa* (Niepielko and Yakoby 2014). **(F)** dpERK activation extending all the way to the posterior in *S. elmoi*. The broken white line denotes the anterior boundary, the white arrow denotes the dorsal midline, the yellow arrow denotes posterior end of the dpERK activation pattern. The star denotes the posterior end. Images D and E are from Niepielko and Yakoby 2014.

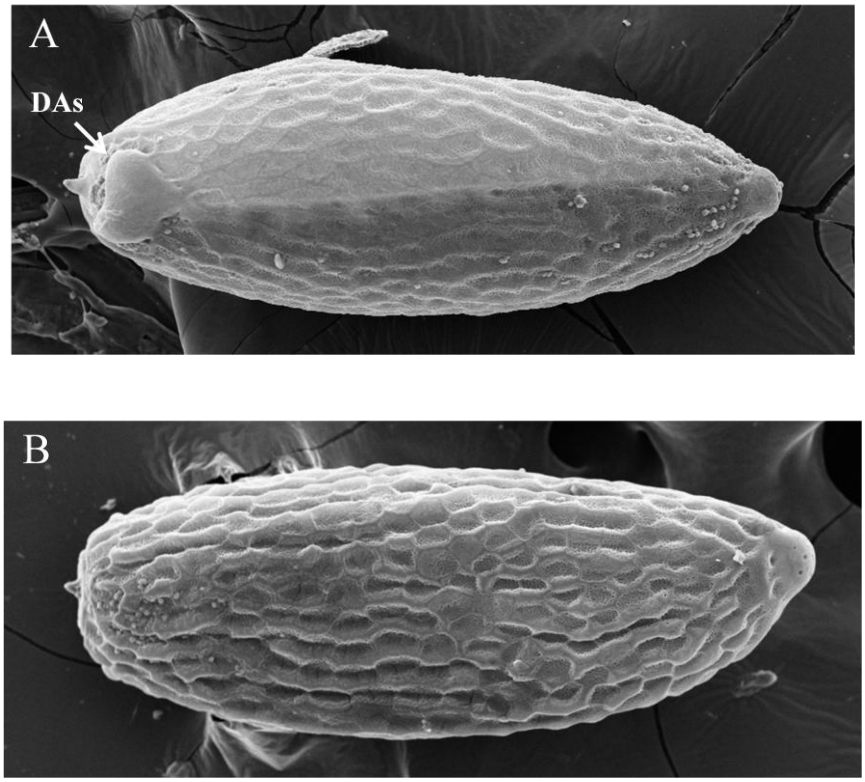


Figure 10 Colchicine treatment disrupts respiratory stripe formation: (A) *S. elmoi* eggshell with disrupted dorsal features including fused dorsal appendages (DAs) (54%, n = 61). **(B)** *S. elmoi* eggshell with dorsal appendages and respiratory stripe missing (28%, n = 61). The remaining phenotypes were wild type (18%, n=61).

Fasiclin – III expression shows change of cell shape in *S. elmoi*

Fasiclin-III (FAS-III) is a known operculum marker in *D. melanogaster*. It was also found to be expressed in the future dorsal ridge domains of *D. willistoni* and *D. nebulosa* [4]. The FAS-III expression pattern in *D. nebulosa* is shown below (Fig. 11A). In *S. elmoi*, FAS-III is also expressed in the future respiratory stripe domain (Fig. 11B). In the area posterior to the dorsal appendages primordia, the *D. nebulosa* cells remained in a typical hexagonal shape with six symmetric edges (Fig 11A, A').

However, in *S. elmoi*, the cells past the DA primordia display an asymmetric shape (Fig 11B, B').

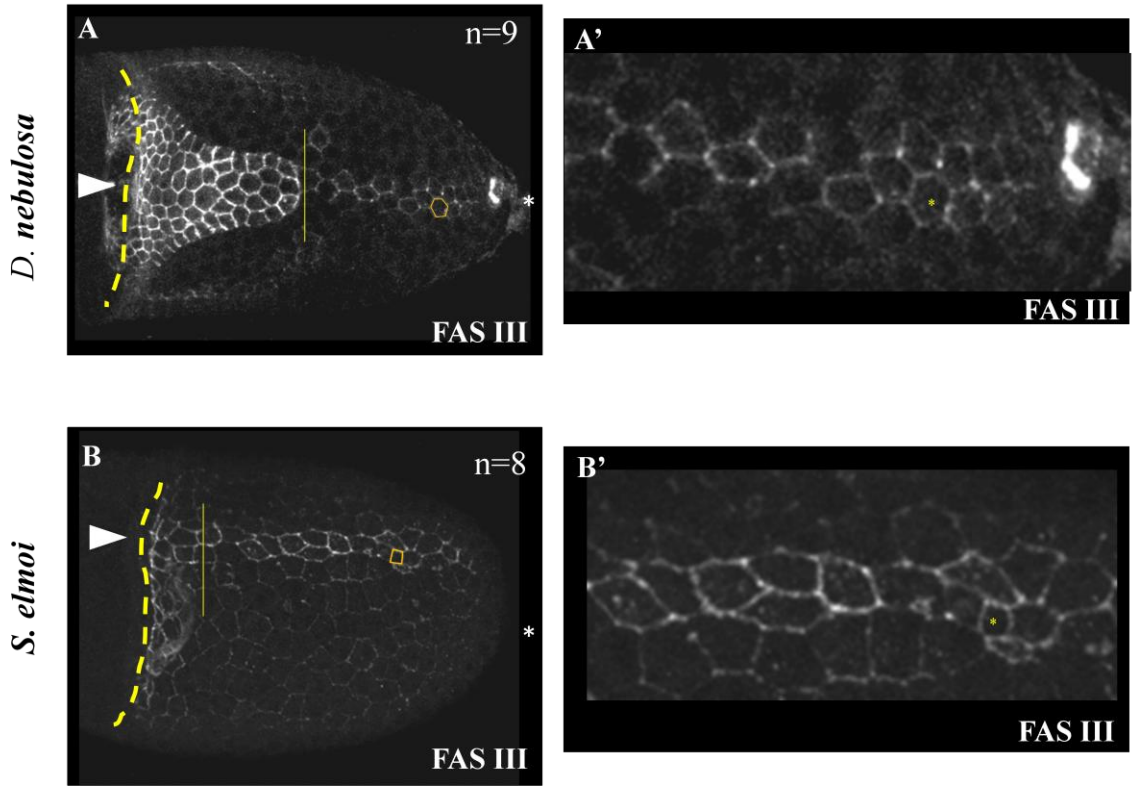


Figure 11 FAS III shows changes of cell shape: (A) FAS-III expression in *D. nebulosa.* **(B)** FAS-III expression in *S. elmoi.* The broken yellow line denotes the posterior boundary of the future dorsal appendage primordia, the white head arrow denotes the dorsal midline. The yellow arrow denotes posterior end of the FAS-III expression pattern. Solid yellow line denotes the approximate posterior boundary of the dorsal appendage primordia. Cells are outlined in yellow to denote number of boundaries. White star denotes the posterior end. **(A', B')** An enlargement of the area behind the dorsal appendage primordia in *D. nebulosa* and *S. elmoi* respectively. Yellow star denotes cell outlined in A or B.

Discussion

Most developmental stages in flies are external, thus flies had to evolve different strategies to protect the developing embryos/larvae from the environment and at the same time generate a functional organ. In particular, *Drosophila* species that lay eggs on rotten fruits evolved tubular respirators that remain exposed to open air when the main body of the egg is sinking into the fruit [49]. The Hawaiian species, like *D. grimshawi*, have remarkably long dorsal appendages (~3 time the length of the egg) since they lay eggs deep in trees bark [6]. Other flies, like the Mediterranean fruit fly (*Ceratitis capitata*) has no appendages since it lays its eggs into citrus fruits [50].

The Yakoby Lab has been studying the formation of a morphological novelty on eggshells, the dorsal ridge, in numerous species of *Drosophila* [4, 27]. In my thesis, I aimed to characterize a new structure found on *Scaptomyza* eggshells, the respiratory stripe. While not investigating its function in respiration, I aimed to document potential mechanisms underlying the formation of the respiratory stripe in these species.

Morphological differences of eggshells among species

The limited information available on the *Scaptomyza* eggshells suggests that these species began to lose their dorsal appendages in favor of respiratory filaments along the dorsal side of the eggshell [46]. Here, I present two *Scaptomyza* species with shortened dorsal appendages and a respiratory stripe along their dorsal side. The dorsal appendages were first described as being elongated tubes to allow for gas exchange when the eggs sunk in rotting fruit [49]. Thus, it would seem logical that as these

species switched to herbivory and lost yeast on fruit as their primary food source, they would begin to lose their need for dorsal appendages as well.

The longitudinal grooves along the lateral sides of the *S. elmoi* eggshell are also interesting(Figs. 4C, 5A, C, E). It is possible that these groves could increase the surface area of the eggshell to allow better gas exchange, ventilation, or isolation from the environment. Investigations into other insect eggshells revealed that another cabbage eating fly, *Erioischia brassicae*, describe similar longitudinal grooves along the lateral sides of the eggshell [48]. Perhaps this is a fundamental trait in flies that prefer such vegetation, however, more investigation is required before this claim can be made. It was found that these grooves in *E. brassicae* allowed the eggshell to float horizontally much like a boat or canoe. The same was found to be the case with *S. elmoi* (Fig. 6). Thus, it is possible that the generation of such structures decrease the need for protruding tubes such as the dorsal appendages. These functional suggestions still need to be determined in future studies.

Reduced dorsal anterior patterning correlates with shortened dorsal appendages

Dorsal appendage formation has been studied during *Drosophila* oogenesis [3, 10, 22, 28-30, 36, 38, 40, 42, 51]. BR is a major regulator of dorsal appendages' formation [3, 10, 40, 42]. The gene is expressed dynamically in the follicle cells. These dynamics are regulated by two independent enhancers. The early enhancer (brE) controls the uniform expression of BR throughout the follicular epithelium. This enhancer is repressed in a dome-like shape at the dorsal anterior of the egg chamber at stage 10A of oogenesis. Then, the late enhancer (brL) is controlling BR expression in the same dorsal anterior cleared domain, in two dorsolateral patches on either side of

the dorsal midline [52]. The shape, number of cells, and number of domains of BR reflect the final numbers and size of the dorsal appendages, which is different among species [36].

The dynamics of BR patterning is regulated by the coordinated action of EGFR and BMP signaling [3, 10]. Specifically, EGFR induces the expression of the transcription factor Mirror (MIRR) in the dome shaped dorsal anterior domain. Consequently, brE is repressed in this domain, and the brL is induced in the same domain of MIRR expression [37, 50]. In addition, EGFR induces the transcription factor Midline (MID) that restricts the posterior border of BR expression [37, 53]. At the same time, BMP signaling represses the brL in the anterior domain, thus restricting its anterior border [10, 36].

Thus, as a first step to understanding the changes in dorsal appendages formation, we monitored the expression of BR. As expected, the number of cells expressing BR in the two *Scaptomyza* species is reduced compared to *Drosophila* species (Fig. 8). At the same time, we noticed quantitative and qualitative changes between the two *Scaptomyza* species. Specifically, the number of BR cells in *S. elmoi* was reduced to a few single cells on either side of the reduced dorsal anterior domain, which reflects the two reduced dorsal appendages on its eggshell (Fig. 8B). In *S. anomala*, which has eggshells with 4 dorsal appendages, the number of BR expressing cells is increased, and each lateral domain of BR split to two domains in preparation to form two dorsal appendages on each side of the dorsal midline (Fig. 8C).

Changes in BMP signaling dynamics

BMP signaling begins when the ligand DPP binds to the type I and type II receptors. The type I receptor TKV is dynamically expressed in oogenesis. While early expression of TKV is uniform, in later stages it acquires an asymmetric pattern of two patches on opposite sides of the dorsal midline [10, 36, 54]. It was also shown that the late expression of TKV is regulated by BR [10]. The diversity of dorsal appendages has been attributed, in part, to the patterning of TKV. It was found that the early expression is conserved across species, however, the later stages of TKV expression could be placed into four classes of patterning [36]. The first class contains TKV expression as seen in *D. melanogaster* which overlaps the cells expressing BR (roof cells). In *D. nebulosa* and other species from this class, it was found that TKV overlaps the BR and also extends into the anterior domain (roof). In species with three dorsal appendages, such as *D. guttifera*, TKV expression is absent from the single middle BR patch found on the egg chamber of these species. The last class contains species such as *D. virilis*. Here, TKV expression remained uniform throughout the follicle cells [36].

In *Scaptomyza*, it was found that early dynamics of BMP signaling were conserved by monitoring P-MAD (Fig 7D, G). During the later stages, both *S. elmoi* and *S. anomala* seem to follow the patterning rules of *D. melanogaster* where P-MAD and BR is present. Such expression, if collected in more species, can also be used to investigate evolutionary relationships as was previously done with TKV in multiple *Drosophila* species [36].

Changes in EGFR signaling dynamics

The importance EGFR signaling in pattering the eggshell has been studied in *Drosophila* species, [2, 10, 27, 28, 30, 39, 51, 55]. In *D. melanogaster*, it was found that not only is GRK required for setting the AP and DV axes, but also activation of genes responsible for patterning the eggshell including BR and RHO which are responsible for dorsal appendage formation [32, 40, 52]. The number of dorsal appendages was found to be correlated with the number of activated EGFR domains [2]. In the *Scaptomyza* species examined here, a reduced activation of EGFR is seen in which correlates with the reduced dorsal appendage phenotype exhibited on their eggshells.

Distributions of GRK were also found to be correlated with the presence and length of the dorsal ridge [27]. For example, *D. cardini* is a species with a dorsal ridge extending all the way to the posterior. It was found that its GRK protein is present in 81% to the posterior end and that *D. melanogaster*, a non-dorsal ridge species, had GRK protein extending only 51% towards the posterior end [27]. While a GRK antibody is currently unavailable for *D. nebulosa* and *S. elmoi* we hypothesize that a similar GRK pattern exists in these species due to similarities in EGFR activation. The question remains as to how EGFR is responsible for generation of different structures on the *Drosophilidae* eggshell such as the dorsal ridge and respiratory stripe. One possibility is that the presence of other signaling pathways may crosstalk or co-regulate the generation these structures, much like EGFR and BMP co-regulate dorsal appendage formation.

Fasiclin-III expression suggests changes in morphogenetic processes

Fasiclin-III (FAS-III) was previously shown to be an operculum marker in *D. melanogaster* [43]. It was later found to also be expressed in the future dorsal ridge domain [4]. Here, FAS-III is expressed in the future operculum and respiratory stripe domains (Fig 11B, B'). Interestingly, while examining the cell shapes of future respiratory stripe domain, they demonstrated deviation from the normal distribution of hexagonal cells seen in this domain in *D. melanogaster* and in species with a dorsal ridge and thus we are lead to believe that changes exist in morphogenesis.

Such changes have been investigated in the *Drosophila* embryo [56-58]. In the generation of the pharynx of the *Drosophila* embryo, cells change their topology or shape as they undergo elongation. In this case, it was found that the EGFR ligand Spitz was in part responsible for the changes in cell shape and that those that acquired a square shape did not intermingle with the surrounding cells [58]. Furthermore, the shape becomes more irregular as cells undergo intercalation as seen in the embryo and wing [57, 59]. Thus, future studies will be needed to investigate the morphogenesis events responsible for the dorsal ridge and respiratory stripe as done for the dorsal appendages [60, 61].

Concluding statements

I have shown that the eggshells of *Scaptomyza* species are unique in that they possess shortened dorsal appendages and a morphological novelty, the respiratory stripe. This structure differs from a dorsal ridge by its height and its interpillar matrix. I have demonstrated that what is known about dorsal appendage and dorsal structure

formation holds true in *Scaptomyza* species. While signaling and patterning can be correlated to morphologies, more understanding of how cell signaling in combination with tissue patterning control the generation of morphologies needs to be investigated.

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