Friedrich-Alexander-Universität Erlangen-Nürnberg

Department Biologie
Abteilung Entwicklungsbiologie
Dr. Ralph Rübsam

Telefon (Büro): +49 9131 85-28069
Telefon (Labor): +49 9131 85-28096
Telefax: +49 9131 85-28040
E-Mail: rruebsam@biologie.uni-erlangen.de

Referee report on the master thesis: „Role of Jun and Fos in oogenesis of the beetle *Tribolium castaneum*" by Vlastimil Smykal, University of South Bohemia, Ceske Budejovice.

Only for a short time functional genetic analyses in insects are no more restricted to *Drosophila*. This has been mainly due to two new developments in the field of insect genetics: the rise of the beetle *Tribolium castaneum* as a genetic and developmental model system and – in parallel – the establishment of systemic RNAi as key technique to efficiently knock down specific gene functions. In his thesis, Vlastimil Smykal made use of these new opportunities. Against the background of functional data yielded in *Drosophila* he asked for the role of the Jun N-terminal Kinase (JNK) pathway during *Tribolium* development, namely during oogenesis. Mr. Smykal clearly formulated the goals of his work in three statements:

1. Cloning the *Tribolium* genes encoding JNK, Jun, Fos
2. Knocking down of JNK, Jun, Fos using systemic RNAi and determining their requirement for *Tribolium* development
3. Analysing the roles of JNK, Jun, Fos in *Tribolium* oogenesis

The thesis` introduction points out the motivation to study oogenesis in *Tribolium* and introduces *Tribolium* as a more basic insect model system compared to *Drosophila*. In addition to transgenesis assays feasible in *Tribolium*, Mr. Smykal correctly explains the key functional technique used in this thesis: systemic RNAi – a technique that is not working in *Drosophila* but is used with great success in *Tribolium*. Next he briefly familiarised the reader with insect oogenesis in order to continue with contrasting the well understood polytrophic meroistic ovary of *Drosophila* to the telotrophic meroistic ovary found in *Tribolium*. This paragraphs needs some minor corrections: page 5, line 2: trophocytes remain in the **anterior** part of the tropharium, not in the posterior part; page 5, line 4: the nutritive cord represents the elongated anterior part of the oocyte, not the intercellular bridge joining it to the nurse cells; page 6, line 10/11: follicle formation in *Drosophila* (and *per definitionem* in all insect ovarioles) is already completed in the germarium (not at the apex of the vitellarium as stated); page 6, line 21: the eggshell's operculum serves to allow hatching of the larva - sperm enters via the micropyle!

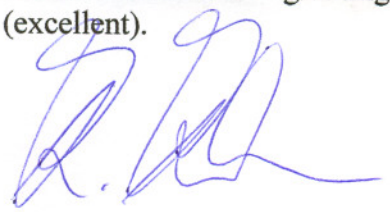
The closing paragraph of the introduction deals with MAP-Kinase cascades, namely the Jun N-terminal Kinase (JNK) pathway and the developmental functions of this pathway in *Drosophila*, among others in oogenesis and in triggering apoptosis as the JNK pathway was shown to activate the cell death effector genes which elicit caspase-mediated apoptosis. In *Drosophila* oogenesis, JNK pathway function was shown to be required in different contexts: stimulating follicle cell divisions prior S6, for migration and differentiation of anterior dorsal follicle cells in mid-oogenesis, and for triggering apoptosis/dumping of nurse cells in late oogenesis.

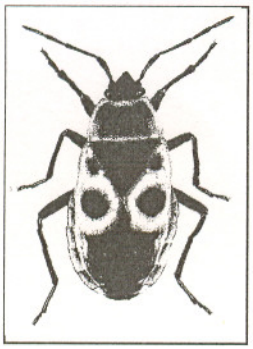
The result paragraph describes the cloning of the *Tribolium jun*, *fos* und *JNK*-orthologs. In comparision with *Drosophila* there were on the one hand some evident sequence variations but on the other hand clear sequence homologies in domains known to be crucial for specific protein functions. Mr. Smykal correctly concluded that all three genes cloned represent *bona fide Tc*-orthologs, each encoded by a single gene. Expression of *jun*, *fos* and *JNK* was determined via RT-PCR throughout development, including dissected ovaries. In ovaries, *jun* and *fos* levels were found to be constantly high whereas *JNK* basal ovarian expression levels were low (however no data on JNK expression in rest of animal!?). Unfortunately the expression analyses were not corroborated by *in situ* – hybridizations. Future work should aim at establishing expression of the genes in question in ovarian tissue! To perform functional analysis, RNAi experiments were added: all dsRNA injections were into one week old adult females. In order to continue the analysis, I strongly recommend additional injections into pre-adult females (pupae, larvae). This would be extremely interesting as a major difference between *Tribolium* und *Drosophila* oogenesis lies in the pre-adult development of the ovary! *jun*- and *fos*- RNAi assays yielded a clearly overlapping phenotype: follicle cell apoptosis starting in mid-oogenesis (convincingly shown by TUNEL-assay, nuclear morphology/chromatin fragmentation (DAPI), breakdown of actin cytoskeleton), suggesting that also in *Tribolium* Jun and Fos proteins cooperate in forming heterodimers and acting together as the canonical Activator protein-1 (AP-1). Loss of *jun* and *fos* function resulting in premature follicle cell apoptosis is an interesting new effect, not known from analysis of the respective *Drosophila* mutants. PH3 staining and BrdU incorporation experiments proved normal follicle cell division activity in *jun*- and *fos*- RNAi ovaries. A very nice complementary set of experiments could demonstrate that apoptosis due to loss of *Tc-jun* and *Tc- fos* requires caspase activity. Vlastimil Smykal performed co-injections of *Tc-ICE* (Tribolium ortholog of effector Caspase-3) dsRNA with *Tc-jun* and *Tc-fos* dsRNAs, respectively. As a result, *Tc-ICE* RNAi restored follicle cell development in *Tc-fos* and *Tc-jun* RNAi ovaries through mid-oogenesis. Future experiments should clarify which later effects of *Tc-jun*, *Tc-fos* or *Tc-ICE* RNAi still abolish the production of proper eggs! Very interstingly, *JNK*-RNAi had no effect on egg laying but on embryonic development (high number of dying embryos), suggesting that activation of *jun* and *fos* during *Tribolium* oogenesis happens via some other kinase.

The discussion considered the relevant literature and gives a sound evaluation of the results. It adds results from another experiment, not mentioned in the results paragraph (why?). *EcR*-RNAi injections lead to a block of oocyte growth. Coinjection of *Tc-jun* dsRNA or *Tc-fos* dsRNA with *Tc-EcR* dsRNA also blocked follicle cell apoptosis. What is the final fate of arrested follicles in *Tc-EcR* -RNAi females?

A preliminary model for *Tc-jun* and *Tc-fos* function is presented postulating that mechanical stress resulting from oocyte growth activates a pathway where the AP-1 transcription factors Jun and Fos ensure cytoskeletal dynamics necessary for epithelial cell shape changes and hence, follicle cell survival. Are there alternative modes of explanation? Does it have to be mechanical stress resulting from oocyte growth?

Vlastimil Smykal's master thesis is clearly and fluently written; it is a pleasure to read it. A minor point of criticism tends to some figures: they are too small and too dark to illustrate the statements made (e.g. Fig. 15A; Fig. 9). Make new, more convincing figures before publishing the work! The results add valuable new data on *Tribolium* oogenesis and encourage to pursue analysis of JNK signalling in *Tribolium* oogenesis and other developmental processes in the beetle. I recommend granting Vlastimil Smykal Msc based on his thesis; I grade this thesis 1 (excellent).





Dr Michal Žurovec
Biologické centrum AV ČR
Entomologický ústav
Branišovská 31
370 05 České Budějovice
Tel: 38777 5283
E-mail: zurovec@entu.cas.cz

Č. Budějovice, 30. května 2008

Posudek na magisterskou práci Vlastimila Smýkala: „Role of Jun and Fos in oogenesis of the beetle Tribolium castaneum".

Tato problematika je v současné době velmi atraktivní, neboť je potřeba srovnat dlouhodobé výsledky studia funkce genů na drozofile s jinými hmyzími druhy, v tomto případě s modelovým broukem potemníkem hnědým (*Tribolium castaneum*). Na rozdíl od drozofily má potemník tzv. telotrofické ovarioly a významně se od octomilky liší i uspořádáním počátečního embryonálního vývoje.

Po obsahové stránce se jedná o magisterskou práci vyjímečné kvality. Práce zahrnuje použití velkého množství molekulárně-biologických metod. Hlavním nástrojem studia pro získávání fenotypických odpovědí se stala RNA interference. Autor získal cDNA genů *jun*, *fos* a *JNK* pomocí RT-PCR, z ní pak připravil dvojřetězcovou RNA, kterou injikoval do samic potemníka a analyzoval fenotypické odpovědi na oocytech těchto brouků. Autor zjistil, že odstranění transkriptů genů *jun* a *fos* poškozuje oogenezi a dochází k degeneraci folikulárních buněk, což se u drozofily nenastává. Pro vysvětlení tohoto jevu autor navrhuje hypotézu, podle které jsou produkty genů *jun* a *fos* potřebné pro odolnost vůči mechanickému stresu, způsobenému expanzí rostoucího oocytu.

Co se týče formální stránky - předložená práce je psaná anglicky obsahuje 42 stran včetně obrázků a přehledu literatury. Práce je psána výbornou angličtinou s minimálním množstvím překlepů. Úvod informuje ucelenou formou o hlavních problémech zvolené problematiky, Cíle práce jsou jasně definovány. Výsledky obsahují obrázky většinou velmi dobré kvality přímo v textu, což usnadňuje orientaci. Kapitola Materiál a metody se nachazí poněkud nezvykle na konci, což ale nevadí. Po formální stránce by práci významně obohatil seznam zkratek jež by usnadnil orientaci ve zkratkami nabitém textu.

K autorovi mám jen několik dotazů a připomínek:

1) Strana 8 – Autor se zmiňuje, že u drozofily existují 3 MAP kinázové kaskády. Ve skutečnosti existují celkem 4, neboť v roce 1998 byl korejským týmem nalezen druhý homolog kinázy p38 a byl nazván p38b (má s dříve popsaným p38a 75 % identity na úrovni aminokyselin).

2) Strana 14 - Autor prověřil pomocí semikvantitativní PCR potlačení exprese *Tc-jun* a *Tc-fos*, avšak potlačení exprese *Tc-JNK* ukázána není. Je možné, že RNAi knockout tohoto genu fungoval méně?

3) Strana 15, obrázek 8 znázorňuje výsledky blokování ovipozice. V obrázku nejsou směrodatné odchylky, hlavně ale chybí údaje o počtu injikovaných samic (tyto údaje pak zcela chybí i v následujících experimentech).

4) Strana 23 - Použití RNAi proti ekdyzonovému receptoru pro blokování růstu oocytů a tím snížení mechanického stresu vedoucímu k apoptóze je problematické v důsledku účinků ekdyzon-respontivních genů na kontrolu apoptózy v oocytech, jak ukázali na drozofile např. Terashima a Bownes (2006).

5) Strana 28 – v Materiálu a metodách by měly byt uvedeny čísla použitých sekvencí z databaze potemníka.

Celkově hodnotím práci velmi kladně. Autor zvládl celou řadu moderních a zajímavých metod buněčné a molekulární biologie a získal důležité a publikovatelné výsledky. Práce splňuje veškeré požadavky na magisterské práce a doporučuji k obhajobě. Navrhují známku výborně.

Michal Žurovec