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## Communication

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# Caffeine effects on AdoR mRNA expression in *Drosophila melanogaster*

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**Abstract:** In this study, we aimed to evaluate whether exposure to caffeine in the early stages of development affect AdoR mRNA expression levels in the fruit fly (*Drosophila melanogaster*) and how this will relate to the developmental success of flies. Adenosine receptors are seen as the most important biochemical targets of caffeine. Simultaneously adenosine signaling orchestrates the development and growth of insects. We demonstrate that AdoR mRNA expression in *D. melanogaster* is persistent from early stages till imago. Strong alterations in AdoR expression were observed in larvae that had been treated with caffeine. However, after the imaginal molt, the differences in AdoR expression between the insects from all of the test groups evened out despite a wide range of developmental success in the groups. Taken together, these results suggest that caffeine affects the expression of its cellular targets even from the early stages of fruit fly development and thus there is a significantly lower larva-to-adult survival rate. Moreover, we also proved that the expression of AdoR undergoes a peculiar reset during the maturation of *D. melanogaster* despite the conditions in which larvae developed.

**Keywords:** methylxanthines, insect development, adenosine receptor

## 1 Introduction

Caffeine is one of the most interesting bioactive compounds from the methylxanthines family. There are numerous reports claiming that caffeine can alter the physiology and behavior of both insects and mammals [1–3]. The effects of the administration of caffeine in animals range from a modulation of the metabolic rate [4,5] and locomotor activity [6–8] to alterations in learning capability [9,10]. Many researches have indicated that caffeine mainly interacts with the adenosine receptors (AdoR) [7,8,11–14]. However, most papers that explore the relationship between caffeine and AdoR are derived from experiments conducted on vertebrate models although this interaction may differ in invertebrates [15] and still remain unclear [1]. Moreover, there have been reports suggesting additional potential targets for caffeine in a cell, i.e. phosphodiesterase (PDE), adenosine deaminase (ADA), ryanodine receptor (RyR) and acetylcholinesterase (AChE) [1]. To date, four types of AdoR in mammals have been discovered (A1, A2A, A2B, A3) [16]. The *D. melanogaster* has only one adenosine receptor (DmAdoR) and it is homologous to the mammalian A2B adenosine receptor [15]. AdoR belongs to the G-protein-coupled receptors (GPCR) family and is responsible for the regulation of the level/intensity of metabolism in a cell [17]. Activation of insect AdoR triggers cAMP synthesis, which subsequently activates fewer instances of regulation in the cell [18]. AdoR is mainly present in the neuronal system of the insect; however, it is also strongly expressed in the digestive and trachea systems during larval development. Overall, AdoR expression during larval development is almost four-fold higher in comparison with the imago [19]. Such an expression pattern suggests that AdoR may play a significant role in the energy management of an intensively consuming and growing organism. Thus, the larvicidal action of caffeine may be multimodal – affecting either the neural or metabolic systems, disrupting feeding and the development of insect on multiple levels. Effective doses of caffeine possible

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disturb cell homeostasis and metabolism regulation [20]. Additionally, caffeine in higher doses is more toxic to larvae than to the imago, as it efficiently inhibits growth and increases mortality rates in the larval stages [21]. Such effectiveness of caffeine against larvae raises the question of whether its mechanism of action involves an interaction with AdoR. It is known that caffeine exposure leads to alterations in the expression of genes encoding the receptor proteins in various models [22–24] including the down-regulation of dopamine receptor (DDA1) gene expression in *D. melanogaster* [25]. Nonetheless, there is currently no data available about AdoR mRNA expression after caffeine treatment in *D. melanogaster* during the whole development cycle (or any other insect model). It is highly possible that caffeine exposure may alter AdoR expression in cells, for example, as part of an adaptation mechanism that involves decreasing or increasing the AdoR density in the cell membrane. The aim of this study was to investigate the influence of caffeine at different concentrations on AdoR mRNA expression in *Drosophila melanogaster* larvae at different time points.

## 2 Materials and methods

Fly stock consisting of the wild-type Canton strain of *Drosophila melanogaster* was obtained from the Department of Genetics at the University of Silesia. The flies were reared at  $23 \pm 2^\circ\text{C}$ , photo phase 12:12 light to dark in Petri dishes filled with a standard culture medium [26]. Prior to the experiment, adult flies were transferred to fresh culture containers and allowed to breed freely for 48 h after which the adults were removed and the newly hatched larvae were transferred to Petri dishes containing different caffeine (Ascent Scientific) concentrations: 0, 0.125, 0.5, 2  $\text{mg} \cdot \text{mL}^{-1}$ . This procedure was applied in order to prevent any inhibitory effects of caffeine on *D. melanogaster* egg hatching [27]. Two hundred larvae per concentration (50 per dish) were left undisturbed for the evaluation of developmental success. Simultaneously two hundred larvae per concentration (50 per dish) were reared for the mRNA expression assays. Insects were collected every 12, 24, 48 h and sacrificed by freezing at  $-70^\circ\text{C}$  (10 insects per dish/time point, four repetition per concentration). Imago were collected as soon as possible after molting (up to one hour after emerging from pupae).

Larvae at an appropriate time point and adults after exposure were frozen and stored at  $-70^\circ\text{C}$  until RNA extraction. The frozen insect samples were homogenized using the FastPrep-24 System with Lysing Matrix A (MP Biomedicals, USA). Total RNA was isolated using a Qiazol®

reagent (Qiagen, Germany) following the recommended procedures. Genomic DNA was removed from RNA through DNase I digestion following the protocol of the DNase I recombinant, RNase-free (Roche, Switzerland). The integrity of the RNA was checked on a RNA Nano Chip (Perlan Technologies, USA). The concentrations and quality of RNA were also measured using Nanodrop ND-2000 (Thermo Scientific, USA). The 260/280 nm absorbance ratios of all of the RNA samples were between 1.8 and 2.2. First strand cDNA was synthesized from 1,558 ng total RNA using a DyNamo cDNA Synthesis Kit (Thermo Scientific, USA). The primers that were used for the quantitative real-time RT-PCR (qRT-PCR) were from Life Technology (USA) – AdoR (Dm02146422\_m1) as the target gene and RpL32 (Dm02151827\_g1) as the reference gene. QPCR was performed in 96-well PCR plates using a LightCycler® 480 (Roche, Switzerland) using the following cycling conditions:  $50^\circ\text{C}$  for 2 min,  $95^\circ\text{C}$  for 10 min and 40 cycles at  $95^\circ\text{C}$  for 15 s and  $60^\circ\text{C}$  for 1 min. The real-time PCR reaction mix (final volume 20  $\mu\text{L}$ ) contained 10  $\mu\text{L}$  2X master mix (DyNamo Flash Probe qPCR Kit, Thermo Scientific, USA), 1  $\mu\text{L}$  cDNA (cDNA final concentration – 20 ng per reaction), 1  $\mu\text{L}$  primer and 8  $\mu\text{L}$  nuclease free water. For analyses of gene expression, Relative Quantification method with calibrator normalized ( $\Delta\Delta\text{Cp}$  algorithm) was used. As calibrator mix RNA (transcribed into cDNA) collected from 10 individuals (12 h larvae) of control group.

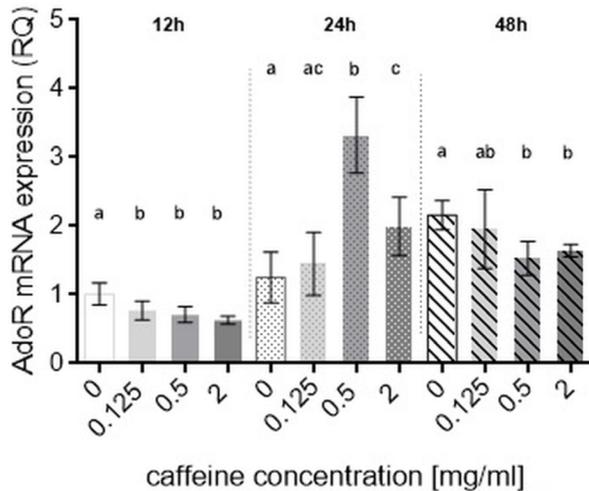
The number of flies that emerged from the pupae was assessed once per day. The cumulative larva-to-adult rate was calculated and analyzed using the Pearson test. Statistical analysis was conducted using Statistica software v12; the one-way ANOVA with Tukey *post hoc* test ( $p < 0.05$ ).

## 3 Results

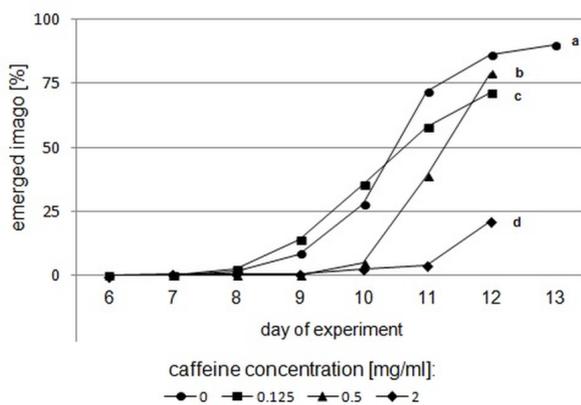
Analysis of the baseline AdoR gene expression in the control group indicated an increase in expression over time. A comparable trend could be observed in the group that had been exposed to a  $0.125 \text{ mg} \cdot \text{mL}^{-1}$  caffeine concentration; thus, we assume that this concentration showed the lowest effectiveness. Evident alterations in AdoR mRNA expression in comparison to the untreated group were observed in the groups that had been treated with higher concentrations – 0.5 and  $2 \text{ mg} \cdot \text{mL}^{-1}$ . Moreover, these concentrations caused a dissimilar (in comparison to lower ones) effect on the temporal pattern of expression. In contrast to the steady increase of mRNA level observed over time for 0 and  $0.125 \text{ mg} \cdot \text{mL}^{-1}$ , the expression pattern

for 0.5 and 2 mg · mL<sup>-1</sup> formed the shape of an inverted U curve with a peak at the 24 h time point. The strongest up-regulation of AdoR expression was observed for 0.5 mg · mL<sup>-1</sup> caffeine concentration at the 24 h time point; however, all of the caffeine concentrations caused down-regulation of the AdoR expression at the other time points.

The larvae-to-adult survival rate decreased significantly with increasing concentrations of caffeine (Fig. 2). In the control group almost 100% of the larvae reached the imago form. The curve representing the emergence of adult flies over time in the group treated with 0.125 mg · mL<sup>-1</sup> caffeine concentration significantly



**Fig. 1.** Adenosine receptor mRNA expression in *D. melanogaster* larvae (mean ± SD). Expression level presented for three time points of caffeine treatment (12 h, 24 h, 48 h), control group (0 mg · mL<sup>-1</sup>) and three different concentrations of caffeine (0.125, 0.5, 2 mg · mL<sup>-1</sup>). Various letters indicate statistically different groups. ANOVA test,  $p < 0.05$ ,  $n = 4$



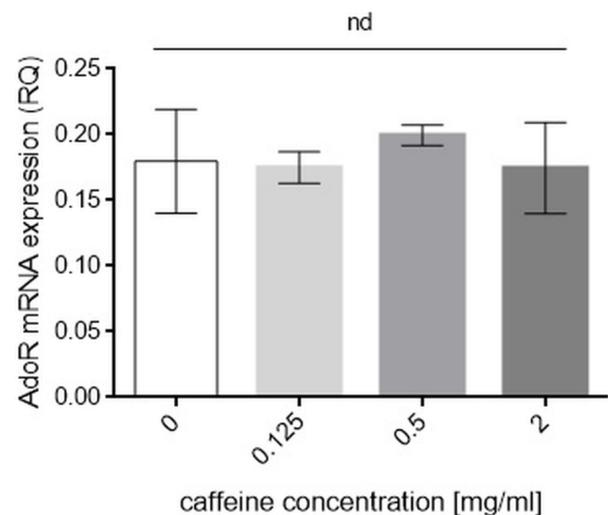
**Fig. 2.** Percentage of larva-to-adult survival for *D. melanogaster* in time. The control and different concentrations of caffeine groups are presented. Various letters indicate statistically different curves, Pearson test was used.

overlaps the one representing the untreated group; however, only 75% of the larvae successfully reached the imago form. The 0.5 and 2 mg · mL<sup>-1</sup> concentrations of caffeine affected the expressions in the larvae to adult the most. In both groups that were treated with the highest caffeine concentrations, the imagos began to emerge two days after those in the control group (development time increased by 30%) and for 2 mg · mL<sup>-1</sup> the number of adult flies that emerged was significantly lower. As is shown in Fig. 1, there was a clear difference between the 0, 0.125 and 0.5, 2 mg · mL<sup>-1</sup> concentrations, which had a much greater efficiency.

The evaluation of AdoR mRNA expression was conducted on emerged imagos, as can be seen from Fig. 3, did not reveal any significant differences between the analyzed groups.

## 4 Discussion

Caffeine is regarded as a natural pesticide of a plant origin [28] that is especially effective against insects undergoing development. In the conducted experiments, caffeine was indeed very effective in disturbing the development of *D. melanogaster*. A significant decrease of the larva to imago rate and an increased time of development was observed, which is consistent with the literature data [21,29,30]. Currently, the AdoR antagonism is perceived as the main route of the action of caffeine [13,31] and only one adenosine receptor isoform (sequence) is known in *D. melanogaster*. Therefore, this receptor was investigated in the presented research [31,32]. The main adaptation



**Fig. 3.** Adenosine receptor mRNA expression in *D. melanogaster* imagos (mean ± SD) for the control group and three different concentrations of caffeine (0.125, 0.5, 2 mg · mL<sup>-1</sup>). ANOVA test,  $p < 0.05$ ,  $n = 4$ .

mechanism to chronic treatment with a compound acting as a receptor antagonist is the up-regulation of the gene expression of the affected receptor [33]. In the control group, a continuous increase of AdoR expression was observed during development. This result suggests the important roles of adenosine and AdoR for the developing larvae [34].

The concentrations used in the present study caused effects that are in agreement with the data from other reports investigating the effects of caffeine on insect development [25,30,35]. Exposure to caffeine in concentrations of up to  $1 \text{ mg} \cdot \text{mL}^{-1}$  slows development and causes moderate toxic effects, whereas higher ones are much more toxic. We showed that the temporal pattern of AdoR expression during chronic treatment varies depending on the caffeine concentration.

The strongest effects were observed 24 h after beginning the caffeine treatment, at which time, after the initial (in 12 h time point) down-regulation, AdoR expression was highly up-regulated. We suppose that such a shape of the temporal pattern of expression may reflect the varying role of adenosine signaling in the subsequent phases of development. The concentration that appears to be the most effective is 0.5 mg as it caused the greatest differences in comparison with the untreated group. We consider it to be optimal for interacting with a purinoergic (or other) system.

There is lack of any precise information about the effects of caffeine (or any other methylxanthine) on AdoR gene expression in insects, however, the topic was briefly covered in rodent models. Dixon et al. (1996) proved an elevated expression of A1 mRNA after caffeine and theophylline treatment in pregnant female rats, in both the mothers and fetuses. However, the same research reported no effects on A2B receptor expression, which is considered to be homologous in both the structure and function to one in *D. melanogaster* [36].

Considering the broader effects of methylxanthines, adenosine A1 receptor antagonists [37] increase the extracellular adenosine levels in the cardiovascular cells in rats and humans, which suggests that the adenosine A1 receptor strictly modulates the concentration of extracellular adenosine. At the same time, there are data that prove that long-term treatment with caffeine (in a dose sufficient to cause an observable tolerance) can increase the number of A1 receptors without causing any change in the A1 mRNA levels. Such data indicates that adaptive changes may occur at a post-translational level [38].

Available data, suggest that exposition to disrupting factors during the development alters gene expression

levels in imago [39,40]. So that, initially, we suspected that the AdoR level of imagos would be permanently changed by chronic caffeine treatment of larvae, but this was not the case. Although we observed low, stable expression of AdoR in the adult specimens from all of the groups, despite the caffeine concentration in the rearing medium. Such results may lead to the conclusion that during pupation, *D. melanogaster* undergoes a reset of the adenosine signalling system. To the best of our knowledge, such a phenomenon has never been described before. Another explanation may be that AdoR expression in the imago stage is fixed [35] or changes can only be observed in the protein density, which would be the same as in case of the A1 receptor in rats [38]. There are no other data about the AdoR level or mRNA in the imago body after caffeine treatment of larvae.

The obtained results indicate that caffeine, even in the lowest concentration, significantly affected the AdoR expression and reduce the maturation success in *D. melanogaster*. Although the observed alterations in AdoR expression may originate from a simple interaction between caffeine molecules and AdoRs, the vast plurality of other targets may take part in the process. Caffeine is known to activate RyRs and triggering the outflow of  $\text{Ca}^{2+}$  ions [31]. High concentrations or chronic exposure to caffeine may inhibit the activity of adenosine deaminase (ADA) [41] as well as cAMP phosphodiesterase (cAMP PDE) [42]. Maintaining a stable, homeostatic adenosine level in the extracellular space is crucial for *D. melanogaster* development [20,34]. It has been proven that prolonged exposure to caffeine can lead to an increase in the adenosine concentration [12,38]. A high concentration of adenosine in hemolymph can lead to the degeneration of the fat body, hemocyte apoptosis and imaginal disc deformation [20]. Therefore, long-term ADA inhibition through persistent exposure to caffeine may cause a deregulation of the adenosine metabolism, which could disrupt the cell metabolism and subsequently could affect AdoR expression [12,43].

Moreover the metabolites of caffeine (theophylline, theobromine, paraxanthine) are also highly bioactive compounds that are capable of causing further effects [44]. This is especially relevant regarding theophylline, which is known to have a higher degree of affinity to AdoR in comparison to caffeine and its other metabolites [32].

The lack of comprehensive data on the influence of methylxanthines on a developing organism creates a broad field for extended investigations. Further research should evaluate the concentrations of the AdoR protein and the expression of other instances that are susceptible to caffeine mentioned earlier.

## 5 Conclusions

We proved that the caffeine treatment does significantly change the expression of AdoR in fruit fly larvae, and whether it is up-regulation or down-regulation is dependent on the concentration and time. The temporal patterns of the alteration of the expression diverged among the groups that had been treated with different concentrations. The main mechanism behind the alterations in the AdoR mRNA level in response to caffeine remains unclear due to the diversity of targets in the adenosine signaling pathway (and further) that caffeine can affect as well as the complicated feedback loops. Nonetheless, accordingly to literature data, we assume that the growth inhibition and lowered maturation success that were observed are probably the results of a disruption of energy management caused by caffeine. We report that adult insects, despite the conditions in which the larvae developed, had no changes in the AdoR expression after imaginal molt, which can suggest the occurrence of either a fixed level of the AdoR expression in *D. melanogaster* adults or a reset of the AdoR expression that has not previously been described.

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**Conflict of Interest:** The authors declare that they have no conflict of interest.

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