An epigenetic change in a moth is generated by temperature and transmitted to many subsequent generations mediated by RNA

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An epigenetic change in a moth is generated by temperature and transmitted to many subsequent generations mediated by RNA

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Running head: RNA mediated paramutation of insect

Key words: phenotype, *Ephestia kuehniella*, paramutation, epigenetic, RNA, heat shock proteins

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Abstract

Epigenetic changes in sexually reproducing animals are transmitted only in a few generations. We have discovered a case where epigenetic change lasts tens of generations. This epigenetic phenomenon occurs in the short antennae (sa) mutation of the flour moth (Ephestia kuehniella). Based on our tests, we demonstrate that the epigenetic phenomenon is probably determined by a small RNA (maybe piRNA, miRNA, tsRNA) and transmitted in this way to subsequent generations through the male and female gametes. The observed epigenetic phenomenon cancels the mutation and creates a wild phenotype. It persists for many generations (40 so far). The epigenetic effect of the flour moth is induced by changes in ontogenetic development, such as increased temperature on pupae development, little nutritious food, different salts in food, or injection of RNA from the sperm of already affected individuals into the eggs. The epigenetic effect may occasionally disappear in some individuals and/or progeny of a pair in the generation chain in which the effect transfers. We consider that the survival of RNA over many generations has practical implications. It is mainly a response to environmental change that is transmitted to offspring via RNA. Such transfer of RNA to subsequent generations may have a greater evolutionary significance than previously thought. Based on some analogies, we also speculate that there is a connection with the SIR2 gene.

Author summary

The inheritance of some traits is controlled to a large extent through RNA rather than DNA, and this is still a major challenge for research. We present findings that this can be caused by environmental factors at the stage of the individual's development, such as temperature or food composition. We consider the most interesting fact that the influence through RNA can persist for tens of generations, but still is rare, with some regularities, it can disappear. Should this be another mean of organisms for adaptive evolution?

Introduction

The epigenetic inheritance seems to be due to the heritable alterations without any change in the sequence of the DNA while the epigenetic information is frequently erased at the start of each new generation. However, in some cases, epigenetic information can be transmitted from parent to progeny (multigenerational epigenetic inheritance) [1]. Exposure to the environmental stressors can induce various epigenetic changes (epimodifications) in mammalian germ cells, which can influence the
developmental trajectory of the offspring across generations [2,3]. The acquired characteristics, can occur through ancestral exposures or experiences and certain paternally acquired traits can be 'memorized' in the sperm as epigenetic information [2].

In our previous study Pavelka and Koudelová [4], a specific phenomenon of phenotypic inheritance (paramutation, epigenetic heredity), which can be studied in the short antennae (sa) morphological mutation of the Mediterranean flour moth [Ephestia kuehniella Zeller (Lepidoptera: Pyralidae)], was assessed for the first time. In the case of the sa paramutation, the transmission to subsequent generations occurs through the male as well as the female. Moreover, the phenomenon reported in this manuscript has other aspects: 1) it is triggered by a change in the ambient environment (e.g., temperature, LiCl in food); and 2) under certain conditions, it rarely returns to the original state. The sa mutation is inherited as a simple autosomal recessive gene and causes considerable shortening of antennae in moths of both sexes At higher temperature, the antennae of sa moths revert to the wild-type (genotype remains the same) which is then transmitted to subsequent generations [4]. In summary, the mutation of short antennae (sa) at high temperature conversions to the long antennae (sa WT) moth. This phenotypical difference appears in future generations even at low temperature. The reversion is relative rare, however when the offspring are reverted, they continue with the sa mutation (short antennae) at low temperature.

Although small, transcriptionally inert, sperm cell with extremely compacted genome and virtually no cytoplasm; contains a plethora of small RNAs anda large number of DNA sequences packaged by histones and a distinctive DNA methylation profile [5]. Furthermore, dysregulation of at least two different microRNAs (miR-1 and miR-124) in sperm and their transmission to the egg have been postulated to be the causes of two cases of intergenerational inheritance in mouse [6,7].

It is still an unanswered question what exactly causes the transmission of epigenetic information to offspring. There are several possibilities: miRNA, tRNA, piRNA, possible methylation, or some other effects. Gapp et al. [8] described that the traumatic stress in early life modified mouse microRNA (miRNA) expression and behavioral and metabolic responses in the progeny.

The tRNA-derived RNA fragments (tRFs, mse-tsRNA) are sometimes responsible for the epigenetic inheritance. The tRFs are the most abundant class of RNAs in mature sperm [9, 3]. Methylation of the tRNA generated by RNA methyltransferase (Dnmt2) was associated with the epigenetic phenomenon in father to offspring transmission [10]. The traumatic stress in early life can cause upregulation of miRNAs in F1, affection of piRNA in particular cluster in sperm. Additonally, piRNA cluster causes the complex changes in stress-coping behaviors, metabolism and stress-induced glucose release in the offspring [8,3].
The role for DNA-bound proteins in epigenetic inheritance seems to be important. Sperm histones (somatic histones) carry posttranslational modifications. For example, the trithorax mark histone H3 lysine 4 trimethylation (H3K4me3) and the polycomb mark histone H3 lysine 27 trimethylation (H3K27me3) are important for the normal development and the link with the maintenance of transcription patterns [5].

Even though this type of inheritance is possible along both the paternal and maternal lines, we focused only on the paternal line. The paternal inheritance is easier to study [4] because the preparation of sperms is more simple than the non-fertilized eggs.

We suppose that the long-lasting epigenetic effect observed in our experiments proves more simple regulatory mechanism in insect cells in comparison to mammals (see [11,12]). In previous studies [13,14], different epigenetic phenomenon in mammals was assessed. These results were in line with our previous study [4] especially in the transmission of characteristics to subsequent generations.

The phenomenon described by us was special due to the fact that it had been induced by a change in the temperature during the development of individuals. However Rassoulzadegan et al.[13] reported a modification of the mouse Kit gene in the progeny of heterozygotes with the null mutant Kit<sup>tm1Alf</sup> (a lacZ insertion). Wild male mice mated with female mutant (heterozygous mouse Kit<sup>tm1Alf</sup>) have offspring with homozygous wild genes and white spots like the mutant female even though they have no allele for these spots. Homozygous wild-type genotype offspring persist, to a variable extent, the white spots characteristic of Kit mutant animals [13].Large amount of an aberrant RNA is produced from the mutant gene (Kit<sup>tm1Alf</sup>), consequently is accumulated in sperm, and thus is transmitted to the embryo. Presence of the aberrant RNA silences the activity of the Kit wild-type gene then so the animals have white spots, even if they do not carry the corresponding mutant gene [15]. Furthermore, the aberrant RNA transport is similar to the Sox9 gene for maus gigantism [7,10]. However, increased expression in the paramutants was not related to a change in miR-124 expression, but to the establishment of a distinct, heritable chromatin structure in the promoter region of Sox9 [7].

Epigenetic information is frequently disappear at the beginning of each new generation. However, in some cases, epigenetic information can be transmitted from parent to progeny (multigenerational epigenetic inheritance). A particularly notable example of this type of epigenetic inheritance is double-stranded RNA-mediated gene silencing (RNAi) in Caenorhabditis elegans. This phenotype caused by RNAi could be inherited for more than five generations [1]. Additionally the effect of piRNAs in Caenorhabditis elegans could survive to at least 20 generations [16].

Between generations epigenetic inheritance is not rare, for example [17] found over 100 cases of epigenetic inheritance in 42 different species (bacteria, protists, plants, animals). However, the
number of generations with epigenetic characteristics (epigenetic memory) are usually restricted. Rassoulzadegan et al., [13] described that mouse effect of white spots, which is caused by miRNA, disappears after 6 generations. In grape phylloxera *Daktulosphaira vitifoliae* epigenetic memory was observed for 15 generations [17](Forneck et al., 2001), however, in this case it was parthenogenetically inherited transfer. It appears that small RNAs do not function without assistance. Buckley et al. [1] described that the defective HRDE-1, encoded by Argonaute protein, directs gene-silencing events in germ-cell nuclei that drive multigenerational RNAi inheritance and promote long-term resistance of the germ-cell lineage.

In previous study [4] evocation of the epigenetic effect by temperature was described. In this study we focus on the cause of particular epigenetic phenomenon in insects, and their persistence through multiple generations. We assessed the influence of diet and dietary salts on epigenetic effect. We demonstrated transmission to the future generations via RNA. We tested the stability of our epigenetic phenomenon and the frequency of the return to the original phenotype.

**Results**

**Injections**

Experiments with insect eggs injected with different components were described in the material and methods.

Descriptive statistics of percentage of $sa^{WT}$ genotype in imagoes of each moth’s pair by all three different types of interventions are summarized in Table 1. Distribution of percentage of $sa^{WT}$ genotype by eight different additives to foods was shown in Figure 1. Overall, homogeneity of means of percentages of $sa^{WT}$ offsprings among all eight groups are rejected (Kruskal-Wallis ANOVA, $P=0.00052$). Dunn’s post-hoc tests (Table 2) showed several significant pairwise differences. It is always possible to see the differences between different additives, where the total RNA from sperm was found and where the additives were without RNA. the experiment with an RNA additive never statistically differs significantly compared to another RNA additive, and again an additive without RNA does not differ significantly from another additive without RNA. For example, total RNA differs from all additives without RNA, but buffer differs statistically only from total RNA. When four additives with RNA and four without RNA content are combined together, differences between means of percentage of $sa^{WT}$ genotype were highly significant between the two groups (Kruskal-Wallis ANOVA,
It means that injection of total RNA isolated from saWT sperm into fertilized eggs (sa x sa) produced a significantly higher percentage of long-antennae offspring.

In the case of sperm and fractions of sperms, the effect of native RNA was demonstrated. The phenotype was statistically significant in each group, even in the control (eggs that were injected only with buffer). In the experimental groups, statistics showed more significant results especially in the groups injected with RNA.

**Epigenetic memory**

During the monitoring of generations of one-pair cultures, we found out that there is a reversion to beginning mutant phenotype by some individuals in each generation (Fig. 3). We statistically assessed whether the number of individuals returning to the original mutant phenotype significantly differing between the generations. The ratio of reverted individuals does not appear to be significantly different. Tracking progeny was conducted for each generation. For precise evaluation of all specimens, four generation (F2, F3, F5 and F12) were randomly selected. In other generations, including F20, the ratios of reverted individuals were similar.

Number of reverted individuals was usually 0–6% per generation and pair. Rarely, the offspring of some pairs reverted in large number (cca 90%). We have tried to determine the point at which there is a reversion of a significant number of individuals in populations. We analysed number of extremely reversed clutches counted as percentage of reversed individuals higher than non-outlier maximum for whole dataset which is 12.6. There is no change in proportion of these extreme clutches between generations F1, F2, F3, F5 and F12 (chi-square = 2.6, df = 4, p = 0.6).

Numbers of extremely reversed and non-reversed clutches are given in Figure 3. There is no change in proportion of these extreme clutches among F1, F2, F3, F5, and F12 generations (chi-square = 2.6, df = 4, P = 0.64).

Five cultures were observed until 40th generation. The long antennae epigenetic effect continued to F40 generation.

**Medium (the effect of salts and poor nutrition)**

The percentage of phenotype changes (sa on saWT) within four different food medium is shown in Figure 2. Kruskal-Wallis ANOVA indicates that differences among group means were significant (P < 0.001). Dunn’s post-hoc tests further showed that three medium (flour, LiCl, and NaCl) had same proportion of phenotypically altered individuals and all these three groups differ from control.
(individuals that were fed with wheat grains) (P values < 0.001).

**Discussion**

The transmission of epigenetic traits across multiple generations is not rare (see Jablonka and Raz [20]). However the effect of 40 generations in sexually reproducing animals duration is unique. This epigenetic influence was dynamic, not completely stable, and increased the variability of the offspring.

In the case of *E. kuehniella*, our results revealed that epigenetic effect was mediated by RNA and persisted for 20 (or 40) generations. It was possible that epigenetic effect would continue further to next generations. It seemed to be a permanent phenomenon because there was no statistical change in proportion of these extreme clutches among F1, F2, F3, F5, and F12 generations. There was no difference in the percentage of reversed clutches between generations.

During the monitoring of one-pair cultures, we found out that there was a reversion to the original mutant phenotype of some individuals in each generation. Number of reverted individuals was usually 0–6% per generation and pair. Rarely, the offspring of some pairs reverted in the large number (ca 90%). It is probably the longest duration of epigenetic influence which has ever been observed in insect. We demonstrated that the change of phenotype was influenced by total RNA isolated from male sperm.

Unlike to the other components (accessory glands, spermatophore sac, denatured sperm with Ribonuclease) separated from male sperm, carrying the epigenetic effect did not have analogous impact. The total RNA differed from all additives without RNA. The influence of geldanamycin comparable to the RNA injection was even stronger than expected, confirming that the effect was due to the small RNA. Geldanamycin inhibits heat shock protein 90 (Hsp90) [21] which is a molecular chaperone essential for activating many signaling proteins in the eukaryotic cell [22]. The link between small RNA within Argonaute proteins (Argonaute proteins bind different classes of small RNAs) and Hsp90 has been demonstrated (Iwasaki et al., 2010). The loading of siRNA duplexes into *Drosophila* Ago2 requires the Dicer-2–R2D2 heterodimer and the Hsc70/Hsp90 chaperone machinery. In the absence of the chaperone machinery, an siRNA bound to Dicer-2–R2D2 associates with Ago2 only transiently [23].

The influence of other environmental factors on the induction of an epigenetic response is obvious, whether the *E. kuehniella* were fed either with flour or LiCl, NaCl additives. Although we
found statistical differences between LiCl, NaCl, and flour in the $sa^{\text{WT}}$ indication, all three investigated groups differ from the spontaneous onset of $sa^{\text{WT}}$ in the optimal diet (milled wheat grains supplemented with a small amount of dried yeast). Similarly, the effect of injections into the eggs was manifested. However, the injection with buffer only, which served as the control, was able to elicit an epigenetic response to some extent because the injection itself is a major intrusion into the developing egg. (see Tab. 1, 2, Fig. 1). The developing embryo was mostly killed immediately after injection (the ratio of mortality was not recorded). The effect of additives on the increased incidence of $sa^{\text{WT}}$ was observed, post-hoc tests show several differences. The additives with RNA and without RNA always differ in pairs, however two additives with RNA or two additives without RNA never differ which led us to combine additives with RNA and additives without RNA together. Not surprisingly, the difference in $sa^{\text{WT}}$ is highly significant. There is a special effect of geldanamycin which manifested itself similarly to RNA additives.

Many studies involved in epigenetic phenomenon concern DNA methylation. The data about the effect of diet on gene methylation and the release of hidden genetic variation by impairment of Hsp 90-mediated buffering systems offer eloquent examples of how epigenetic mechanisms might affect gene-environment interactions [24]. However the change of methylation to demethylation is usually due to the transition through the male gamete [25,26].

In our previous study [4], the first effect of temperature on E. kuehniella was observed. A similar effect of temperature was observed in Caenorhabditis elegans. A majority of the SynMuv B mutants grown at high temperature were irreversibly arrested at the L1 stage. High temperature arrest (HTA) is accompanied by upregulation of many genes characteristic of the germ line, including genes encoding components of the synaptonemal complex and other meiosis proteins [27]. Unfortunately, the link to small RNAs is unclear. This phenomenon has been documented in mammals [13]. As the RNA is not degraded and continues to act in the cells, there might be a relationship with RNAs generating interference RNA (RNAi) (see [15]. In this process, RNA methyltransferases seem to be also essential. Research in animal models has shown that RNAs can be inherited and that RNA methyltransferases can be important for the transmission and expression of modified phenotypes in the next generation [28,10]. Furthermore, RNA methyltransferases increase the stability of small RNA as cytosine-5 methylation [29,10].

Similar phenomenon conditioned by miRNAs found in lepidopterans has been observed for example in plants where a temperature-dependent epigenetic memory from the time of embryo development expresses in norway spruce. This epigenetic machinery thereafter influences the timing
bud phenology [30].

Nevertheless, reversion of mutant phenotype occurs spontaneously also in the part, or rarely in all the offspring of studied flour moths (Fig.2). We suppose that it means simple diminishing of epigenetic influence on the basis of gradual decrease of small RNAs (maybe piRNA) during ontogeny under particular threshold. So, an individual posses epigenetic trait, but all its tissues contain no small sRNAs causing epigenetic effect any more. It could occur during ontogeny of some individuals, whereas others could retain the same concentration of small sRNAs. Occasionally, in rare cases could some small sRNAs disappear only in the parental tissues where eggs and sperm develop, while the individuals and their other tissues display epigenetic phenotype. The offspring then do not display epigenetic phenotype.

The epigenetic effect in flour moths is maintained even after many generations (our experimental culture has 20 monitored generations and total 40 observed). The piRNAs at *Caenorhabditis elegans* can trigger highly stable long-term silencing lasting at least 20 generations. Once established, this long-term memory becomes independent of the piRNA trigger but remains dependent on the nuclear RNAi/chromatin pathway [16]. Insects benefit more from the pronounced variability of the progeny because they produce much larger numbers of offspring and are physiologically more influenced by environmental conditions than mammals.

Epigenetic memory in mice lasts only six generations [13], in voles is the memory documented only to F3 generation [31]. However, the situation is different in insects. The studied epigenetic trait (developed antennae) is involved in finding of sexual partner (see [4]) and lasts for many generations. It was not observed in mammals. Such long lasting epigenetic effect could be considered potentially adaptive [17,32]. It is possible that epigenetic phenomena could have evolutionary consequences that increase variability in offspring.

Many studies have demonstrated that epigenetic mechanisms, including DNA methylation and histone modification, not only regulate the expression of protein-encoding genes, but also miRNAs [33]. Conversely, another subset of miRNAs controls the expression of important epigenetic regulators, including DNA methyltransferases, histone deacetylases, and polycomb group genes. This complicated network of feedback between miRNAs and epigenetic pathways appears to form an epigenetics-miRNA regulatory circuit, and to organize the whole gene expression profile [33,34].

Our results show that the phenomenon persists for many generations (beyond the 20 monitored). A number of individuals revert to the genetically conditional mutant phenotype. Generations, for which accurate images existed, were processed statistically. There is no change in proportion of these extreme clutches between generations. The number of reverted individuals is 10-
15% and there is no statistical difference of the percentage in between the generations. The studied epigenetic phenomenon effects about 85-90% of the population. The population variability is ensured. This effect is therefore evolutionarily significant. It is possible that switching between states occurs because certain small RNA in the fertilized eggs are lower than the critical concentration required. However, the mechanism can be far more complex.

The most common manifestation of epigenetic change it can be seen in the sirtuin genes. The similarity with our phenomenon is primarily in the long-term inheritance genotype, which is occasionally interrupted for reasons which remain unclear. It is still an unanswered why this mechanism is triggered by a change in temperature. The SIR2 gene, for example, could activate the production of small RNAs. White–opaque switching in the human fungal pathogen *Candida Albicans*, results from the alternation between two distinct types of cells [35,36](. Switching is probably caused by the SIR2 (silent information regulator) gene, which seems to be important for phenotypic switching [37]. Silent Information Regulator (SIR) proteins are involved in regulating gene expression and some SIR family members are conserved from yeast to humans [38].

SIR genes have many functions. Sirtuins are evolutionary conserved NAD(+)-dependent acetyl-lysine deacetylases and ADP ribosyltransferases dual-function enzymes involved in the regulation of metabolism and lifespan [39]. Sirtuins are hypothesized to play a key role in an organism’s response to stresses (such as heat or starvation). A calorie restriction turns on a gene called PNC1, which produces an enzyme that rids cells of nicotinamide, a small molecule similar to vitamin B3 that normally represses Sir2. The gen PNC1 is also stimulated by other mild stressors known to extend yeast lifespan, such as increased temperature or excessive amounts of salt [40].

Based on the similarity, we speculate that SIR proteins in addition to various known functions (e.g. silence genes - see Ralser et al [41]) might also catalyse the formation of small double-stranded RNA, according to damaged genes to be silenced. SIRT regulation is multifaceted, but not yet considered to be associated with RNA. We present the hypothesis that insects can initiate the creation of an RNA against harmful genes.

SIR genes linked with small RNAs have been reported. It was shown that small interfering RNA-mediated SIRT7 knockdown leads to reduced levels of RNA Pol I protein, but not messenger RNA, which was confirmed in diverse cell types [42].

For our best knowledge, the effect of small RNAs in *E. kuehniella* generations was observed for the first time in this manuscript. We mapped reversion of mutations to the wild phenotype and observed that only in a small proportion of offspring and rarely on the whole population mutant phenotype occurred.
Materials and Methods
Flour Moth Rearing and Handling

Animals and breeding

For experiments, the strain of the Mediterranean flour moth (*Ephestia kuehniella* Zeller, Lepidoptera: Pyralidae) homozygous for the autosomal recessive mutation short antennae (*sa*) was used. The strain was derived from a mutant Qy strain that was obtained from the stock cultures of W. B. Cotter, Jr. (Albert B. Chandler Medical Center, University of Kentucky, Lexington, KY) and has been kept in single-pair cultures at the Institute of Entomology (České Budějovice, Czech Republic) since 1991.

Stock cultures were reared in constant temperature rooms (20°C ± 1°C) at a 12-h: 12-h light/dark regime, and at a humidity level of about 40 %. Experimental and control cultures were kept at either 20°C ± 1°C at the same conditions. New generations were done with single-pair cultures. Pairs were collected during copulation and placed individually into empty Petri dishes (6 cm in diameter). Females laid eggs for 3-4 days, then imagos were removed and Petri dishes with eggs were put into plastic boxes with food. Hatching larvae migrated from the dish to the food where they completed their development. Larvae were fed with milled wheat grains supplemented with a small amount of dried yeast.

An ethical note. The authors state that all experiments were conducted on insects (*Ephestia kuehniella*), none were conducted on vertebrates, including humans, or on cephalopods. Insects were killed in a container with 96% ethanol.

**Procedure**

In order to determine the epigenetic effect we utilised multiple paramutant animals, kept in normal breeding conditions. We observed the ratio of short and long antennae in every generation. The exact number of animals with a specific phenotype was observed in F12. The ratio of phenotypes was relative constant, and other generations were simply observed visually without a complete count being made.

In this study, only short antennae (*sa*) and prolonged antennae (*sa^WT*) with changed phenotype were distinguished. The first generation (*sa^WT* phenotype) of the line served to preparation of the changed phenotype (*sa* mutation). This mutation was turned out at 25°C ± 1°C, subsequent generations with the wild-type phenotype were then nursed at 20°C to exclude additional influence of temperature, and to monitor epigenetic feature only. The *sa* males of the wild phenotype (*sa^WT*) were used for all
subsequent generations. Each male of a $sa^{WT}$ phenotype ($sa$ genotype) was mated to a randomly chosen virgin female of the same phenotype and genotype.

In order to determine what is contained in the epigenetic information, the male spermatophore (from $sa^{WT}$ male) was analyzed and its individual parts were then injected into the previously fertilized eggs. The eggs were from the original $sa$ line, male and female $sa$ phenotype, i.e. the line without epigenetic information.

The experimental design was governed by the method of exclusion: at first the impact of separate ejaculate components ($sa^{WT}$ males) was tested, then we focused on the sperm - first on fractionated proteins, then on all of the sperm with RNase, and finally total RNA from the sperm $sa^{WT}$ males was used. Similarly, eggs were injected with a solution of geldanamycin.

**Isolation of separate parts of spermatophore**

Immediately after the copulation ended ($sa^{WT}$ male and female), the female was dissected. The following components from male spermatophore were separated for injection: (1) the spermatophore sac from the bursa copulatrix, (2) the seminal fluid from the spermatophore containing both the eupyrene and apyrene sperm, and (3) the secretion of male accessory glands from the bursa copulatrix. Ten samples of each category were stored at −70°C, and later homogenized and mixed with injection buffer (5 mM KCl, 0.1 mM NaH$_2$PO$_4$, pH 6.8) (Rubin and Spradling, 1982). Separated parts of spermatophore were prepared in room temperature only sperm into which RNase was added after homogenization (inactivated after 3 hours by incubating at 95°C for 30 seconds) were used for injections. Sperm proteins were purified using Sep-Pak® (Cartridges for solid Phase Extraction) Waters Corporation according to the manufacturer's instructions. The acquired components were mixed together with injection buffer [18] and individual dissolutions were divided into several aliquots and deep frozen at −70°C. These aliquots have been used successively to inject fertilized eggs. Total RNA was extracted from sperm using the RNA Blue reagent according to the manufacturer's instructions (Top-Bio, Czech Republic). Inactivation test of some heat shock proteins was done by geldanamycin solution that is able to block the proteins. All chemicals were supplied by Sigma-Aldrich (Sigma-Aldrich s.r.o. Prague, Pobrezni 46 Czech Republic).

**Injecting Eggs**

Total RNA, homogenized protein or geldanamycin were dissolved in injection buffer and approximately 0.2 - 1 fmol of RNA, 0.05 μg proteins or 0.5μg geldanamycin (ca 0.3 μl of injection buffer) were injected into the ventral side of the posterior domain of *Ephestia* embryos, similar to
Drosophila or Chymomyza [19]. One-tree day old fertilized eggs were injected (Tab 1).

The environmental effect of food

Larvae (sa) were fed with milled wheat grains supplemented with a small amount of dried yeast with added NaCl or LiCl to 1.1 on 1mg of food. In another case, the larvae were fed only plain flour (Tab 1).

Monitoring of following generations

Over 40 lines carrying epigenetic information for twenty generations were monitored. We counted a proportion of epigenetic trait (saWT) and nonepigenetic trait (sa) for individuals in generation. F₆ – F₁₁ and F₁₃ – F₂₀ generations were just observed without precise counting.

Statistical Analyses

We counted percentage of wild saWT offspring (imagoes) of each moth’s pair (clutch) in each group that were defined by three different types of interventions: (a) additive to food (eight groups), (b) additive by RNA content (two groups), or (c) type of medium (four groups). To compare mean percentage of saWT genotype among groups in each type of intervention, we performed non-parametric Kruskal-Wallis ANOVA followed by Dunn post-hoc tests. Ordinary one-way ANOVA cannot be used as the data does not met ANOVA assumptions (normality by groups and homogeneity of variances). P-values of post-hoc tests were adjusted by Benjamini-Hochberg correction to control the false discovery rate.

We have tried to determine the point at which a significant number of individuals in populations. It was reversed was defined as the overall non-outlier maximum (12.6% of reversed individual). The number of extremely reversed clutches was in each generation counted as the number of clutches with percentage of reversed individuals higher than the threshold of 12.6%. To compare number of reversed and non-reversed clutches we used goodness-of-fit chi-square test. All tests were performed in R version 4.0.2 (2020 The R Foundation for Statistical Computing).

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References:
1. Buckley BA, Burkhart KB, Gu SG, Spracklin G, Kershner A, Fritz H, Kimble J, Fire A, Kennedy S. A nuclear Argonaute promotes multigenerational epigenetic inheritance and germline immortality. Nature 2012; 489:447-51; PMID: 22810588; https://dx.doi.org/10.1038/nature11352

2. Chen Q, Yan W, Duan E. Epigenetic inheritance of acquired traits through sperm RNAs and sperm RNA modifications. Nat Rev Genet. 2016 Dec;17(12):733-743. doi: 10.1038/nrg.2016.106. Epub 2016 Oct 3. PMID: 27694809; PMCID: PMC5441558.

3. Bohacek J, Rassoulzadegan M. Sperm RNA: Quo vadis? Semin Cell Dev Biol. 2020 Jan;97:123-130. doi: 10.1016/j.semcdb.2019.07.005. Epub 2019 Jul 12. PMID: 31299279.

4. Pavelka J, Koudelová J. 2001 Inheritance of a temperature-modified phenotype of the short antennae (sa) mutation in a moth, *Ephestia kuehniella* (Lepidoptera: Pyralidae). J Hered. 2001; 92: 234-42; PMID: 11447238

5. Casas E, Vavouri T. Sperm epigenomics: challenges and opportunities. Front Genet. 2014 Sep 18;5:330. doi: 10.3389/fgene.2014.00330. PMID: 25278962; PMCID: PMC4166955.

6. Wagner KD, Wagner N, Ghanbarian H, Grandjean V, Gounon P, Cuzin F, Rassoulzadegan M. (2008) RNA induction and inheritance of epigenetic cardiac hypertrophy in the mouse. Dev Cell. 14(6):962-9.

7. Grandjean V, Gounon P, Wagner N, Martin L, Wagner KD, Bernex F, Cuzin F, Rassoulzadegan M. The miR-124-Sox9 paramutation: RNA-mediated epigenetic control of embryonic and adult growth. Development. 2009 Nov;136(21):3647-55. doi: 10.1242/dev.041061. PMID: 19820183.

8. Gapp K, Jawaid A, Sarkies P, Bohacek J, Pelczar P, Prados J, Farinelli L, Miska E, Mansuy IM. Implication of sperm RNAs in transgenerational inheritance of the effects of early trauma in mice. Nat Neurosci. 2014 May;17(5):667-9. doi: 10.1038/nn.3695. Epub 2014 Apr 13. PMID: 24728267; PMCID: PMC4333222.

9. Kiani J, Grandjean V, Liebers R, Tuorto F, Ghanbarian H, Lyko F, Cuzin F, Rassoulzadegan M. RNA-mediated epigenetic heredity requires the cytosine methyltransferase Dnmt2. PLoS Genet. 2013 May;9(5):e1003498. doi: 10.1371/journal.pgen.1003498. Epub 2013 May 23. PMID: 23717211; PMCID: PMC3662642.

10. Garcia-Fernández J, Holland PW. Archetypal organization of the amphioxus Hox gene cluster. Nature 1994; 18: 563-6; PMID: 20404028; https://dx.doi.org/10.1258/ebm.2009.009251

11. Garcia-Fernández J, Holland PW. Archetypal organization of the amphioxus Hox gene cluster. Nature 1994; 18: 563-6; PMID: 20404028; https://dx.doi.org/10.1258/ebm.2009.009251

12. Morris K.V. (ed.) RNA and the Regulation of Gene Expression: A Hidden Layer of Complexity.
13. Rassoulzadegan M, Grandjean V, Gounon P, Vincent S, Gillot I, and Cuzin F. RNA-mediated non-mendelian inheritance of an epigenetic change in the mouse. Nature 2006; 441: 469-74; PMID: 16724059; https://dx.doi.org/10.1038/nature04674

14. Cuzin F, and Rassoulzadegan M. The making of an organ: RNA mediated developmental controls in mice. Organogenesis 2010; 6: 33–36; PMID: 20592863; https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2861741/

15. Soloway PD. Genetics: paramutable possibilities. Nature 2006; 441: 413-4; PMID: 16724044; https://dx.doi.org/10.1038/441413a

16. Ashe A, Sapetschnig A, Weick EM, Mitchell J, Bagijn MP, Cording AC, Doebley AL, Goldstein LD, Lehrbach NJ, Le Pen J, Pintacuda G, Sakaguchi A, Sarkies P, Ahmed S, Miska EA. piRNAs can trigger a multigenerational epigenetic memory in the germline of C. elegans. Cell 2012; 150: 88-99; PMID: 22738725; https://dx.doi.org/10.1016/j.cell.2012.06.018

17. Forneck A, Walker MA, Blaich R. Ecological and genetic aspects of grape hyloxera Daktulosphaira vitifoliae Fitch (Hemiptera: Phylloxeridae) performance on rootstock hosts. Bulletin of Entomological Research 2001; 91: 445–451; PMID: 11818039;

18. Rubin GM, and Spradling AC. Genetic transformation of Drosophila with transposable element vectors. Science 1982; 218: 348-53; PMID: 6289436;

19. Pavelka J, Shimada K, Koštál V. TIMELESS: a link between fly's circadian and photoperiodic clocks? Eur J Entomol. 2003; 100: 255–265;

20. Jablonka E, Raz G.Transgenerational epigenetic inheritance: prevalence, mechanisms, and implications for the study of heredity and evolution. Q Rev Biol. 2009; 84:131-76. PMID:19606595

21. Whitesell L, Mimnaugh EG, De Costa B, Myers CE, Neckers LM (August 1994). Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. Proc. Natl. Acad. Sci. U.S.A. 91: 8324–8.

22. Pearl LH, Prodromou C. 2006 Structure and mechanism of the Hsp90 molecular chaperone machinery. Annu Rev Biochem. 2006; 75: 271-94; PMID:16756493; https://dx.doi.org/10.1146/annurev.biochem.75.103004.142738

23. Iwasaki S, Sasaki HM, Sakaguchi Y, Suzuki T, Tadakuma H, Tomari Y. Defining fundamental steps in the assembly of the Drosophila RNAi enzyme complex. Nature 2015; 521:533-6; PMID: 25822791; https://dx.doi.org/10.1038/nature14254

24. Vercelli D. Genetics, epigenetics, and the environment: switching, buffering, releasing. J Allergy Clin Immunol. 2004; 113: 381-6; Review; PMID: 15007332;
25. Ariel M, Robinson E, McCarrey JR, Cedar H. Gamete-specific methylation correlates with imprinting of the murine Xist gene. Nat Genet. 1995; 9: 312-5; PMID: 7773295; https://dx.doi.org/10.1038/ng0395-312

26. Biliya, S. and Bulla, L.A. Genomic imprinting: The influence of differential methylation in the two sexes. Experimental Biology and Medicine 2010; 235: 139-147; PMID: 20404028; https://dx.doi.org/10.1258/ebm.2009.009251

27. Petrella LN, Wang W, Spike CA, Rechtsteiner A, Reinke V, Strome S. synMuv B proteins antagonize germline fate in the intestine and ensure C. elegans survival. Development 2011; 138:1069-79; PMID: 21343362; https://dx.doi.org/10.1242/dev.059501

28. Liebers R, Rassoulzadegan M, Lyko F. Epigenetic regulation by heritable RNA. PLoS Genet 2014; 10:e1004296; PMID: 24743450; https://dx.doi.org/10.1371/journal.pgen.1004296

29. Tuorto F, Liebers R, Musch T, Schaefer M, Hofmann S, Kellner S, Frye M, Helm M, Stoecklin G, Lyko F. RNA cytosine methylation by Dnmt2 and NSun2 promotes tRNA stability and protein synthesis. Nat Struct Mol Biol. 2012; 19: 900-5; PMID: 22885326; https://dx.doi.org/10.1038/nsmb.2357

30. Yakovlev IA, Fossdal CG, Johnsen Ø. MicroRNAs, the epigenetic memory and climatic adaptation in Norway spruce. New Phytol. 2010; 187:1154-69; PMID: 20561211; https://dx.doi.org/10.1111/j.1469-8137.2010.03341.x

31. Francis D., Diorio. J., Liu D. and Meany M.J. 1999. Nongenomic transmission across generation of maternal behavior and stress responses in the rat. Science 286: 1155–1158.

32. Loxdale, H.D. Lushai, G. Rapid changes in clonal lineages: the death of a ‘sacred cow’. Biological Journal of the Linnean Society 2003; 79: 3–16; DOI: 10.1046/j.1095-8312.2003.00177.x

33. Sato F., Tsuchiya S., Meltzer S.J., Shimizu K. MicroRNAs and epigenetics. FEBS JOURNAL 2011; 278: 1598-1609; PMID: 21395977; https://dx.doi.org/10.1111/j.1742-4658.2011.08089.x

34. Wei J., Xie L., Taron M., Rose R., Liu B.R. 2010 Epigenetic alterations of tumor marker microRNAs: towards new cancer therapies. Drug News Perspect. 2010; 23: 655-61; https://dx.doi.org/10.1358/dnp.2010.23.10.1560143

35. Sonneborn A, Tebarth B, Ernst J. Control of white-opaque phenotypic switching in Candida albicans by the Efg1p morphogenetic regulator. Infect. Immu. 1999; 67: 4655–4660; PMID:
TABLE 1. Mean, median, and standard deviation (SD) of percentage of saWT imagoes of each moth’s pair by three different types of interventions.

| Type of intervention | Number of imagoes | Number of moth's pairs | Mean | Median | SD |
|----------------------|-------------------|-------------------------|------|--------|----|
| Additive                                      | 390 | 7  | 27.2 | 11.8 | 30.2 |
|----------------------------------------------|-----|----|------|------|------|
| Buffer                                       | 353 | 5  | 48.5 | 51.7 | 14.7 |
| Geldanamycin                                 | 636 | 17 | 52.1 | 51.7 | 29.0 |
| Sperm                                        | 187 | 6  | 18.6 | 18.6 | 11.8 |
| Accessory gland                              | 456 | 7  | 43.9 | 52.2 | 26.4 |
| Homogenized fractions of sperm               | 152 | 5  | 8.0  | 7.7  | 2.1  |
| Homogenized spermatophore sac                | 164 | 10 | 22.4 | 17.9 | 18.9 |
| Sperm and RNase                              | 140 | 7  | 63.2 | 66.7 | 24.6 |
| Total RNA                                    | 1585| 36 | 52.1 | 52.1 | 25.9 |
| Additive RNA content                         | 893 | 28 | 20.2 | 20.2 | 19.8 |
| Food medium                                  | 340 | 8  | 90.2 | 92.1 | 5.6  |
| Flour                                        | 485 | 11 | 84.7 | 93.1 | 17.7 |
| LiCl                                         | 244 | 12 | 89.9 | 88.7 | 7.3  |
| NaCl                                         | 1363| 15 | 11.0 | 10.0 | 6.8  |
| Wheat grains                                 |     |    |      |      |      |
Table 2. Dunn’s pairwise comparison of percentage of saWT imagoes between eight types of additives to eggs. P-values are adjusted with Benjamini-Hochberg correction.

|                                  | Buffer | Geldan.# | Sperm# | Accessory gland | Homogen. fractions of sperm# | Homogen. sperm. sac | Sperm and RNase |
|----------------------------------|--------|----------|--------|----------------|-----------------------------|-------------------|-----------------|
| Geldanamycin#                    | 0.218  |          |        |                |                             |                   |                 |
| Sperm#                           | 0.099  | 0.959    |        |                |                             |                   |                 |
| Accessory gland                  | 0.788  | 0.146    | 0.041* |                |                             |                   |                 |
| Homogenized fractions of sperm# | 0.294  | 0.814    | 0.783  | 0.175          |                             |                   |                 |
| Homogenized spermatophore sac    | 0.296  | 0.040*   | 0.009**| 0.500          | 0.041*                     |                   |                 |
| Sperm and RNase                  | 0.925  | 0.168    | 0.041* | 0.814          | 0.206                       | 0.296             |                 |
| Total RNA#                       | 0.041* | 0.596    | 0.500  | 0.032*         | 0.378                       | 0.008**           | 0.032*          |

* Statistically significant at 0.05 level
# Additive with RNA content

Figures:

Fig. 1. Boxplots of percentage of saWT offspring between eight different types of additives to the eggs. The central thick line is median, box corresponds to lower and upper quartiles and whiskers correspond to non-outlier minimum and maximum. Circles are raw values of Y variable in each clutch. Grey and white fill corresponds to additives with and without RNA content, respectively.

Fig. 2. Boxplots of percentage of saWT offspring between four different food medium. The central thick line is median, box corresponds to lower and upper quartiles and whiskers correspond to non-outlier minimum and maximum. Circles are raw values of Y variable in each clutch.

Fig. 3. Absolute and relative number of extremely reversed clutches by generation. Extremely reversed clutch was defined as a clutch with a percentage of reversed individuals higher than