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EPIGENETIC STUDIES IN ECOLOGY AND EVOLUTION

Paternal intergenerational epigenetic response to heat exposure in male Wild guinea pigs

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Abstract

Epigenetic modifications, of which DNA methylation is the best studied one, can convey environmental information through generations via parental germ lines. Past studies have focused on the maternal transmission of epigenetic information to the offspring of isogenic mice and rats in response to external changes, whereas heterogeneous wild mammals as well as paternal epigenetic effects have been widely neglected. In most wild mammal species, males are the dispersing sex and have to cope with differing habitats and thermal changes. As temperature is a major environmental factor we investigated if genetically heterogeneous Wild guinea pig (*Cavia aperea*) males can adapt epigenetically to an increase in temperature and if that response will be transmitted to the next generation(s). Five adult male guinea pigs (F0) were exposed to an increased ambient temperature for 2 months, i.e. the duration of spermatogenesis. We studied the liver (as the main thermoregulatory organ) of F0 fathers and F1 sons, and testes of F1 sons for paternal transmission of epigenetic modifications across generation(s). Reduced representation bisulphite sequencing revealed shared differentially methylated regions in annotated areas between F0 livers before and after heat treatment, and their sons' livers and testes, which indicated a general response with ecological relevance. Thus, paternal exposure to a temporally limited increased ambient temperature led to an 'immediate' and 'heritable' epigenetic response that may even be transmitted to the F2 generation. In the context of globally rising temperatures epigenetic mechanisms may become increasingly relevant for the survival of species.

Keywords: adaptation, *Cavia aperea*, DNA methylation, environmental factor, global change, plasticity, temperature increase

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Introduction

To cope with environmental factors, (wild) species need appropriate adaptive traits to survive and reproduce (Penuelas *et al.* 2013; Kilvitis *et al.* 2014). The regulation of genes and their expression is fundamental for immediate adaptation processes in the same generation. In addition, the inheritance of responses to experienced changes (adapted traits) is fundamental for long-term adaptational memory. The mechanism regulating gene expression and conferring such immediate and inherited adaptation is 'epigenetic response' (Jablonka & Raz 2009).

Current research mainly has focused on maternal epigenetic response and transmission of (environmental) 'experiences', to which animals were exposed to during crucial developmental phases; either during pregnancy when the intrauterine environment provides direct contact of the foetus to the mother, and during postnatal maternal care (e.g. Weaver *et al.* 2004; Szyf *et al.* 2005; Dolinoy *et al.* 2006a,b; Ma *et al.* 2015). Due to the close relationship of mother and offspring in combination with epigenetic reprogramming processes in the blasto-

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cyst, the paternal side – and thus the father's role in a potential epigenetic adaptation – has long been neglected. Only recently a small number of studies considered paternal intergenerational epigenetic effects. Those studies were carried out e.g. for isogenic mice, that were fear-conditioned to the odour acetophenone (Dias & Ressler 2014) and for rat and mouse strains after exposure to drugs (Vyssotski 2011; Vassoler *et al.* 2013) and after chronic nutrition changes (Carone *et al.* 2010; Ng *et al.* 2010; Wei *et al.* 2014). Current research has mainly focused maternal effects that were studied in isogenic lab animals, such as inbred mice and rat strains, which were exposed to external changes under laboratory conditions. Those studies have the advantage of great numbers of genetically identical animals and thus facilitate deciphering epigenetic mechanisms, but they may not reflect the responses to external changes as they may occur in a genetically heterogeneous mammal living in the wild.

Such necessary ecological epigenetic studies on wild species are, however, still rare (Richards 2006; Pertoldi & Bach 2007; Bossdorf *et al.* 2008). Although less convenient, they are mandatory to understand adaptation processes on a molecular level (Kilvitis *et al.* 2014), where diversity is represented by genetic and epigenetic variations (Johannes *et al.* 2009). While the former reflects long-term evolutionary adaptations, the latter can be threefold: a short-term response to external changes (immediate, short-time lasting), a long-term response without transmission (immediate, long-time lasting), and a long-term response that is transmitted to the next generation (inherited).

In wild mammal species, males more often have to cope with rapid environmental fluctuations than females, because males predominantly disperse, while females are often phylopatric. This also holds true for the Wild guinea pig, *Cavia aperea*, living in harem structures with one dominant male, defending several females from other males (Asher *et al.* 2008). The non-dominant, roaming males thereby need to rapidly adapt to new habitats and temperatures before finding accepting female(s). Therefore, a paternal transmission through epigenetic effects may contribute to a fitness increase of the offspring and may thus be of evolutionary importance. That fitness increase would be even more pronounced if the paternal response was transmitted over more than one generation.

Climate changes have always impacted and accompanied the evolution of species. They pose a great challenge to existing species whose long-term survival depends on their short-term responses as well as on their ability to convey heritable phenotypic plasticity to descendant generations. Even though the annual average mean of global temperature is recently rising, the immediate and heritable epigenetic responses to heat exposure have not been studied in mammals and hence not in a wild, genetically heterogeneous mammal.

To study whether there is such a paternal contribution to an epigenetically adaptational response to increasing temperature in a wild species, we temporally exposed adult male Wild guinea pigs to an increased ambient temperature and allowed them to mate (with the same females) before and after the heat exposure. We then examined if they and their sons – one group sired before, the other after the fathers heat treatment – showed altered DNA methylation patterns compared with the situation prior to the heat exposure (Fig. 1).

As a mammal species, the wild guinea pig is a homoeostatic animal, which compensates external thermal fluctuations (thermoregulation) to maintain its internal body temperature (homeostasis). To study the epigenetic responses to heat, we here focused on the body's main metabolic, heat producing and thus thermoregulatory organ, the liver. To further determine if the father's response in terms of altered DNA methylation was potentially even transmitted to the F2 generation, we also analysed the germ cells in the testes of 7 day old F1 sons (sired after heat treatment of fathers). We focused on regions that are of main interest due to their regulatory functions, such as CpG islands (CGIs), genes and gene promoters. *Hyper*methylation of promoter regions is correlated with gene silencing, while *hypo*methylation is correlated with gene activation (Bird 2002; Skinner 2011). 70% of mammalian promoter regions are associated with CGIs, genomic regions rich in CG content and a main regulatory site for gene silencing (Deaton & Bird 2011).

Materials and methods

Animal care and treatment

All husbandry and experimental procedures were approved of by the German Committee of Animal Welfare in Research (permit no. V3-2347-35-2011). Wild guinea pigs (*Cavia aperea*) originating from Argentina and Uruguay (Asher *et al.* 2008) were obtained from F. Trillmich (University of Bielefeld) and housed at the IZW field station in Niederfinow, Germany. All animals were fed guinea-pig pellets (Altromin Spezialfutter GmbH & Co. KG). Water and hay were provided *ad libitum* and supplementary apples, peppers or carrots were given daily. Vitamin C was added to the drinking water once a week. To avoid male competition, males were held as singles and were always separated by single females kept in separate cages between them in a way that social interaction between male and female Wild guinea pigs was possible, but direct contact was prevented. Indoor cages

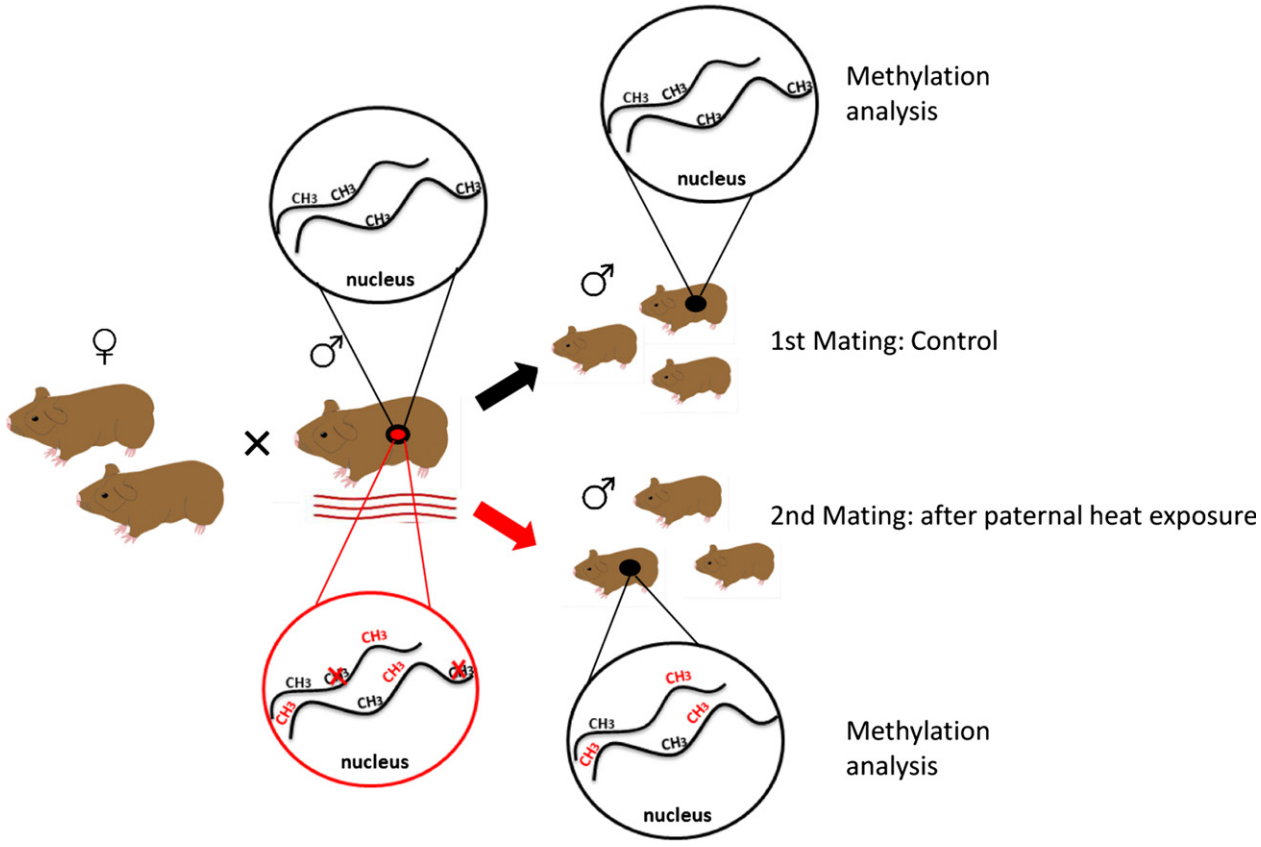

Fig. 1 The experimental set-up shows that male Wild guinea pigs (F0) were mated to the same two females Wild guinea pigs before and after exposure to increased temperature. Potential changes in methylation of nuclear DNA are indicated by red CH_3-groups. Methylation patterns were analysed from DNA of liver biopsies taken from fathers (prior ($F0L_C$) and after heat exposure ($F0L_H$)), as well as from DNA of whole livers and testes of sons sired before (control: $F1L_C$, $F1T_C$) and after heat exposure ($F1L_H$, $F1T_H$). A red X indicates loss of methyl-group.

(80 × 80 cm^2) were filled with bedding of wood shavings and opaque tubes for cover. Wild guinea pigs (*Cavia aperea*) are polyoestric and the duration of spermatogenesis is 2 months. Thus, during a 2 month period, the indoor cages were placed on a heating plate (Candor GmbH Leipzig) which heated the floor to a temperature of 30 °C (Fig. 1). The slightly cooler edges of the heating plate were fenced off by a mesh, installed in 5 cm distance from the cage wall, reducing cage size to 70 × 70 cm^2. Control group animals were housed in short-tunnel-connected indoor-outdoor-enclosures (1.3 m^2) under natural photoperiod and temperature. The experiment to assess the influence of environmental stressors was carried out with five adult males kept exclusively indoor (60 days, 24 h; 30 °C). Outside temperature and natural light were not manipulated.

Mating and tissue sampling

The five male F0 Wild guinea pigs were born in mid-November 2010 and the 10 F0 females in April–May 2011. The parent group consisted of five Wild guinea pig F0-males (F-J) and ten F0-females (average genetic dissimilarity among animals ~0.17%). Each male was mated twice with two females. Mating took place in January/February 2012 [control group, $F0_C$; average ambient temperature: Jan. 3.4 °C; Feb. 0.8 °C] and in September 2012 [heat group, $F0_H$; average ambient temperature: 19.5 °C; male animals were kept at kept at 30 °C]. In order to achieve mating, males were introduced to the females' cage, and after an observed mating, males were transferred back to their own cage. Because the males mated with the same two females before and after heat exposure, the offspring produced per female were direct siblings. Biopsies of livers (L) of F0-fathers ($F0L_C$ and $F0L_H$) were taken straight after each mating round. At day 7 after birth [ambient temperature: $F1_C$: March/April 2012: 14 °C; $F1_H$: 19.5 °C], we harvested whole livers (L) and testes (T) of F1 sons from the first mating ($F1L_C$ and $F1T_C$; $N = 16$) and of F1 sons from the second mating ($F1L_H$ and $F1T_H$; $N = 18$). Livers and testes were homogenized, snapped

frozen in liquid N_2 and stored at −80 °C until DNA isolation (see below). As males are the dispersing sex in this species, we focused on the male offspring and did not investigate the female offspring.

Reduced representation bisulphite sequencing

We performed Reduced Representation Bisulphite Sequencing (RRBS) (Meissner *et al.* 2005) to profile DNA methylation changes among fathers and offspring grouped by fathers (labelled F to J) [Table S1, Supporting information: $F0L_C$ and $F0L_H$ samples (consisting of $F0L_C$-F to $F0L_C$-J and $F0L_H$-F to $F0L_H$-J)] and offspring grouped by fathers [Table S2, Supporting information: $F1L_C$ and $F1L_H$ (consisting of $F0L_C$-F1 to $F0L_C$-J3 and $F0L_H$-F1 to $F0L_H$-J1) and Table S3, Supporting information: $F0T_C$ and $F0T_H$ samples (consisting of $F0TL_C$-F to $F0T_C$-J and $F0L_H$-F to $F0L_H$-J)]. Liver samples were sequenced individually ($F1L_C$ and $F1L_H$; $N = 34$), DNA of the sons' testis samples were pooled by father before sequencing ($F1T_C$ and $F1T_H$; $N = 10$).

RRBS was performed using the EpiQuest genomic service of ZymoResearch. Genomic DNA (200–500 ng) was digested with restriction enzymes TaqI (60U, NEB) and MspI (30U, NEB), and size selected (40–120 bp and 120–350 bp). After 3′-end fill-in using dNTPs (NEB) and GoTaq polymerase (Promega) and purification of the DNA fragments (DNA Clean & Concentrator™-5 kit; Zymo Research), 5mC-adapters (Illumina) were ligated to the fragments. Fragments were then bisulphite-treated (EZ DNA Methylation-Direct™ Kit; ZymoResearch), purified and size-selected again (130–210 bp and 210–460 bp) on a 4% NuSieve 3:1 agarose gel. After recovery from gel (Zymoclean™ Gel DNA Recovery Kit, ZymoResearch), 50 bp paired-end sequencing was performed on a HiSeq2000 (Illumina).

Adapter trimming was followed by trimming of nucleotides with $Q < 20$ from the 3′end of reads using the software TRIM GALORE (v.0.3.3, Babraham, Bioinformatics, Cambridge). Reads were mapped against an in-house-generated *C.aperea* reference sequence (Weyrich *et al.* 2014) using BISMARK MAPPER (Krueger & Andrews 2011) (v.0.7). We allowed two mismatches in the first 28 nucleotides of each read. For each given read, the best alignment was kept. If more than one 'best alignment' was found the read was discarded as nonunique. We corrected for cytosine-phosphate-guanines (CpGs) that were filled in at the enzyme cutting sites. Genomic coverage was calculated as number of covered nucleotides/genome size. The average sequencing depth was calculated as number of mapped reads × read length/covered genomic nucleotides.

DNA methylation level analysis and group comparison

The bisulphite conversion rate was calculated as the number of mapped nonmethylated CpGs divided by the total number of mapped CpGs. After bisulphite treatment and alignment to the reference sequence, cytosines (Cs) in a read that mapped to a C in the reference, were assumed to have been methylated cytosines (mCs). Thymines that mapped to a C position were regarded as Cs that had been unmethylated before, which were converted to U by bisulphite and substituted by bisulphite substituted to T by subsequent PCR. Accordingly, the methylation ratio of each cytosine position was calculated as the number of reads mapping to this position and carrying a C, divided by the number of reads carrying either C or T at this position.

Methylation ratio was calculated by the equation:

$$\frac{C}{C+T} = \text{methylation ratio per specific mC site}$$

Because nonmethylated Cs were deaminated to T by bisulphite, this equation translates to

$$\frac{mC}{mC+C} = \text{methylation ratio of one per specific mC site}$$

Methylation level determination

We compared methylation states of fathers ($F0L_C$ vs. $F0L_H$) and sons, respectively, before and after heat treatment ($F1L_C$ vs. $F1L_H$ and $F1T_C$ vs. $F1T_H$). Significance of differences was determined using Fisher's exact test. Strong *hyper* and strong *hypo*methylation was defined as absolute methylation difference >30% (Gu *et al.* 2010).

Single cytosine methylation comparisons

All Cs with at least 5× coverage were counted, as well as mCs and those in CpG context. A two-side *t*-test was applied to ratios of mC and C; and mCpG and CpG sites, respectively.

To study the within-group and in-between group variance: we calculated the variance of the methylation ratios per CpG site using individual F1L samples and applied the paired Wilcox-test.

Differentially methylated regions (DMRs)

Because methylation changes of single CpGs (mCpGs) are thought to occur more likely due to genetic variability (Johannes *et al.* 2009; Radford *et al.* 2014), we clustered mCpGs to identify differentially methylated regions (DMRs) using the software METHPIPE (Song *et al.*

2013) allowing a maximum distance of 100 bp between two CpGs. For DMR calculation the input data set was generated (i) by using only CpG positions with read coverage in all samples and (ii) by using methylation ratios with a coverage at least 5× per CpG position in each sample. This approach generated a conservative data set, strongly restricting the number of CpG sites. Identification of DMR congruence between at least four fathers was performed by using BEDTOOLS [v. 2.15.0; intersect and multiinter; (Quinlan & Hall 2010)]. Genome annotations were performed as described earlier (Weyrich *et al.* 2014). We selected for DMRs located in CpG islands, promoter regions and CDS, which occurred in all three sample groups: $F0L_H$, $F1L_H$ and $F1T_H$ (henceforth called 'annotated DMRs'). Differences between corresponding control and heat samples ($F0L_C$ vs. $F0L_H$, $F1L_C$ vs. $F1L_H$ and $F1T_C$ vs. $F1T_H$) were assessed in pairwise comparisons which for F1 sons were grouped according to their father. Results were visualized using R (with customized functions by T. Girke; http://faculty.ucr.edu/~tgirke/Documents/R_BioCond/My_R_Scripts/overLapper.R; Fig. S1, Supporting information).

To generate a shuffled data set we used the R sample command, which is reordering the methylation ratios of $F1L_C$ vs. $F1L_H$ (sons grouped by father F-J) for 100 times and recalculated DMRs as described above.

To investigate the inherited component we made use of a 'hierarchical cluster analysis' (using R; hclust), and compared the distances of the amount of shared DMRs in livers among fathers ($F0L_C$ vs. $F0L_H$), sons ($F1L_C$ vs. $F1L_H$) and between fathers and sons. DMRs were first normalized:

Normalized DMRs = No. shared DMRs between two groups/(total No. DMRs group 1 × total No. DMRs group2)* 1 000 000

Using those normalized numbers of shared DMRs ($F0L_C$ vs. $F0L_H$, $F1L_C$ vs. $F1L_H$) we applied a 'hierarchical cluster analysis'. As distance measure we used the formula:

Distance = 200 – normalized numbers of shared DMRs

in which 200 was greater than the highest number of normalized shared DMRs. As clustering agglomeration, we used the method 'complete linkage'.

Gene ontology terms (GO terms)

Gene ontology terms for *Cavia porcellus* were taken from the Gene Ontology Annotation (GOA) database (http://www.ebi.ac.uk/GOA). Selected gene ontologies that were not yet annotated for the *Cavia porcellus* genome were adopted from mice using GOA and AmiGO (http://amigo.geneontology.org) databases.

To search for thermoregulation genes we used AmiGO gene ontology terms of thermoregulation genes (GO: 0001659) and applied those to mCpG sites with significant differences among groups (Fisher's exact test). Results (Fig. S2, Supporting information) were plotted using R (v. 3.0.2) GGPLOT2 (v.1.0.0).

Results

Total DNA methylation changes

The overall single cytosine methylation level did not significantly differ between control and treatment group neither for $F0L_C$ vs. $F0L_H$, $F1L_C$ vs. $F1L_H$ nor $F1T_C$ vs. $F1T_H$. On average, ~10% of all cytosines were methylated, out of which ~70% were in a CpG context, and only 2% in either a CHG or a CHH context, as expected (Weyrich *et al.* 2014).

Immediate and inherited epigenetic response

To identify an ecologically relevant, shared and thus rather 'general' response to heat, we looked for DMRs detected in at least four fathers and their offspring (grouped by father). Father samples taken before and after heat exposure ($F0L_C$ and $F0L_H$) varied in 1,831 DMRs, of which 758 were located in annotated regions [promoters, coding sequences, CpG islands (CGIs)], reflecting a general 'immediate response' (or 'epigenetic plasticity') to heat exposure. The comparison between liver samples of $F1L_C$ with $F1L_H$ yielded 471 DMRs (incl. 245 in annotated regions), which we regard as a general 'inherited response' (or 'transgenerational epigenetic plasticity') to the heat treatment of the sires. For changes which bear the potential to be transmitted even to the F2 generation, we found 2484 DMRs (incl. 940 in annotated regions) in premature testis cells between $F1T_C$ and $F1T_H$. These results are supported by a random approach, shuffling the $F1L_C$ vs. $F1L_H$ methylation ratios 100 times, which resulted in <2 DMRs per calculation.

Using a hierarchical cluster analysis, no increased direct inheritance from one father to his sons was detectable. A variance test comparing within-treatment and between-treatment groups resulted in highly significant differences among all groups.

Annotated DMRs

In all five fathers (F-J) and their offspring (grouped according to father) as well as in both tissues ($F0L_H$, $F1L_H$, and $F1T_H$) we detected 27 shared annotated regions including at least one annotated DMR (Fig. 2). This finding indicates the existence of a shared and thus general paternal epigenetic response to heat.

To analyse the general heat response in more detail, we further investigated the annotated DMRs detected in at least four fathers and offspring grouped by fathers (N = 116; Fig. 2; Table S4, Supporting information). Out of these 116 annotated DMRs, 45 were located in CDSs, 11 in promoter regions and 60 in CGIs (Table S4, Supporting information).

Epigenetically responding genes

Among the annotated DMRs we detected male-specific genes, and genes with function in heat response, as well as with importance in gene regulation, which we further described in more detail (Table 1; see Table S5, Supporting information for the complete list.)

Among the gene promoters and CDS regions were the heat shock proteins AlphaB-crystallin (*A9lpa2*) and Heat shock protein beta (*Hspb2*). Both belong to the heat shock protein family 20 (*HSP20*), whose members are activated as an immediate response to heat and other environmental stressors to protect the correct protein structure (Javid *et al.* 2007). *A9lpa2* itself is involved in morphogenesis, apoptosis, the response to hypoxia, protection against gamma radiation and, as shown here, in response to temperature increase. Interestingly, its promoter was *hypo*methylated in $F0L_H$ and $F1T_H$, but *hyper*methylated in $F1L_H$. This oppositional epigenetic information is indicative of 'epigenetic compensation'

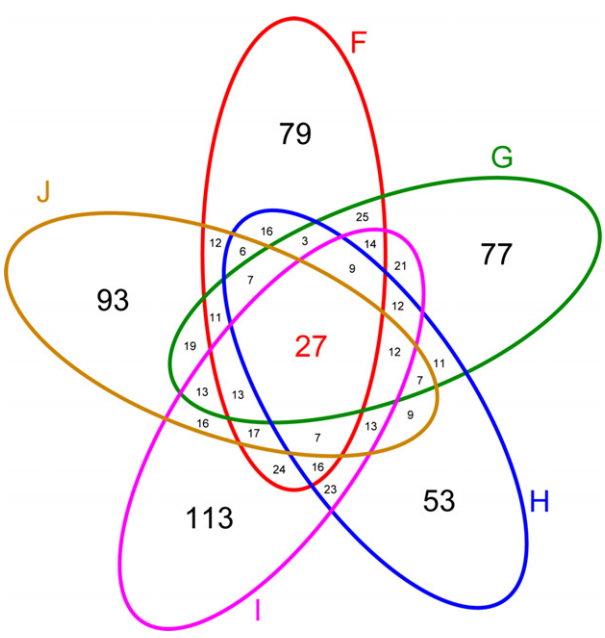

Fig. 2 Annotated differentially methylated regions after paternal heat exposure. The Venn diagramm shows the number of annotated regions (CpG islands, gene coding regions and promoters) in which DMRs were detected in $F0L_H$ DNA, $F1L_H$ DNA and $F1T_H$ DNA sorted according to the five fathers (F-J).

processes (Vyssotski 2011) in the next generation. *Hspb2*-CDS, which is located on the complementary strand of the *A9lpa2*-promoter was showing the same methylated level. Besides acting in response to heat and stress *Hspb2* is also involved in somatic muscle development.

A male-specific gene which showed promoter-methylation changes is the seminal vesicle polypeptide (*Svp*) gene, important for maintaining the integrity of spermatozoa in the ejaculate (Fautsch *et al.* 1997). Here, *Svp* was *hypo*methylated in $F0L_H$ fathers and *hyper*methylated in $F1T_H$. Methylation occurred in very specific patterns indicating a 'composition of specific switches' (Fig. S1, Supporting information). Interestingly, in $F1L_H$ *hypo* and *hyper*methylation was detected.

In addition, we detected several genes that are important in gene regulation, such as *Rara* (Retinoic acid receptor) with transcription factor activity important during spermatogenesis (Wolgemuth & Chung 2007), *Sox13* (Sex determining region *Y*-related high mobility group (HMG)-box 13), a member of the SOX gene family, and the transcription factor *Wiz* (Widely interspaced zinc finger motifs).

Sox13 codes for a transcription factor, involved in regulating embryonic brain and spinal cord development, organogenesis, as well as cell fate determination. In this study, we detected *hypo*methylation in *Sox13* CDS in $F0L_H$, $F1L_H$ and $F1T_H$, indicating enhanced gene activity. *Wiz* stabilizes a complex of two histone methyltransferases (G9 and GLP), methylating lysine 9 in histone 3 (H3K9) at euchromatic regions (Ueda *et al.* 2006). H3K9 methylation acts on gene transcription, heterochromatin formation, DNA repair and recombination, and often changes in concert with DNA methylation (Rose & Klose 2014). As additionally *Wiz*' siRNA inhibits transcription of both *Wiz* and *G9a*, the methylation dependency found here thus combines the three epigenetic mechanisms (DNA methylation, microRNA, histone modification), suggesting their close interaction. Furthermore, the Fibrillarin-Like 1 gene (*Fbll1*) was differentially methylated in all groups, having the ability to methylate RNAs as well as proteins.

Interestingly, methylation patterns also changed in an imprinted gene. The imprinted *Gnas* gene codes for the Guanine Nucleotide-binding protein, which is involved in hormonal adenylate cyclase regulation and in a variety of cellular responses, as well as in GTP binding and GTPase activity regulation. The transcripts are maternally, paternally, and biallelically derived and regulated from four different promoters (Hayward *et al.* 1998; Weiss *et al.* 2000). One transcript is an antisense transcript which is paternally expressed and involved – together with another, likewise paternally expressed transcript – in imprinting regulation. *Gnas* showed tissue-dependent imprinting: we detected a general

Table 1 Selected promoter and genes with DMRs after comparing $F0L_C$ vs. $F0L_H$, $F1L_C$ vs. $F1L_H$ and $F1T_C$ vs. $F1T_H$

Gene name (Ensembl ID)	$F0L_C$ vs. $F0L_H$	$F1L_C$ vs. $F1L_H$	$F1T_C$ vs. $F1T_H$	No. of fathers	Regulatory region	CGI	GO terms†
A9lpa2 (ENSCPOG00000012928)	5	4	4	4	Promoter	No	Response to hypoxia, muscle organ development, negative regulation of gene expression, regulation of cell death, negative regulation of intracellular transport, response to hydrogen peroxide, activity involved in apoptotic process, apoptotic process involved in morphogenesis, cellular response to gamma radiation
Fbll1 (ENSCPOG00000025737)	5	4	5	4	CDS	Yes	Methylation, rRNA processing, tRNA processing
Gnas (ENSCPOG00000009248)	5	4	5	4	CDS	Yes	Genetic imprinting, DNA methylation, tissue homeostasis, energy reserve metabolic process, GTP catabolic process, signal transduction, G-protein coupled receptor signalling pathway, adenylate cyclase-activating dopamine receptor signalling pathway, postembryonic development, multicellular organism growth, response to drug, skin development
Hspb2 (ENSCPOG00000012929)	4	4	4	4	CDS	No	Response to heat, response to stress, somatic muscle development
Rara (ENSCPOG00000007730)	5	5	5	5	CDS	Yes	Germ cell development, spermatogenesis, regulation of gene expression, transcription, DNA-templated, protein phosphorylation, signal transduction, Sertoli cell fate commitment, positive regulation of cell proliferation, response to estradiol, negative regulation of tumour necrosis factor production, positive regulation of interleukin (Il13, Il4, Il5), multicellular organism growth, apoptosis, steroid hormone mediated signalling pathway, cell differentiation, positive regulation of T-helper 2 cell differentiation, positive regulation of cell cycle, regulation of translational initiation, retinoic acid receptor signalling pathway, positive regulation of binding
Sox13 (ENSCPOG00000006604)	4	5	4	4	CDS	No	Regulation of gene expression, sequence-specific binding, DNA template regulation of gamma-delta T cell differentiation
Svp (ENSCPOG00000025237)	5	5	4	4	Promoter	Yes	Copulation, DNA binding, transcription
Wiz (ENSCPOG00000009735)	5	4	4	4	Promoter and CDS	Yes	Positive regulation of nuclear cell cycle DNA replication, protein stabilization, protein heterotrimerization, metal ion binding

Selection of genes and gene promoters with annotated DMRs occurring after comparison of $F0L_C$ vs. $F0L_H$ DNA methylation patterns, as well as $F1L_C$ vs. $F1L_H$ and $F1T_C$ vs. $F1T_H$ in at least four fathers groups (F-J), in both generations (F0, F1) and both tissues (liver, testes). Genes were selected for male-specificity, function in heat response, and gene regulation. Their potential regulatory impact was derived from GO terms. Promoters consisting of or being located within a CpG island (CGIs) are marked with either Yes or No.
†GO terms were not yet annotated for Cavia and were adopted from mice (http://www.ebi.ac.uk/QuickGO; http://amigo.geneontology.org).

*hypo*methylation in $F0L_H$ and $F1L_H$, whereas in $F1T_H$ it was *hyper*methylated with a slightly shifted DMR.

Thermoregulation genes

To further investigate the general response to heat, we examined the 19 known mammalian thermoregulation genes that are annotated in the guinea pig genome for significant methylation changes of single CpGs among control and heat groups, and which were not recognized by the METHPIPE software (see Table S6, Supporting information; for all genes recognized by MethPipe and genes including mCpGs in $F0L_C$ vs. $F0L_H$, $F1L_C$ vs. $F1L_H$ and $F1T_C$ vs. $F1T_H$). Surprisingly, 13 of the 19 thermoregulation genes displayed significant mCpG changes (Fig. S2, Supporting information). One gene, *Slc27a1,* was also detected by METHPIPE and therefore assured twice. We also found 12 genes with mCpG changes in their promoters and/or CDS (Fig. S2, Supporting information): 10 in $F0L_C$ vs. $F0L_H$ and $F1T_C$ vs. $F1T_H$, 12 in $F1L_C$ vs. $F1L_H$, 9 we found in all three comparisons. In F0 and F1 animals, the highest numbers of mCpG changes per gene were found for *Adrb2*, *Dbh*, *Stat3, Slc27a1* and *Cidea*.

Discussion

Main findings

We demonstrated immediate and inherited paternal epigenetic response with a potential adaptation reaction that occurred in response to increased ambient temperature in a wild genetically heterogeneous mammal species, the Wild guinea pig. We implicate a strong ecological relevance, because we identified shared patterns of DNA methylation changes within and across generations as well as in different organs, indicating a general response to the exposure. To the best of our knowledge this is the first study showing epigenetic changes to temperature increase in a mammal species.

Paternal epigenetics of ecological relevance

So far, only a few studies considered paternal intergenerational epigenetic effects (Carone *et al.* 2010; Ng *et al.* 2010; Vyssotski 2011; Vassoler *et al.* 2013; Dias & Ressler 2014; Wei *et al.* 2014). They show e.g. that nutrition changes to a high fat diet on one hand caused abnormal DNA methylation patterns in daughters' pancreas (Ng *et al.* 2010), while on the other hand nutrition changes to a low protein diet altered expression of cholesterol genes in sons (Carone *et al.* 2010). Fear-conditioning of fathers to the odour acetophenone caused lower methylation in the receptor gene *Olfr151* in sperm DNA, leading to an increased expression of the receptor in the animals noses, and enhancing sensitivity to the smell in fathers as well as in naïve sons and grandsons exposed to the smell (Dias & Ressler 2014). To achieve a comprehensive understanding of transgenerational epigenetic effects, paternal effects need to be studied also, as well as their role in adaptation.

Although the exact mechanisms of transgenerational epigenetic inheritance and the differences between maternal and paternal effects are still unclear, the phenomenon itself is progressively confirmed (Weaver *et al.* 2004; Champagne 2008; Carone *et al.* 2010; Ng *et al.* 2010). Advantages in examining paternal effects are that effects during pregnancies and behaviourally induced effects can be excluded (in many wild mammal species males are not actively involved in rearing the offspring), simplifying the detection underlying molecular mechanisms (Curley *et al.* 2011).

The majority of studies on epigenetics have focused on medical research in humans and model species, while studies on ecologically relevant traits in non-model species are still rare (Richards 2006; Pertoldi & Bach 2007; Bossdorf *et al.* 2008; Lea *et al.* 2015), but mandatory to comprehend the complexity of adaptation processes in a constantly changing environment.

Immediate and inherited epigenetic response

The DMRs identified between $F0L_C$ vs. $F0L_H$ reflect the 'immediate response'. Epigenetic changes thus represent a mechanism to facilitate rapid adaptation. As our experimental setup was designed to also include the offspring, the duration of the heat exposure was determined by the length of spermatogenesis which in Wild guinea pigs is 2 months. Thus, we do not know the minimum exposure time necessary to initiate the 'immediate response' but expect it to be even shorter than 2 months.

Contrasting the comparisons of naïve sons vs. heat-exposed fathers and fathers before vs. after heat-exposed, we detected both different and shared DMRs (Fig. 2). The random approach supported the treatment specific reaction, because using randomized data resulted in <2 DMRs per calculation. The shared DMRs indicate a general adaptation to temperature. We believe that the paternal transmission through epigenetic effects may prepare the sons for increased external temperature based on the fathers' experiences. Thus, wild dispersing males implement paternal epigenetic pattern with ecological relevance, which may have an impact on evolutionary processes. The functions of the underlying genes in which DMRs were detected support these conclusions. A closer relation between methylation patterns of fathers to their own sons in

comparison to other fathers' sons was not detected. This may be explained by the fathers close relation among each other (brothers) and again supports the general response to temperature increase.

However, a within-group and between-group comparison using the variance of methylation ratio per position was significantly different in all groups. These results were likely caused by the great amount of data points, even after reduction to the annotated DMR data set (Fig. 2).

The consideration of female offspring in future studies will be worthwhile as gender-cross-inheritance has been detected e.g. in Spargue–Dawley founder rats, where daughters but not sons showed impairment of glucose tolerance after paternal exposure to high fat diet (Ng *et al.* 2010).

Interestingly, the greatest amount of DMRs (2484 DMRs) was found in testis of sons before and after the fathers-heat treatment ($F1T_C$ vs. $F1T_H$), while liver samples of identical animals revealed only 471 DMRs, those results strongly point to a tissue-specific difference. An explanation may be the early postnatal stage of the testis of the 7 days-old animals, in which spermatogonia occur differentiated and undifferentiated (Kubo *et al.* 2015), attended by a differentially DNA methylation, due to its role in this differentiation process. Further analysis will be needed to specify annotated regions and their functions. Here, we like to point out that F0 males grew ~7.5 months older until the second mating, giving the chance that DNA methylation may have changed in age-effective manner.

Specific differential methylation and general response

The investigation of the CpG sites revealed methylation changes in methylation patterns in numerous loci. Interestingly, the genome-scale cytosine-methylation level was not significantly changed among control and heat groups ($F0L_C$ vs. $F0L_H$, $F1L_C$ vs. $F1L_H$, $F1T_C$ vs. $F1T_H$). We found differentially methylated regions in annotated regions (annotated DMRs), between both generations (F0 and F1) as well as between both organs (liver and testis).

These findings thus demonstrated (i) specific paternal epigenetic transmission, and (ii) a general epigenetic response to heat. We further assume a potential greater relevance of DMRs in contrast to single cytosine-methylation and a similar DNA methylation mechanism directed to specific loci, revealing a general response among individuals (see Fig. 2). The specificity of the mechanism was further supported by the function of the genes found, to be impacted in response to temperature increase, including heat shock proteins, thermoregulation genes, male-specific genes and genes important in gene regulation. One example for a male specific gene is *Svp*. Besides stabilizing spermatozoa, additional functions are assumed in other tissues where *Svp* is also expressed, such as liver, lung and kidney (Hagstrom *et al.* 1996). In our study, *Svp* might demonstrate a male-specific effect heat exposure has on the 'immediate' and 'inherited' paternal epigenetic pattern.

Because control and heat groups in fathers and sons shared numerous genes with changes in their methylation levels, the heat experiences of the father appeared to be transmitted through those genes to the offspring. The offspring might thus be better adaptable in case of thermal changes. To study the inherited adaptation in more detail, an extended study, also exposing F1 generations to temperature changes would be needed.

Interestingly, by close inspection of *hypo* and *hyper*methylation of selected DMRs we observed compositions of DMRs, exhibiting certain patterns. Even though the number of individuals is too limited to provide statistical strength, we postulate that those patterns reflect a combination of 'switches' controlling epigenetic regulation of subsequent gene expression in a (exposure) time dependent manner. Thus, short exposure to increased temperature will only onset a certain number of 'switches', not yet sufficient to effect gene regulation. Extended exposure may then lead to the accumulation of onset 'switches' up to the threshold above which gene expression in impacted. Our results indicated a more complex mechanism to the often-assumed simplified single position switch. Therefore, using a genetically heterogeneous species rather than inbred strains or cells facilitated to study epigenetic responses in a naturally setting and may have provided new insights into the mechanism itself. Here, we demonstrated on a molecular level that the methylation level of heat-induced rapid compensatory 'immediate response' in fathers was partly reversely transferred to the next generation as heritable epigenetic modifications. This supports the 'transgenerational epigenetic compensation' concept (Vyssotski 2011), stating that epigenetic inheritance will promote compensation of disturbed functionality across generations and may entail reverse changes in naïve offspring. An alternative/additional explanation might be an insufficient transgenerational persistence of modifications, meaning a change in the developing or developed offspring.

Epigenetic response to heat among taxa

Environmental temperature acts as a strong selection factor, shaping the phenotype, behaviour and physiological processes of prokaryotes and eukaryotes (Sonna *et al.* 2002). Even though, the epigenetic response to heat exposure has not been studied in mammals, investigations have been done for other species, including plants, corals, insects (fruit fly), chicken and fish.

In the model plant Arabidopsis, exposure to heat resulted in transcriptional activation of repetitive elements that are epigenetically controlled (Pecinka *et al.* 2010). Interestingly, these changes occur without a change in the DNA methylation patterns and cause only slight changes in the histone modifications. The activation is likely to be controlled by chromatin reassembly, with recovery in the same generation, but impairment in the next generation. Also, the plant flowering locus is epigenetically regulated during the temperature-dependent vernalization process (Bouché *et al.* 2015). In this issue, James Dimond and Steven Roberts show that in response to heat in three reef building coral species genes expression changed associated with a decrease in DNA methylation (Dimond & Roberts 2015). In the fruit fly *Drosophila melanogaster*, heat shock and osmotic stress induced phosphorylation of transcription factor 2 (*dAFT-2*) and also caused an impaired chromatin structure by releasing ATF-2 from the heterochromatin for several generations (Seong *et al.* 2011). In chicken an 'immediate response' was seen in the frontal hypothalamus, where thermal heat conditioning changed gene expression of the brain-derived neurotropic factor (*Bdnf*) after exposure and re-exposure which coincided with changes in CpG methylation in the *Bdnf* promoter region (Yossifoff *et al.* 2008). A wild life study comparing artic and tropical fish species has revealed greater global DNA methylation (using Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC)) in warm than in cold temperatures (Varriale & Bernardi 2006).

An example of crucial impact of global temperature increase is seen in several vertebrates, including turtles, crocodiles and some fish species, where sex-determination is a temperature-dependent process (Valenzuela & Lance 2004). In European sea bass females, promoter methylation occurred at the gonadal aromatase (cyp19a), the enzyme that converts androgen to estrogen (Navarro-Martin *et al.* 2011).

Thus, temperature has a strong impact on environmental processes. However, epigenetic mechanisms, such as the DNA methylation machinery itself, differ among taxa (e.g. invertebrates vs. vertebrates) (He *et al.* 2011) constraining a direct comparison among them and to our data. In contrast with mammals DNA methylation levels are very low in Drosophila, and in plants methylation occurs not only in a CG, but also quite strongly in a CHG and CHH context, whereby fishes and amphibians show a higher DNA methylation than mammals and birds (Jabbari *et al.* 1997). In male mammals, temperature increase may reduce reproductive fitness directly by impairing spermatogenesis, induction of apoptosis and DNA damage, and reduction of sperm quality (Falk & Issels 2001; Pagani *et al.* 2007; Sharpe 2010).

Conclusion

In conclusion, we hypothesize that the observed inherited epigenetic plasticity after paternal heat exposure is ecologically relevant, by providing the offspring with improved long-term resilience to environmental temperature increase. It is noteworthy that $F0_H$ sires and naïve $F1_H$ sons differed in the majority of their epigenetically affected genes and regions. An explanation might lie in the different developmental stages (ontogenesis), and/or in differences in mechanisms required for either short-term or long-term epigenetic processes with a potential adaptational reaction in response to temperature increase.

Wild guinea pigs live in a wide range of habitats at different altitudes and temperatures, reflecting the ability of this species to adapt. Thus, while our study extends the understanding of the paternal epigenetic response to heat, we also hope it will initiate similar research on other mammalian species to eventually decipher the underlying mechanism in more detail. This response mechanism may be critically important for species survival as global temperatures are rising.

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Competing interests

The authors declare no competing financial interests.

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K.J., J.F. and A.W. designed the study. K.S. and F.G. performed animal handling and biopsy sampling. D.L., M.J., T.Z.C. and F.H. performed data analysis. A.W. and J.F. wrote the manuscript. K.J. contributed scientific discussion and reviewing of the manuscript. RRBS library preparation and sequencing was done at Zymo Research (Irvine, USA). All authors reviewed the manuscript and approved the final version.

Data accessibility

Next generation sequencing data were uploaded to the National Center for Biotechnology Information Short Reads Archive (http://www.ncbi.nim.nih.gov/sra) and will be publicly accessible under the SRA study accession number SRP048942 in.fastq file format. Raw data, coverage, methylation ratios (file names: F0L_coverage_MethLevels, F1L_coverage_MethLevels, F1T_coverage_MethLevels), DMR tables, including GO terms (files name: Venn_data_genes_table_F0LF1LF1T_withGO) are accessible on Dryad http://dx.doi.org/10.5061/dryad.0f8q1. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to A.W. (Weyrich@izw-berlin.de; alexandraweyrich@gmx.de).

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 DMRs located in the Seminal vesicle polypeptide (*Svp*) gene.

Fig. S2 Thermoregulation pathway genes showing single mCpG changes detected in heat group compare to control.

Table S1 Sample list and Reduced Representation Bisulfite Sequencing (RRBS) results of livers of fathers before and after heat treatment ($F0L_C$ and $F0L_H$).

Table S2 Sample list and Reduced Representation Bisulfite Sequencing (RRBS) results of livers of sons sired before and after heat treatment of their fathers ($F1L_C$ and $F1L_H$).

Table S3 Sample list and Reduced Representation Bisulfite Sequencing (RRBS) results of testis of sons sired before and after heat treatment of their fathers ($F0T_C$ and $F0T_H$).

Table S4 Number of annotated regions with DMRs per father group.

Table S5 Annotated regions with DMRs per father group after comparing $F0L_C$ vs. $F0L_H$, $F1L_C$ vs. $F1L_H$ and $F1T_C$ vs. $F1T_H$.

Table S6 Number of genes in CpGs and DMRs.