Large-scale identification of differentially expressed genes during pupa development reveals solute carrier gene is essential for pupal pigmentation in *Chilo suppressalis*

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ABSTRACT

Insects undergo metamorphosis, involving an abrupt change in body structure through cell growth and differentiation. Rice stem striped borer (SSB), *Chilo suppressalis*, is one of the most destructive rice pests. However, little is known about the regulation mechanism of metamorphosis development in this notorious insect pest. Here, we studied the expression of 22,197 SSB genes at seven time points during pupa development with a customized microarray, identifying 622 differentially expressed genes (DEG) during pupa development. Gene ontology (GO) analysis of these DEGs indicated that the genes related to substance metabolism were highly expressed in the early pupa, which participate in the physiological processes of larval tissue disintegration at these stages. In comparison, highly expressed genes in the late pupal stages were mainly associated with substance biosynthesis, consistent with adult organ formation at these stages. There were 27 solute carrier (SLC) genes that were highly expressed during pupa development. We knocked down *SLC22A3* at the prepupal stage, demonstrating that silencing *SLC22A3* induced a deficiency in pupa stiffness and pigmentation. The RNAi-treated individuals had white and soft pupa, suggesting that this gene has an essential role in pupal development.

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1. Introduction

Insect development is characterized by metamorphosis that involves abrupt changes in body structures. It has been reported that the balance between ecdysteroid and juvenile hormone (JH) is crucial in regulating metamorphosis (Cho et al., 2014; Jindra et al., 2013; Yamanaka et al., 2013). The orchestrated actions of ecdysteroid and JH, and their downstream physiological responses regulate development during metamorphosis (Riddiford, 2012). In the process of transforming from larvae to adult, two developmental programs are activated by ecdysteroid in pupa, the destruction of larval tissues by programmed cell death and the formation of adult tissues by cell differentiation (Yin and Thummel, 2005).

The regulation mechanism of pupa development has been well studied in *Drosophila melanogaster*, *Bombyx mori*, *Tribolium castaneum*, etc (Jindra et al., 2013; Riddiford, 2007, 2008, 2012; Truman and Riddiford, 2007). As a model organism, *D. melanogaster* serves as an important system to identify genes involved in metamorphosis development (Jones et al., 2010). JH has a key role in the prepupal development of *D. melanogaster* (Riddiford et al., 2010). Many genes such as protein tyrosine phosphatase PTP52F was confirmed to be indispensable for larva-pupa transition in *Drosophila* (Santhanam et al., 2013). The silkworm *B. mori* is another model insect which has been well studied to uncover the mechanism of metamorphosis. As well known, JH also has a key role in larval-pupal transformation of the cecropia silkworm (Riddiford, 1972). Fifty-seven genes were specifically expressed in the intersegmental muscle of larvae whereas another 54 genes were in the pupa of *B. mori*. Twelve proteins were also found to be significantly upregulated during pupation in *B. mori* (Zhang et al., 2007a). A cDNA microarray analysis indicated that the expression of *Bombyx* genes were clustered into 12 patterns from

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the first instar larvae to pupa (Zhang et al., 2007a,b). Homeodomain proteins participate in regulation of embryonic development. A study indicated that they also regulate the larval-to-pupal transition by interacting with another homeodomain transcription factor, BmAbd-A (Deng et al., 2012).

Recently, RNA sequencing (RNA-Seq) has been used to screen genes associated with developments in several non-model insects. In *T. castaneum*, a large-scale and unbiased iBeetle screen identified many novel genes participating in oogenesis, embryogenesis and metamorphosis (Schmitt-Engel et al., 2015), which is a good supplement to *Drosophila* study. Transcriptome analysis of wing disc from 6-day-old fifth instar larvae (L5D6), prepupae (PP) and pupae (PO) identified thousands of genes involved in larva-to-pupa metamorphosis development (Ou et al., 2014). A transcriptome-wide analysis of gene expression of five cycle stages of bumblebee *Bombus terrestris* showed that genes involved in amino acid storage, metabolism, immunity and olfaction were differentially expressed (Colgan et al., 2011). Developmental transcriptome analysis of the small white butterfly *Pieris rapae* yielded 849 genes that involved in seven canonical development signaling pathways (Qi et al., 2016). Similar strategy has also been applied to study *Aethis lepigone*, an important insect pest of maize (Li et al., 2013), and the synanthropic fly *Chrysomya megacephala* (Wang et al., 2015), finding hundreds of or thousands of differentially expressed genes involved in larvae or pupa development.

Rice striped stem borer (SSB), *Chilo suppressalis* Walker, is one of the most notorious and destructive rice pests. It is widely distributed from Asia to the Pacific, causing huge rice yield losses (He et al., 2013). Here, we used a customized microarray to study the gene expressions at seven developmental time-points during pupal development of SSB, identifying 622 differentially expressed genes. *SLC22A3* was selected for further study and it was shown to have an essential role in pupal pigmentation in SSB.

2. Materials and methods

2.1. Insect rearing and specimen preparation

Insects were collected from a rice field in Cangnan County in Zhejiang Province, China. The insects were kept on rice seedlings at 28 ± 1 °C under a 16-h photoperiod and >80% relative humidity. For microarray analysis, insects at seven developmental time-points during pupal development were selected, including aging larval (aL), prepupal (pP), early pupal (eP), compound eye formation (cE), pretarsal formation (pT), pupal elongation (Pe), and adult (Ad) stages (Fig. 1). aL is late larvae which stop eating and begin to find dry place for pupation. pP is the insects at wandering stage. eP is early pupae within 10 min and the colour of the pupae is still white. cE is the pupa whose compound eye became brown under the dissecting microscope. pT is the pupa when pretarsus can be observed under the dissecting microscope. Pe is the pupa just before molting, when the pupae become golden and much longer. Ad is the adult molting within 8 h. Four individuals (two males and two females) were used for each time-point as one biological replicate. There is a “Y” shaped feature at the abdomen end of female pupae but not male. By this feature, we can identify females and males. Insects were frozen in liquid nitrogen and stored at -70 °C before use. All of the experiments were repeated in triplicate.

2.2. Microarray probes design

We developed a transcriptome of SSB (SRA050703) (Yin et al., 2014), producing 22,197 unigenes by using Trinity with the default parameters (Grabherr et al., 2011). The contigs were annotated by

using BLASTX against the GenBank non-redundant (nr) database with an E-value cutoff set to 10^{-5} . We designed a customized standard 12×135 K microarray with a NimbleGen hybridisation system (Roche, Swiss) to study the expression of 22,197 SSB unigenes. Six different probes that ranged from 45- to 60-mers were designed based on the sequences of unigenes.

2.3. Microarray experiment

The microarray experiment was carried out following the procedure described previously (Guo et al., 2005) with a little modification. All experiments were carried out in CapitalBio Co. Ltd (Beijing, China).

2.3.1. cRNA preparation

Total RNA was extracted using Trizol reagent and purified using NucleoSpin® RNA clean-up (Macherey-Nagel, Germany). Eberwine's linear RNA amplification method and subsequent enzymatic reaction was used for cDNA synthesis and labelled with a fluorescent dye (Cy3-dCTP). The CapitalBio cRNA Amplification and Labeling Kit (CapitalBio, China) were used for cRNA preparation.

2.3.2. cDNA synthesis and labelling

Briefly, 2 µg amplified RNA was mixed with 4 µg random nanomer, denatured at 65 °C for 5 min, and cooled on ice. Then, 5 µL of 4× first-strand buffer, 2 µL of 0.1 M DTT, and 1.5 µL CbcScript II reverse transcriptase were added. The mixture was incubated at 25 °C for 10 min, then at 37 °C for 90 min. The cDNA products were purified using a PCR NucleoSpin Extract II Kit (Macherey-Nagel) and vacuum evaporated to 14 µL. The cDNA was mixed with 4 µg random nanomer, heated to 95 °C for 3 min, and snap cooled on ice for 5 min. Then, 5 µL Klenow buffer, dNTP, and Cy3-dCTP (GE Healthcare, United Kingdom) were added to final concentrations of 240 µM dATP, 240 µM dGTP, 240 µM dTTP, 120 µM dCTP, and 40 µM Cy-dCTP. Next, 1.2 µL Klenow enzyme was added, and the reaction was performed at 37 °C for 90 min. Labelled cDNA was purified with a PCR NucleoSpin Extract II Kit (Macherey-Nagel) and re-suspended in elution buffer.

2.3.3. Hybridisation, washing, and scanning

The experiments were carried out according to the NimbleGen Expression user's guide and performed at CapitalBio Corporation (Beijing, China). Labelled samples were dried and re-dissolved in the hybridisation solution. Hybridization was performed at 42 °C for 14 h with NimbleGen Hybridization System, Array were washed in wash buffer I and II and III supplied by NimbleGen and dried in NimbleGen Microarray Dryer. After drying, the arrays were scanned using an MS200 scanner (NimbleGen) with 2 µm resolution, and NimbleScan software (NimbleGen) was used to extract the fluorescent intensity raw data from the scanned images (see manufacturer's user guide).

2.4. Data analysis

The expression data of the probes were normalized using quantile normalisation (Bolstad et al., 2003) and expression data of the genes were generated using the Robust Multichip Average (RMA) algorithm with default parameters (Irizarry et al., 2003a,b). To identify differentially expressed genes (DEGs), Significant Analysis of Microarray (SAM) software (version 3.02) (Tusher et al., 2001) was used with a threshold of false discovery rate of <5%. The cutoff was set as q-value (%) = 0 and fold change ≥ 2 . Hierarchical clustering with the average linkage method was performed with Cluster 3.0 software (Eisen et al., 1998) and cluster results were visualised with the TreeView program (Eisen et al., 1998). Insect

Fig. 1. Heat map of differentially highly expressed genes in seven developmental stages during pupal development. These genes are bundled into six groups. From top to bottom, there were 79 genes up-regulated at the pretarsal formation stage in the first row. 182 genes were highly expressed from aging larval to early pupal stages in the second row. There were 154, 70 and 79 genes highly expressed at compound during eye formation, early pupal and aging larval stages in the next 3 rows, respectively. In the last row, 58 genes were highly expressed in the pupal elongation and adult stages.

pathway analysis was conducted using iPathCons in InsectBase (Yin et al., 2016; Zhang et al., 2014).

2.5. RT-PCR and qPCR

Total RNA was isolated using Trizol reagent followed by RNase free DNase I treatment (Roche Diagnostics, Mannheim, Germany). The cDNA was synthesised using PrimeScript® RT Master Mix kit (Takara, Japan) with oligo (dT) and 500 ng total RNA. The primers for reverse-transcription PCR (RT-PCR) were designed using Primer Premier 5.0 and are found in Table S1. Premix Taq® Version 2.0 kit (Takara) was used for the PCR reactions. The primers for quantitative real time PCR (qPCR) were designed with an online tool (<http://www.idtdna.com/Scitools/>) and synthesised in GenScript (Nanjing, China). The primer sequences are found in Table S1. The qPCR reactions were performed using an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). A SYBR Premix Ex Taq kit (Takara) was used with a standard PCR protocol of denaturing at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 31 s. Melting curves were determined by heating the sam-

ple to 95 °C for 15 s, followed by 60 °C for 15 s, and again heating to 95 °C for 15 s. The actin gene was used as the internal control. Data were analyzed using the $2^{-\Delta\Delta C_t}$ method (Pfaffl, 2001).

2.6. RNA interference

The siRNAs used in the RNAi experiments were chemically synthesised by GenePharma Co., Ltd. (Shanghai, China). The siRNA sequences are found in Table S1. The double-stranded siRNAs were purified by high-performance liquid chromatography, and dissolved in diethylpyrocarbonate-treated water (Milli-Q-grade) to a final concentration of 4 mg siRNA/mL H₂O. Then 1 µL (4 µg) of siRNA was injected into the larvae or pupa using a microneedle. The needles were pulled from glass capillaries (1.0 mm outer diameter and 0.50 mm inner diameter) using a micropipette puller (Model P-87, Sutter Instruments Co., Novato, CA). To avoid leakage of siRNAs, needles were held still at the injection point for 30 s. The shuffled siRNAs were used as the negative control. Thirty insects were used for each treatment and all of the experiments were per-

formed in triplicate. A *t*-test was used for statistical analysis and the significance level was set to a *p*-value of <0.05.

3. Results

3.1. Gene expression patterns during pupal development

A customized NimbleGen microarray was used to examine the expressions of all 22,197 SSB transcripts at seven developmental time-points during pupal development (Fig. 1). All seven stages were analyzed with three biological repeats. An unsupervised clustering showed biological repeats of different stages had high reproducibility (Fig. S1). The results indicated that gene expression in pupal development could be classified into two subgroups. One is an early pupal subgroup, consisting of aging larval, prepupal, early pupal, and compound eye formation stages. These four stages shared similar gene expression patterns. The other is a late pupal subgroup, including pretarsal formation, pupal elongation and adult stages. The gene expressions of these three stages were clustered together. This result is consistent with the present understanding of pupal development. The larvae tissues are disintegrated during early pupal stages whereas adult organs form in the late pupal stages.

3.2. Differentially expressed genes in pupal development

To identify DEGs in pupal development, the ratio of the highest signal value (rank1) to the second highest (rank2) was calculated for each gene. When the cutoff was set with a *q*-value = 0 and a ratio >2, there were 622 DEGs at different developmental time-points. Of them, 79, 70, 154, and 79 genes were highly expressed at aging larval, early pupal, compound eye formation, and pretarsal formation stages, respectively (Figs. 1 & 2). There were 182 genes co-expressed highly at aging larval, prepupal, and early pupal

stages, while 58 genes were co-expressed highly at the pupal elongation and adult stages (Fig. 2). The differentially expressed genes at each developmental stage are listed in Supplemental file 1.

Among these genes, chitinases and cuticle proteins genes were upregulated at aging larval and early pupal stages. Hormone receptor 4 (*HR4*), a central regulator of genetic responses to ecdysteroid at the onset of metamorphosis (King-Jones et al., 2005), was highly expressed at the compound eye formation stage. The functions of *BR-C*, *E74*, *FTZ-F1* and *Kr-h1* have been well studied in the metamorphosis development (Kayukawa et al., 2014; Stilwell et al., 2003; Sultan et al., 2014). We examined the expression patterns of these genes from microarray data and found that they were in general consistent with previous reports, showing the microarray data was reliable for further analysis. Among which, *BR-C* highly expressed from aging larvae and decreased rapidly after pupation, which is consistent with the expressional pattern in the red flour beetle and silkworm (Parthasarathy et al., 2008; Reza et al., 2004). *E74* highly expressed around pupation. *FTZ-F1* was expressed throughout the pupa, with especially high-expression the late pupal stage. The transcript of *Kr-h1* was abundant at the prepupal stage but was undetectable from early pupae to middle pupae. It gradually increased from late pupae to adult, and the transcript level was the highest at adult stage (Fig. S2).

Pathway analysis of DEGs was conducted using iPathCons (Zhang et al., 2014). In total, 83 pathways were identified, 22 of which could be classified into three categories of energy metabolism, sensory system and hormone-related pathways (Table S2). The highly expressed genes at the aging larval, prepupal and early pupal stages mainly involved protein digestion and absorption (ko04974), amino sugar and nucleotide sugar metabolism (ko00520), and carbohydrate digestion and absorption (ko04973). These pathways are closely associated with substance metabolism, which is consistent with the physiological process of tissue disintegration at these stages. In contrast, the highly expressed genes

Fig. 2. The patterns of differentially highly expressed genes. The peak(s) means the stages at which the transcript abundances of the genes were the highest. The gene numbers were indicated at the peak(s).

at the pupal elongation and adult stages were signal transduction pathways such as taste transduction (ko04742), phototransduction (ko04744), retinol metabolism (ko00830) and carotenoid biosyn-

thesis (ko00906). There were 9 DEGs in hormone-related pathways. We selected them for qPCR validation and the expression of eight genes were consistent with the microarray data (Fig. 3).

Fig. 3. qPCR validation of differentially highly expressed genes. aL: aging larvae; pP: prepupae; eP: early pupae; cE: compound eye formation stage; pT: pretarsus; Pe: pupae elongation stage; Ad: adult. Error bars indicate standard deviation (s. d.).

3.3. Knockdown of *SLC22A3* led to white and soft pupa

Among the list of DEGs, there were 27 solute carrier (*SLC*) genes (Table 1). Among these *SLC* genes, *SLC22A3* was the most highly expressed gene at the early pupal stage with a ratio of 39.62. *SLC22* is known to participate in the absorption and excretion of drugs, xenobiotic, and endogenous compounds in mammal intestines, liver, and/or kidney (Pelis and Wright, 2014). We chose *CsuSLC22A3* to study its function in pupal development of SSB. Phylogenetic analysis of this gene with other *SLC* subfamily genes indicated that *CsuSLC22A3* shared high relatedness with the homo-

logs in other lepidopteran insects (Fig. S3). Pfam domain analysis showed that *CsuSLC22A3* had the conserved domains of *SLC* gene family such as major facilitator superfamily-1 (MSF_1) and Sugar (and other) transporter (Sugar_tr) (Fig. S4).

We used qPCR to examine the expression of *CsuSLC22A3* during larval, pupa and adult development, suggesting that the highest expression appeared at the fifth day in the fifth instar larvae (Fig. 4). It was highly expressed at the early pupa stage, which was consistent with the microarray analysis (Fig. 5a). We successfully knocked down *CsuSLC22A3* gene by injecting small interfering RNA (siRNA) at the prepupal stage. The transcript abundance of

Table 1
Differentially expressed solute carrier family genes in different stages.

Development Stages	GeneID	Ratio	Homologous genes in blastx results
Aging larvae	Singletons104470	6.87	Solute carrier family 22 member 2 (<i>SLC22A2</i>)
	Singletons24463	5.59	Solute carrier family 22 member 15 (<i>SLC22A35</i>)
	Singletons28023	2.55	Solute carrier family 2, facilitated glucose transporter member 8 (<i>SLC2A8</i>)
	Singletons9176	2.22	Proton-coupled amino acid transporter 1 (<i>SLC36A1</i>)
Aging larvae- prepupae- early pupae	Singletons21491	5.66	Proton-coupled amino acid transporter 1 (<i>SLC36A1</i>)
	Singletons8035	3.03	Organic cation transporter protein (<i>Orct</i>)
	Singletons3013	2.50	Solute carrier family 22 member 3 (<i>SLC22A3</i>)
	Singletons81470	2.42	Organic cation transporter-like protein (<i>Orct2</i>)
Early pupae	Singletons102752	39.62	Solute carrier family 22 member 3 (<i>SLC22A3</i>)
	Singletons23617	2.67	Organic cation transporter protein (<i>Orct</i>)
	Singletons110766	2.60	Sodium-independent sulfate anion transporter (<i>SLC26A11</i>)
	Singletons24544	2.11	Solute carrier family 2, facilitated glucose transporter member 8 (<i>SLC2A8</i>)
Compound eye formation stage Pretarsus	Singletons8003	2.05	Solute carrier family 46 member 3 (<i>SLC46A3</i>)
	Singletons99734	7.64	Sodium-independent sulfate anion transporter (<i>SLC26A11</i>)
	Singletons110102	6.65	Sodium-independent sulfate anion transporter (<i>SLC26A11</i>)
	Singletons114116	6.58	Solute carrier family 2, facilitated glucose transporter member 8 (<i>SLC2A8</i>)
Pupae elongation stage-adult	Singletons114849	5.98	Solute carrier family 2, facilitated glucose transporter member 8 (<i>SLC2A8</i>)
	Singletons113765	5.50	Solute carrier family 22 member 11 (<i>SLC22A31</i>)
	Singletons10224	3.88	Sodium-independent sulfate anion transporter (<i>SLC26A11</i>)
	Singletons27909	2.47	Long-chain fatty acid transport protein 4 (<i>SLC27A4</i>)
	Singletons15995	7.83	Solute carrier family 2, facilitated glucose transporter member 2 (<i>SLC2A2</i>)
	Singletons24811	3.97	Monocarboxylate transporter 6 (<i>SLC16A5</i>)
	Singletons78036	2.95	Zinc transporter 8 (<i>SLC30A8</i>)
	Singletons23250	2.90	Monocarboxylate transporter 13 (<i>SLC16A13</i>)
	Singletons109185	2.77	Solute carrier family 2, facilitated glucose transporter member 8 (<i>SLC2A8</i>)
	Singletons26902	2.65	Organic cation transporter protein (<i>Orct</i>)
	Singletons115293	2.49	Monocarboxylate transporter 9 (<i>SLC16A9</i>)

Fig. 4. The expression profile of *CsuSLC22A3* gene during larva, pupa and adult development. The results indicated that this gene were highly expressed before molt.

Fig. 5. RNA interference of *CsuSLC22A3* induced a soft and milky pupa. (a) qPCR validation of *SLC22A3* gene. (b) The microarray signal values of *CsuSLC22A3* genes. (c) Abnormal phenotype of RNAi-treated individuals. Thirty insects were used for each treatment. All experiments were conducted in triplicate.

RNAi-treated individuals was only 40% of the control level (T-test, $p < 0.05$) (Fig. 5b). Knockdown of *SLC22A3* gene led to deficiency in skeletonisation and pigmentation. A total of 90% of the RNAi-treated individuals showed abnormal phenotypes of minor stiffening at 24–48 h after pupation, resulting in a white and soft epidermis on the abdomen, back and head. Then the pupa shrivelled and died one week after RNAi treatment (Fig. 5c). This result suggests that *SLC22A3* has an essential role in pupal pigmentation and stiffness.

4. Discussion

Pupa is a unique stage in the life cycle of holometabolous insects, in which the larval tissues disintegrate and the adult organs appear (Buszczak and Segreaves, 2000; Truman and Riddiford, 1999). Studying gene regulation in pupal development should be helpful for better understanding programmed cell death and adult organ formation. It is also useful to find environmental friendly targets for RNAi pest control since metamorphosis exists only in insects and other several animal groups (De Loof et al., 2014; Kaneko and Hiruma, 2014). Though the molecular mechanism of insect metamorphosis has been well studied in larvae, less studies have been carried out in *C. suppressalis*. Here, we identified 622 DEGs during pupation, pupa development and eclosion of SSB, which add the knowledge of genes associated with insect metamorphosis. The results indicated that the genes associated with substance metabolism, protein degradation, and cuticle digestion were highly expressed in the early pupal stages, whereas genes associated with signal transduction organisation were highly expressed in the late pupal stages, indicating that the active biological processes in the early pupal stages were different from those in the late pupal stages. Uncovering the roles of these genes may provide new insights into programmed cell death, cell proliferation, and tissue stem cells.

Among these DGEs, a high number of *SLC* genes were highly expressed during pupal development. The *SLC* gene family codes for membrane transport proteins that are responsible for transporting a variety of solutes, including charged and uncharged organic molecules, inorganic ions, and ammonia (Cheeseman, 2009). It has been reported that *SLC6* acts as a nutrient-related transporter (Meleshkevitch et al., 2009; Miller et al., 2008; Rudnick et al., 2014). Mutations of *SLC2A9* were responsible for sex-linked translucent larval skin of the silkworm (Cheeseman, 2009; Kiuchi et al., 2011). Thus, we silenced *SLC22A3* in the early pupa of SSB, which led to an abnormal pupal epidermis. A similar phenotype appeared when N- β -alanyldopamine (NBAD) was decreased in the epidermis of the Mediterranean fruit fly (Wappner et al., 1995b). In *B. mori*, disrupting the biogenesis of NBAD resulted in white pupa (Kiuchi et al., 2011; Wappner et al., 1995a) and melanic pupa (Dai et al., 2015; Wappner et al., 1996a,b). Since the *SLC22A3* gene is an organic anion transporter (Koehler et al., 1997), we hypothesized that *SLC22A3* might be involved in transporting NBAD or its precursor into the cuticle membrane, which requires further validation.

We identified a set of genes involved in larval-pupal and pupal-adult transition. It has been reported that complete loss of JH in *mod*-mutated silkworms induced precocious metamorphosis with fewer larval-larval moults, yielding small pupa and small adults (Daimon et al., 2012). Since precocious metamorphosis significantly shortens the larval stage and reduces yield loss, it is possible to control insect pests by inducing pupation at the early larval

stage. This method can control the pest without destroying the insect population and would be an ecologically friendly strategy. The identified 622 DGEs would be helpful in finding safe and efficient targets for RNAi pest control.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jinsphys.2016.12.007>.

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