DNA methylation plays a crucial role during early *Nasonia* development

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Abstract

Although the role of DNA methylation in insect development is still poorly understood, the number and role of DNA methyltransferases in insects vary strongly between species. DNA methylation appears to be widely present among the social hymenoptera and functional studies in *Apis* have suggested a crucial role for de novo methylation in a wide variety of developmental processes. The sequencing of three parasitoid *Nasonia* genomes revealed the presence of three *Dnmt1* (Dnmt1a, Dnmt1b and Dnmt1c) genes and one *Dnmt2* and *Dnmt3* gene, suggesting a role of DNA methylation in *Nasonia* development. In the present study we show that in *Nasonia vitripennis* all *Dnmt1* messenger RNAs (mRNAs) and *Dnmt3* mRNA are maternally provided to the embryo and, of these, *Dnmt1a* is essential during early embryogenesis. Lowering of maternal *Dnmt1a* mRNA results in embryonic lethality during the onset of gastrulation. This dependence on maternal *Dnmt1a* during embryogenesis in an organismal group outside the vertebrates, suggests evolutionary conservation of the function of *Dnmt1* during embryogenesis.

Keywords: DNA methyltransferase, Dnmt, DNA methylation, epigenetics, embryogenesis, development, maternal effect, RNAi, *Nasonia vitripennis*, insect.

Introduction

DNA methylation is the covalent addition of a methyl group to predominantly CpG dinucleotides, and is mediated by DNA methyltransferases. In vertebrates, DNA methylation represents one of the key epigenetic modifications that have been associated with the regulation of gene expression and chromatin structure, in particular during early development (Geiman & Muegge, 2010). This process is involved in many regulatory functions, including X-chromosome inactivation, DNA repair and stability, cell differentiation, alternative splicing, and the establishment of parent-of-origin-specific gene expression (Schwartz & Ast, 2010). In mammals, differentially methylated clusters are established during gametogenesis by the ‘de novo’ methyltransferase DNMT3, while imprinting is maintained by the methyltransferase DNMT1 (Hermann et al., 2004). The DNMT2 methyltransferase is mainly involved in tRNA methylation (Goll et al., 2006).

In contrast to vertebrates, *Drosophila melanogaster* lacks both *Dnmt1* and *Dnmt3* genes and, accordingly, has a sparingly methylated genome without a clearly defined functional significance of methylated sites. Initially, this called into question whether there was any evolutionary or functional role of DNA methylation in insects (Mandrioli & Borsatti, 2006; Phalke et al., 2009; Krauss & Reuter, 2011); however, the discovery of a functional methylation system in the honeybee, consisting of two *Dnmt1* genes and one copy each of *Dnmt2* and *Dnmt3*, provided the first indication for the existence of both de novo and maintenance methylation in insects (Wang et al., 2006). Subsequently, an increasing number of insect genomes have been sequenced, showing a large diversity in the number of *Dnmt* genes present (Lyko & Maleszka, 2011). DNA
methylation genes have been found among multiple species of wasps, bees and ants representing the social hymenoptera, although functional studies have been largely confined to the honeybee (Kronforst et al., 2008). Methylation in adult honeybees (Apis mellifera) seems to be restricted to CpG dinucleotides and occurs mainly in gene bodies, particularly in the proximity of alternatively spliced exons, suggesting a role in splicing regulation (Lyko et al., 2010). Kucharski et al. (2008) showed that siRNA-mediated silencing of the expression of Dnmt3 in newly hatched honeybee larvae gave rise to the development of adult worker bees with developed ovaries, which is normally associated with queen development only. Further roles for Dnmt3 in the honeybee have been shown in the regulation of behaviour and long-term memory (Maleszka et al., 2009; Lockett et al., 2010). In addition, the observed lethality associated with Dnmt3 silencing in embryos suggests a crucial role during embryonic development (Kucharski et al., 2008). Altogether, these results indicate that de novo DNA methylation is crucial for a wide variety of developmental processes in Apis, and it is of interest whether this feature is general throughout the hymenopteran insects.

The Nasonia species group has emerged as a new important hymenopteran model system for investigating complex life history traits and development. The sequencing of the Nasonia genome revealed three Dnmt1 genes (Dnmt1a, Dnmt1b and Dnmt1c), one Dnmt2 gene and one Dnmt3 gene. Dnmt1a and Dnmt1b are structurally very similar, while Dnmt1c is less similar to both Nasonia Dnmt1a and Dnmt1b, but has the invariant cysteine required for catalytic activity (Werren et al., 2010). Of the three Dnmt1 homologues present on the Nasonia genome, Dnmt1a also shows sequence similarity to Dnmt1 homologues in other species, including humans (Werren et al., 2010). This, in conjunction with the fact that all three Dnmt genes were present in the last common ancestor of both invertebrates and vertebrates, suggests an ancestrally conserved function for DNA methylation (Werren et al., 2010; Lyko & Maleszka, 2011). Moreover, despite the broad diversity of sequences shown to be methylated, and the complex, dynamic patterns in which they occur on the vertebrate genome, gene body methylation is suggested to represent an ancient property of the eukaryotic genome (Zemach et al., 2010).

Being a hymenopteran species, Nasonia has haplodiploid sex determination, in which males emerge from haploid unfertilized eggs and females are diploid. Recently, it was shown that Nasonia sex determination involves maternal provision of transformer (Nvtra) messenger RNA (mRNA) to the eggs, combined with parental imprinting to regulate zygotic transformer (Nvtra) expression (Verhulst et al., 2010). In addition, Park et al. (2011) showed distinct patterns of methylated genes in the genome of Nasonia, among them the Nvtra gene, which may function as regulators of gene function. During embryonic development in Nasonia, the early pronucleus initially goes through several rounds of rapid mitoses without cytokinesis to form the syncytium (Bull, 1982; Pultz & Leaf, 2003; Pultz et al., 2005), followed by the first onset of zygotic transcription 5 h after egg laying (at 25°C) (Verhulst et al., 2010). Conservation of DNA methylation patterns during these early mitoses would therefore suggest maternal provision of Dnmt mRNA. To evaluate this, we examined the effect of parental RNA interference (pRNAi) of Dnmt gene expression in the present study. The results show an important role for maternal provision of Dnmt1a in early embryonic development, suggesting a crucial function for DNA methylation during early Nasonia development.

Results

Maternal input of Dnmt mRNA
Quantitative real-time PCR (qPCR) analysis showed that both Dnmt1a and Dnmt1c genes are expressed in the ovaries and that the corresponding mRNAs are maternally provided to the eggs. In contrast, Dnmt1b appeared to be nearly absent in ovaries and early embryos (Fig. 1). The maternally provided amount of mRNA for Dnmt1a and Dnmt1c is about 3–5-fold higher than that of maternally provided Nvtra, that was chosen as a reference. However, the normalized expression levels of Dnmt1a and Dnmt1c do not significantly differ from one another (Kruskal–Wallis one-way ANOVA on ranks). Dnmt3 mRNA is also maternally provided, although at lower level than Dnmt1a and Dnmt1c.

Parental RNA interference of Dnmt1c and Dnmt3
Because Dnmt1c showed the highest level of maternal input, pRNAi in the white pupal stage (Lynch & Desplan,
2006) was applied to reduce maternal provision of Dnmt1c mRNA. qPCR analysis showed that Dnmt1c mRNA levels were significantly lowered in injected females, compared with the controls (one-tailed t-test, \( P < 0.01 \), Fig. 2). Dnmt1c pRNAi did not, however, lead to a decrease in Dnmt1a expression levels (Fig. 2), even though the target region of Dnmt1c shows similarity to that of Dnmt1a, demonstrating the specificity of the pRNAi knockdowns. After emergence, injected females showed no aberrant phenotype; they were able to mate and oviposit and produced normal clutch sizes. This result shows that expression of Dnmt1c is not essential for development from the white pupal stage onward. Moreover, the lowering of Dnmt1c maternal input had no apparent effect on the development of their offspring. Apparently, maternal provision of the maintenance DNA methyltransferase Dnmt1c mRNA is also not essential for embryonic development.

Combination of the pRNAi of Dnmt1c and Dnmt3 showed results identical to those of Dnmt1c alone. Neither the development of the treated females nor the development of the offspring were affected by the injections, although the levels of both mRNAs were significantly lowered (one-tailed t-test, \( P < 0.01 \), Fig. 2). These results show that expression of Dnmt3 is not essential from the white pupal stage onward and that maternal provision of Dnmt3 mRNA is not essential for embryonic development.

Parental RNA interference of Dnmt1a and Dnmt1c

Parental RNAi of Dnmt1a and Dnmt1c combined also had no effect on the development and phenotype of the injected females. The effectiveness of pRNAi of Dnmt1a and Dnmt1c in the injected pupae was confirmed by qPCR (one tailed t-test, \( P < 0.01 \), Fig. 3). This result shows that the normal high level of expression of Dnmt1a is not essential for development from the white pupal stage onward. However, none of the offspring of the injected females were viable, indicating that depletion of maternally provided Dnmt1a and Dnmt1c together results in embryonic lethality. As was shown in the previous section, silencing Dnmt1c alone or in combination with Dnmt3 did not result in any aberrant phenotype in the treated females or their offspring, and it was concluded, therefore, that this lethality was caused by the depletion of maternally provided Dnmt1a mRNA. To confirm this, the pRNAi experiment was repeated with Dnmt1a double-stranded (ds)RNA alone. As this resulted in the same embryonic lethality, we concluded that the successful knockdown of Dnmt1a expression (one-tailed t-test, \( P < 0.01 \), Fig. 4) in

Figure 2. Relative expression levels in adult (4-day-old) females of Dnmt1a (■) and Dnmt1c (▲) after parental RNAi of Dnmt1c, and Dnmt1c combined with Dnmt3. Sterile water was used as an injection control. Error bars represent se.

Figure 3. Relative expression levels in adult (4-day-old) females of Dnmt1a (■) and Dnmt1c (▲) after parental RNAi knockdown of Dnmt1a combined with Dnmt1c. Sterile water was used as an injection control. Error bars represent se.

Figure 4. Relative expression levels in adult (4-day-old) females of Dnmt1a after Dnmt1a (■) parental RNA interference (pRNAi) or water-injected controls (▲). Error bars represent se.
the white pupal stage results in reduction of maternally provided Dnmt1a mRNA to the eggs and, subsequently, to developmental arrest and lethality. pRNAi of males in the white pupal stage did not have any effect on their further development or their offspring (data not shown). This confirms that the observed pRNAi phenotype is caused by a maternal effect.

We previously showed that maternal input of Nvtra mRNA in combination with epigenetic control of zygotic tra expression, starting 5 h after fertilization, is essential for female-specific splicing of Nvtra and doublesex (Nvdsx) mRNA (Verhulst et al., 2010). Therefore, we determined whether silencing of Dnmt1a and Dnmt1c combined had an effect on the sex-specific splicing of Nvtra and Nvdsx mRNA. The female-specific splicing of Nvdsx is dependent on the presence of an active NvTRA, which is produced from the female but not from the male splice form of Nvtra. Unfertilized haploid eggs will develop as males, while fertilized diploid eggs start the female developmental route 7 h after egg laying. Surprisingly, as is shown in Fig. 5, 12-h-old embryos produced by Dnmt1a and Dnmt1c dsRNA-injected females showed normal splicing of both Nvtra and Nvdsx mRNA and control embryos. This developmental series of DAPI-stained embryos showed that both sets of embryos went through a normal developmental programme initially, forming a normal syncytium and regular cellularization. DAPI staining on embryos produced after knockdown of maternally provided Dnmt1a mRNA and control embryos showed that 0–1 h after egg laying (Fig. 6A, E), no mitosis had occurred in developing embryos of both groups while normal positioning of the pronucleus was observed (Bull, 1982). No effects of developmental arrest were observed at 4 h after egg laying (Fig. 6B, F), when embryos of both groups had gone through several rounds of nuclear mitosis without cytokinesis to form the syncytium (Pultz & Leaf, 2003; Pultz et al., 2005). The nuclear divisions appeared synchronous in both groups, pole cells migrated
normally to the posterior end of the embryo and nuclear migration to the periphery of the embryos marked the start of the blastula stage (Bull, 1982; Pultz & Leaf, 2003). The first differences were observed 10–12 h after the eggs were deposited (Fig. 6C, G), when small invaginations could be seen in nearly 100% of the control embryos, marking the start of segmentation and onset of gastrulation. These invaginations could not be observed in the embryos from the injected females. Instead, all of the embryos stopped developing at this same developmental stage.

Discussion

The identification of Dnmt genes in several insect species (The Honeybee Genome Sequencing Consortium, 2006; Wang et al., 2006; Walsh et al., 2010; Werren et al., 2010; Xiang et al., 2010; Zemach et al., 2010) has directed attention to the possible role of DNA methylation in establishing the complex insect phenotype. For several insect species, patterns of genomic methylation have been reported, indicating a relatively low percentage of methylated sites compared with vertebrates (see Lyko et al., 2010 and references therein), and methylation sites that are mainly located in gene bodies (Kucharski et al., 2008; Werren et al., 2010; Park et al., 2011). However, functional analysis of DNA methylation has thus far only been reported for the honeybee (Kucharski et al., 2008; Maleszka et al., 2009; Lockett et al., 2010; Lyko et al., 2010). In the present study, we demonstrate an essential function for maternally provided Dnmt1a mRNA in Nasonia development.

The hymenopteran Nasonia, like the dipterans Drosophila and Musca form so-called ‘long germ’ embryos. Although Nasonia diverged from diptera more than 200 million years ago, like Drosophila it starts developing in a syncytial environment in which germ patterning is strongly dependent on maternal factors (Pultz & Leaf, 2003; Rosenberg et al., 2009). Lynch et al. (2006) and Lynch & Roth (2011) showed that the Nasonia zygote relies heavily on maternal input of mRNA gradients for axis formation and germline determination. In addition, Verhulst et al. (2010) showed that maternal input of Nvtra mRNA, in combination with specific zygotic Nvtra transcription and Nvtra autoregulation of female-specific splicing, is essential for female development of Nasonia embryos, while males develop as a result of maternal imprinting that prevents zygotic Nvtra transcription. These combined results led to the hypothesis that maternal provision of Dnmt mRNA may play an important role in Nasonia development. Indeed, our results indicate that all Dnmts, except Dnmt1b, are maternally provided. Strikingly, only knockdown of maternally provided Dnmt1a mRNA resulted in an observable phenotype, where embryonic development is arrested 10–12 h after egg laying, coinciding in time with the onset of gastrulation (Bull, 1982). This finding suggests that maintenance of DNA methylation patterns is an important feature of early Nasonia development and is in part mediated by maternal provision of Dnmt1a.

Despite this pronounced effect on development, no disturbance of sex-specific splicing of either Nvtra or the downstream Nvdsx gene was observed. Since this sex-specific splicing is dependent on the zygotic expression of Nvtra, our results indicate that the activation and expression of zygotic Nvtra does not critically depend on maternally provided Dnmts. Moreover, pRNAi using all Dnmt dsRNA never led to the production of diploid males during the present study, whereas this was a crucial finding in our previous study on sex determination using Nvtra dsRNA. As there was no zygotic expression of Dnmt1 genes to maintain maternally determined methylation patterns, these observations may contradict the suggestion of Park et al. (2011) that the preferential methylation pattern of CpG sites of exon 2 of Nvtra plays a role in Nasonia sex determination by marking alternative splicing sites of this gene. Alternatively, the residual Dnmt mRNA levels after pRNAi may be sufficient to maintain this pattern. It should be noted, however, that the underlying mechanism of splicing regulation by DNA methylation is unknown (Glastad et al., 2011). Clearly, this issue requires more detailed research.

Another important result is our finding that maternal provision of Dnmt3 mRNA is not essential for either early development or sex determination of the offspring, despite the fact that in the honeybee larval expression of this gene is involved in caste determination (Kucharski et al., 2008). Perhaps the effect of Dnmt3 in the honeybee only becomes apparent in the larval stage and can hence be independent of maternal provision. Moreover, the function of Dnmt3 in caste formation in A. mellifera is context-dependent and its absence does not necessarily reflect an abnormal developmental situation. It would be interesting to know if Dnmt3 possesses such context-dependent function in Nasonia as well. In addition, in the honeybee the germline does not appear to be specified maternally (Dearden et al., 2006; Rosenberg et al., 2009), in contrast to that of Nasonia (Lynch et al., 2011). Since Nasonia development is heavily dependent on maternal factors, this raises the question why no apparent phenotypic effect is observed when maternal provision of Dnmt3 and Dnmt1c is prevented. A possible explanation could be that these genes are less important until the onset of maternal zygotic transition (MZT). In D. melanogaster some 30% of maternal mRNAs degrade markedly at MZT, while others are more stable (De Renzis et al., 2007). Since Nasonia has a relatively long syncytial pre-blastoderm stage (Pultz et al., 1999), perhaps zygotic expression for Dnmt1c and Dnmt3 is

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Figure 6. Blue-fluorescent 4′,6-diamidino-2-phenylindole (DAPI) staining on embryos produced by Dnmt1a double-stranded (ds)RNA-injected females and embryos produced by females injected with sterile water as a control. (A, E) 0–1 h after egg laying, no mitosis has occurred in the developing embryo, both groups show normal positioning of the pronucleus (→) (Bull, 1982). (B, F) 4 h after egg laying, embryos of both groups have gone through several rounds of nuclear mitoses without cytokinesis to form the syncytium (Pultz et al., 2005). The nuclear divisions appear synchronous in both the Dnmt1a-injected and control group. Pole cells (→) have normally migrated to the posterior end of the embryo in both the dsRNA- and water-injected groups. Nuclear migration to the edge of the embryos is beginning, marking the start of the blastula stage, just prior to cellularization and activation of the zygotic genome (Bull, 1982; Pultz & Leaf, 2003). (C, G) After 12 h, small invaginations can be observed in the water-injected embryos (→) marking the start of segmentation and onset of gastrulation. These invaginations are not observed in the Dnmt1a parental RNA interference (pRNAi)-treated embryos. The embryos from this group stopped developing at this time point. (D, H) After 18 h, a clear difference can be observed between the water-injected and Dnmt1a pRNAi embryos. Whereas the embryos from the water-injected control group have advanced well into gastrulation, no further development could be observed for the Dnmt1a-injected group.

Experimental procedures

Insect strains

The Nasonia vitripennis wild-type laboratory strain AsymC, which is cured from Wolbachia, and the recessive red eye colour mutant strain stDR were used throughout the experiments. All wasp culturing was done on pupae of Calliphora vicina flies at 25°C, unless stated otherwise.

RNA extraction and cDNA conversion

Total RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. All isolated total RNA was subsequently reverse transcribed according to protocol with a mix of one part oligo-dT and six parts random hexamer, both provided with the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA).

Quantitative Real-Time PCR

Quantitative real-time PCR analysis was done using 2 μl of a 10-fold cDNA dilution and ABSolute™ QPCR SYBR Green ROX (500 nM) Mix (Abgene, Hamburg, Germany) on an Applied Biosystems 7300 Real Time PCR System (Foster City, CA, USA) with 300 nM qPCR primers for Dnmt1a, Dnmt1b, Dnmt1c and Dnmt3 (see Table 1). The transformer gene expression was used as reference, using 300 nM of primers Nvtra_qPCR_F1 and Nvtra_qPCR_R1 that amplify a non-sex specific part of the transcript (Verhulst et al., 2010). All primers sets (Table 1) were developed to contain at least one exon–exon spanning primer using PerlPrimer (Marshall, 2004). Primer sets showed no amplification from the genomic DNA template. qPCR profile was as follows: 95°C for 15 min, 40 cycles of 95°C for 15 s, 58°C for 30 s and 72°C for 30 s. Standard curve for 1:10,000 fold cDNA dilutions were run to determine linearity and primer efficiencies. A standard ABI7300 dissociation curve was applied to control for nonspecific amplification. LinregPCR (Ramakers et al., 2003) was used for calculating starting concentrations of both genes. Statistical analysis was performed using SPSS 16.

Expression analysis of Dnmt in ovaries and early embryos by quantitative real-time PCR

Sixty females from the stDR line were individually setup as virgin and given one fly pupa every other day for a period of 3 days. Thereafter, these females were allowed to oviposit for 3 h at 25°C in an egg-laying chamber to facilitate embryo collection. Seven replicates of 50 embryos per time point were collected in 100% ethanol at 2°C in approximately 30 min and stored at –80°C until RNA extraction. Also, seven samples of three ovaries per sample of 4-day-old females were collected in 100% ethanol at 2°C and stored at –80°C until RNA extraction. Relative expression levels of Dnmt1a, Dnmt1b, Dnmt1c and Dnmt3 in embryos and ovaries were calculated by comparing these starting concentrations to starting concentrations of the reference gene Nvtra, which was previously shown to be maternally provided (Verhulst et al., 2010).

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Table 1. Overview of the primers used during this study

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**RNA interference**

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*Represents exon–exon spanning primers.

Desplan (Lynch & Desplan, 2006). A dsRNA fragment of 693 bp was used for Dnmt1a, a fragment of 700 bp for Dnmt1c and a fragment of 488 bp for Dnmt3. At either the 5' or 3' end of the fragment a T7 promoter was placed using designed primers for all three Dnmt-specific dsRNAs (see Table 1 for primer sequences). These fragments were transcribed in both directions using the Megascript RNAi kit (Ambion, Austin, TX, USA) according to protocol to generate dsRNA. Approximately 75 white female stg/m pupae were injected with a mixture of 4 μg/μl of Dnmt1c dsRNA and 2 μg/μl of Dnmt3 dsRNA mixed with 10% red food colouring dye. Another 75 white female pupae were injected in the abdomen with a mixture of 4 μg/μl of Dnmt1a dsRNA and 2 μg/μl of Dnmt1c mixed with 10% red dye. Injections were performed with Femtotips II needles (Eppendorf, Hamburg, Germany) under continuous injection flow. The embryos were then transferred to glass microscopy slides and analyzed on a Zeiss AxioObserver Z1 using a 380 nm filter with 200 × magnification. Pictures were taken in the Z-sectioning mode with an interval of 0.8–1.2 μm and subsequently analyzed with ImageJ (National Institutes of Health, Bethesda, MD, USA).

**Expression analysis**

Expression levels of Dnmt1a, Dnmt1b, Dnmt1c and Dnmt3 after RNAi were assessed in the abdomen of adult females 4 days after emergence i.e. 10–11 days after dsRNA injection (n = 7). They were compared with the Dnmt expression levels in the abdomens of adult females of the same age that were injected with sterile water (n = 7). Relative expression levels of Dnmt1a, Dnmt1b, Dnmt1c and Dnmt3 in water-injected females and Dnmt1a, Dnmt1c + Dnmt3 or Dnmt1a + Dnmt1c dsRNA-injected females and their offspring were calculated by comparing Dnmt starting concentrations to starting concentrations of the reference gene Nvtra.

**DAPI staining of embryos**

Embryos were collected in 1.5 ml Eppendorf tubes containing 1 ml heptane + 300 μl phosphate-buffered saline and fixed by adding a 20% formaldehyde solution to a final concentration of 5%, followed by shaking at 100 rpm for 30 min. A series of washings with methanol was used to dehydrate the embryos for storage at −20°C. After embryos of all time points were collected, the embryos were rehydrated by gradually replacing the methanol by PBT buffer. For the DAPI stain itself, one drop of vectashield mounting medium with DAPI (Vector Laboratories, Inc. Burlingame, CA, USA) was added and incubated overnight at 4°C. The embryos were then transferred to glass microscope slides and analyzed on a Zeiss AxioObserver Z1 using a 380 nm filter with 200 × magnification. Pictures were taken in the Z-sectioning mode with an interval of 0.8–1.2 μm and subsequently analyzed with ImageJ (National Institutes of Health, Bethesda, MD, USA).

**Splice form analysis of Nvtra and Nvdx**

Sex-specific fragments of Nvtra in 12-h-old embryos from untreated and RNAi-injected stg/m females were analysed by RT-PCR. Three samples containing ~30 embryos per treatment were collected in 100% ethanol and stored in −80°C until RNA extraction. One μl of cDNA was used in a PCR with Nvtra primers, NvTra_F2 and NvTra_R3, that are located in exon 2 and 3 respectively and give a fragment of 228 bp in females and three fragments of of 514 bp, 460 bp and 282 bp in males and Nvdx primers NvdxU_F3 and NvdxR1 that are located in exon 4 and 6 respectively and give a product of 543 bp in males and 435 bp in females (Verhulst et al., 2010).

For amplification and cDNA integrity control Elongation factor 1α (EF1α) was amplified with primer set EF1α_F1 and EF1α_R1 (Verhulst et al., 2010) yielding a product of 174 bp in both males and females. The PCR profile used was 35 cycles of 95°C for 15 s, 55°C for 30 s and 72°C for 45 s. PCR fragments were visualized on a 1.5% non-denaturing agarose gel and stained with ethidiumbromide.
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References


