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Application of the LUminometric Methylation Assay to ecological species: tissue quality requirements and a survey of DNA methylation levels in animals

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Abstract

The LUminometric Methylation Assay (LUMA) measures global DNA methylation. LUMA depends on digestion of DNA with methyl-sensitive and methyl-insensitive restriction enzymes, followed by pyrosequencing. Until recently, LUMA has been principally used for biomedical research. Here, we use chickens as a model to investigate sample quality issues relating to LUMA and then apply the method to ecological species. First, we assessed the effect of tissue storage conditions on DNA methylation values. This is an important consideration for ecological species because samples are not always ideally preserved and LUMA is sensitive to poor DNA quality. We found that good quality LUMA data could be obtained from chicken liver and brain tissues stored at 21 °C for at least 2 and 12 h, respectively. Longer storage times introduced nonspecific peaks to pyrograms which were associated with reduced DNA methylation. Repeatedly, freezing and thawing the tissues did not affect LUMA data. Second, we measured DNA methylation in 12 species representing five animal classes: amphibians (African and Western clawed frog), reptiles (green anole lizard), fish (yellow perch, goldfish, lake trout), mammals (American mink, polar bear, short-beaked common dolphin, Atlantic white-sided dolphin) and birds (chicken, Japanese quail). We saw a pattern of high DNA methylation in fish (84–87%), and intermediate levels in mammals (68–72%) and birds (52–71%). This pattern corresponds well with previous measures of DNA methylation generated by HPLC. Our data represent the first CpG methylation values to be reported in several species and provide a basis for studying patterns of epigenetic inheritance in an ecological context.

Keywords: DNA methylation, DNA quality, epigenetics, LUminometric Methylation Assay, methods, wildlife

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Introduction

DNA methylation refers to the addition of methyl groups to cytosine (C), and occasionally other residues, in the genetic code. In vertebrates, methylation is distributed throughout the genome and is thought to occur almost exclusively on C residues that are followed by a guanine (G) (Law & Jacobsen 2010). This is referred to as CpG methylation. The principal function of DNA methylation is to regulate the expression of genes by controlling access of transcriptional machinery to promoter regions. All known vertebrate genomes exhibit substantial amounts of CpG methylation, but the percentage of the genome that is methylated varies tremendously between species (Bird 2002). Although DNA methylation is increasingly studied across taxa, there remains a lack of information on basic patterns of DNA methylation in most species.

DNA methylation is one of the two main mechanisms of epigenetic inheritance that are currently best understood (the other is chromatin remodelling). Epigenetics refers to factors affecting gene expression that are heritable but occur outside of changes to the DNA sequence itself. Epigenetics may explain how environmental factors such as diet, parental care, stress and exposure to toxins can have lasting and even multigenerational effects on health outcomes (Faulk & Dolinoy 2011). This concept is currently the subject of intensive research in the biomedical field with a focus on cancer and the foetal origins of disease. The effect of environmental factors on epigenetic inheritance is equally important to the

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ecological sciences, but much less research has been done in this area.

As interest in epigenetics grows, there is an increasing need to examine epigenetic modes of inheritance in non-model organisms. Recent publications in the areas of ecology (Bossdorf *et al.* 2008) and ecotoxicology (Head *et al.* 2012; Vandegehuchte & Janssen 2013) suggest that epigenetics may be critical to furthering our understanding of how genetic variability and environmental stress interact to produce phenotypic change. As little is known about epigenetic modes of inheritance in most species, characterization of DNA methylation will be important for this endeavour.

With this in mind, a principle goal of our study was to apply LUMA to diverse animal species, many of which have not been previously studied with respect to DNA methylation. Although many different methods for measuring global DNA methylation exist (Ammerpohl *et al.* 2009), LUMA is a convenient choice because it requires a relatively small DNA sample (<1 μg), has a short processing time and is easily adapted to multiple species. A potential limitation of LUMA is that it only measures CpG methylation within the recognition sequences of the restriction enzymes (CCGG) and thus will not give information about methylation outside of this sequence or about methylation on other nucleotides. Additionally, LUMA requires high-quality DNA that is free of 5′ overhangs.

Given this requirement for high-quality DNA, we first addressed the issue of sample quality: a critical consideration for the application of analytical methods to ecological species. As ecological samples are usually collected in the field, it is not always possible to maintain the high quality of sample preservation and storage technique that is routine in a laboratory setting. Methylated DNA is very stable, but the DNA fragmentation that can occur when tissues are stored at increased temperature can be problematic for LUMA. Here, we address this issue by establishing a timeline for storage of samples destined for LUMA analysis, testing freeze–thaw conditions and discussing issues relating to the use of fragmented DNA for LUMA. We then use LUMA to measure DNA methylation in 12 species representing five animal classes: mammals, fish, amphibians, birds and reptiles. Our focus is on liver and brain tissue because these tissues are routinely studied by our group and are central to a number of physiological functions relating to responses of organisms to environmental stress.

Methods

Sample collection – live specimens

Fertilized chicken (*Gallus gallus*) and Japanese quail (*Coturnix japonica*) eggs were purchased from the Michigan State University Poultry Farm (East Lansing, MI, USA). Both were incubated with turning at 37.5 °C and 60% humidity as previously described (Rutkiewicz & Basu 2013). The embryos were euthanized at 1 day prehatch (embryonic days 19 and 15 for chicken and quail, respectively). Adult goldfish (*Carassius auratus*) were purchased from Ozark Fisheries (Stoutland, MO, USA) and euthanized upon receipt. All animal sacrifice was carried out according to protocols approved by the University of Michigan's University Committee on Use and Care of Animals (UCUCA).

Sample collection – donated and archived tissues

Tissue samples from Western clawed frog (*Xenopus tropicalis*), African clawed frog (*Xenopus laevis*) and anole lizard (*Anolis carolinensis*) were generously donated (see Acknowledgements). Samples from lake trout (*Salvelinus namaycush*), yellow perch (*Perca flavescens*), American mink (*Neovison vison*), Atlantic white-sided dolphin (*Lagenorhynchus acutus*), short-beaked common dolphin (*Delphinus delphis*) and polar bear (*Ursus maritimus*) were obtained from archived tissues in our possession. These originated from various other projects that our group has been involved with and were stored at −80 °C prior to use.

Sample collection – storage conditions

Tissues from all live specimens were flash frozen in liquid nitrogen (LN2) within minutes of collection. Archival tissues originating from other studies were collected as follows: lake trout (frozen on dry ice), yellow perch (flash frozen in LN2), mink (flash frozen in LN2), polar bear (flash frozen in LN2) and dolphins (frozen on dry ice within a few hours of death).

Sample-handling tests

Brain cerebral cortex and liver from embryonic chicken were used to test postmortem stability of DNA methylation as measured by LUMA. This experiment was designed to address effects of the variable conditions under which environmental samples are often collected and preserved. Chicken embryos were sacrificed on embryonic day 19. The cerebral cortex and liver were removed and each cut into 8–12 pieces of approximately 20 mg each. One piece of each tissue type was immediately flash frozen in LN2 (control), while the other pieces were kept at 21 °C (room temperature) for 0.5, 2, 6, 12, 24, 48 or 72 h. This was done for three different individuals, producing a sample size of n = 3 for each tissue type and timepoint. After the initial time at increased temperature, all tissues were stored at −80 °C. We initially also kept a set of tissues at 30 °C, but saw no differences

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between these and the tissues kept at 21 °C, and so we proceeded with only the room temperature set.

We also carried out a freeze–thaw (F/T) experiment. Three pieces of chicken brain tissue were flash frozen in LN2 immediately after dissection and stored at −80 °C. One piece of tissue was used as a control, while the others were subjected to one or two F/T cycles. A F/T cycle was defined as removing the tissue from storage and allowing it to sit thawed on the bench at room temperature for 1 h before being replaced in the freezer.

DNA isolation

DNA was isolated from tissues using the Qiagen DNeasy kit (Qiagen) according to manufacturer's directions. All samples were treated with RNase (Qiagen). We found this step to be essential for accurate quantification of DNA. This was especially true for transcriptionally active tissues such as liver. DNA was quantified on a NanoDrop 2000 spectrophotometer (Thermo Scientific).

LUMA assays

LUminometric Methylation Assay was performed based on the original method described in Karimi *et al.* (2006) as detailed in Pilsner *et al.* (2010) and Basu *et al.* (2013). The method is described in Fig. 1. Briefly, each 20 μL reaction contained DNA, 2 μL 10X Tango Buffer (Fermentas), 2.5 U EcoRI and 5U of either HpaII or MspI (all enzymes supplied by New England Biolabs). Each sample was analysed four times (in duplicate with HpaII and in duplicate with MspI). Samples were digested at 37 °C for 4 h followed by a 20-min heat inactivation at 80 °C. Annealing buffer (Biotage) was added to each sample at a volume of 15 μL, and 30 μL of the resulting mixture was aliquoted into a pyrosequencing plate. The amount of DNA loaded into the restriction digest was kept constant within a species but ranged from 300 to 1000 ng between species to meet the minimal peak height requirements. Theoretically, variable DNA loading should not influence DNA methylation values as the data are normalized to the EcoRI peak. We tested this assumption by analysing a pooled chicken brain sample in various amounts ranging from 300 to 1200 ng of input DNA, and found no effect on DNA methylation values (Table 1). The minimal acceptable pyrogram peak height was set at 10 relative light units, which corresponds to a signal-to-noise ratio of approximately 5. Species with higher per cent methylation values require more DNA to meet this requirement due to the smaller size of the HpaII peak, which appears only when the internal C of the recognition site is unmethylated.

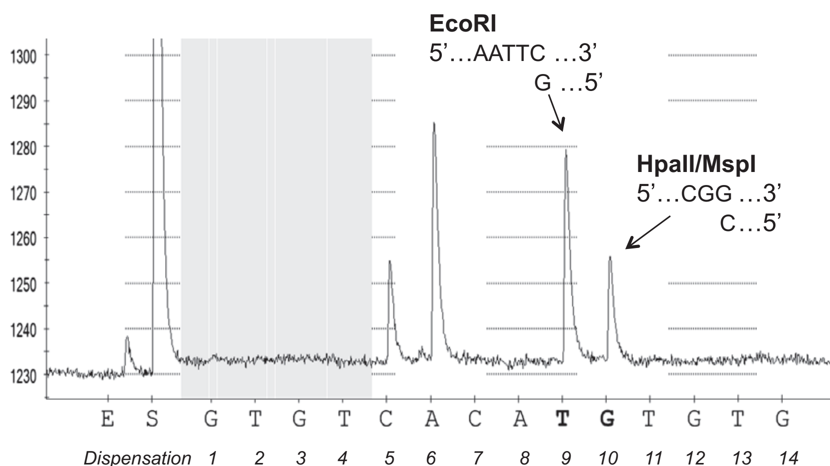

Fig. 1 Illustration of the LUminometric Methylation Assay (LUMA) initially described by Karimi *et al.* (2006). DNA is digested in parallel reactions with EcoRI (recognition site G/AATTC) and isoshizomeric restriction enzymes, *Hpa*II or *Msp*I. Both cleave at the recognition site 5′-C/CGG-3′, but *Hpa*II cuts only when the internal C is unmethylated, whereas *Msp*I is insensitive to methylation status at the internal C (note: neither enzyme can cut when the external C is methylated). Overhangs generated by the restriction digest are quantified by pyrosequencing. After enzyme and substrate are added, nucleotides are incorporated one by one in the order represented on the *x*-axis. The pyrogram peak heights are proportional to the amount of each nucleotide incorporated. In the method updated by Bjornsson *et al.* (2008), nucleotide dispensation begins with GTGT to eliminate nonspecific overhangs. The next set of incorporations, CACA, fills in G of the *Hpa*II/*Msp*I overhangs, and the T of the EcoRI overhangs as well as nonspecific G or T overhangs in the sample. Dispensations 5 and 6 (C and A) can be used for quantification, but as they also may include nonspecific signals, it is preferable to use dispensations 9 and 10 (T and G). Per cent methylation is calculated as: $1-[(HpaII(\mathrm{G})/EcoRI(\mathrm{T}))/(MspI(\mathrm{G})/EcoRI(\mathrm{T}))] \times 100$, where G and T are the peak heights for *HpaII* or *MspI* (methylation) and *EcoRI* (input DNA), respectively. Data generated by LUMA represent the percentage of CpG sites within the *Hpa*II/*Msp*I recognition site that are methylated (excluding *Hpa*II/*Msp*I recognition sites with a methylated external C residue as neither enzyme will cut in this context).

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Table 1 Quality control tests for LUMA. A pooled DNA sample from cerebral cortex of day 19 chicken embryos was used for all tests, and each sample was run in duplicate (technical standard deviation shown in parentheses). Unless otherwise specified, 600 ng of DNA was used for all tests. No significant differences in DNA methylation levels were detected for individual tests or between tests (ANOVA, $P > 0.05$)

Test	DNA methylation* Mean% (stdev)
DNA Loading (ng)	
300	55.3 (1.7)
600	54.8 (1.4)
900	56.5 (0.37)
1200	55.0 (1.0)
Between plate variability	
Plate 1	56.8 (1.0)
Plate 2	56.2 (1.0)
Plate 3	57.4 (0.01)
Plate 4	55.7 (0.78)
Within plate variability	
Sample 1	56.1 (0.16)
Sample 2	53.6 (0.25)
Sample 3	56.0 (3.8)
Freeze–Thaw (F/T)†	
Control (no F/T)	54.9 (0.60)
1 (F/T)	55.3 (2.4)
2 (F/T)	55.5 (0.23)

*Per cent DNA methylation as assessed by LUMA. This refers to the percentage of CpG sites within the HpaII/MspI recognition site (CCGG) that are methylated within the genome.
†A single freeze–thaw (F/T) cycle was defined as removing the tissue from −80 °C storage and allowing it to sit thawed on the bench at room temperature for 1 h before putting it back in the freezer.

Overhangs generated by the restriction digest were quantified via pyrosequencing on a Pyromark Q96 MD instrument, using Pyromark Gold Q96 reagents (Qiagen). This is a specialized and costly instrument, but at many institutions, time on pyrosequencers can be rented or borrowed from other laboratories or core facilities. The dispensation order for nucleotides was GTGTCA CATGTGTG. For a subset of samples, we also tested an elongated dispensation programme designed to further eliminate nonspecific peaks. For this subset, the dispensation order was GTGTGTGTCACACACATGTGTG.

Methylation values were calculated according to the formula: 1-[(*HpaII*(G)/*EcoRI*(T))/(*MspI*(G)/*EcoRI*(T))] × 100, where G and T are the peak heights for *HpaII* or *MspI* (methylation) and *EcoRI* (input DNA), respectively (dispensations 9 and 10 in Fig. 1). This method estimates the percentage of CpG sites within the MspI/HpaII recognition site that are methylated, and can be used as an approximation for percentage DNA methylation in the genome. As this is only an approximation (LUMA does not measure every methylated cytosine in the genome) considerations such as the relative proportion of methylated CG dinucleotides within the genome that are part of MspI/HpaII recognition sites are important when directly comparing LUMA to other methods for measuring DNA methylation.

Pyrograms that visually exhibited nonspecific peaks were rejected. Nonspecific peaks were defined as peaks generated from nucleotide incorporations that do not correspond to the restriction digest overhangs (Fig. 1). A repeat analysis was performed if the coefficient of variation between duplicate samples was >5%.

Quality control tests

LUminometric Methylation Assay was validated using a standard curve created from lambda DNA. Lambda DNA is unmethylated and can be methylated to close to 100% with CpG methyltransferase (M.SssI, NEB). Methylated and unmethlyated DNA were mixed in different proportions to create DNA standards with theoretical methylation values of 0%, 25%, 50%, 75% and 100%. The resulting standard curve was linear with a slope close to 1 ($y = 0.95x–1.36$, $r^2 = 0.99$, $P < 0.001$). Actual values were within 10% of expected values for all standards (Fig. S1, Supporting information).

To assess variability between and within plates, we performed repeat analyses of pooled DNA isolated from cerebral cortex of day 19 chick embryo. The coefficient of variation for per cent DNA methylation in these pools was consistently below 3% (Table 1). Other groups have also found LUMA to be very reproducible. For example, one group found the average assay variance to be 2% (Bjornsson *et al.* 2008).

Statistical analysis

Differences in mean DNA methylation values between quality control groups (Table 1) were evaluated using one-way analysis of variance (ANOVA). Variation between DNA methylation values in samples left at room temperature for various times (0.5–72 h) and control samples (flash frozen in liquid nitrogen) was assessed via one-way ANOVA with Bonferroni post hoc analysis. Pearson correlation was used to assess the relationship between actual and theoretical methylation values for the lambda DNA standard curve. In all cases, a P value of 0.05 or lower was considered significant.

Results and discussion

Sample quality

The first goal of this project was to investigate sample-handling and DNA quality requirements for LUMA. We used a laboratory model species, the chicken, for this

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purpose. In a F/T experiment, we determined that freezing and thawing brain tissues up to three times had no effect on DNA methylation values (Table 1). We also investigated the effect of leaving tissue samples at room temperature to mimic sample collection conditions that might occur in the field. Liver and brain samples were left at room temperature (21 °C) for various amounts of time (0.5–72 h). DNA isolated from these tissues showed signs of time-dependent degradation, as apparent on an agarose gel (Fig. 2A). DNA isolated from control tissues was visible as a sharp band of high molecular weight while DNA isolated from tissues left at room temperature appeared as a smear that increased with increasing time. Nonspecific peaks appeared in pyrograms after liver samples were left at room temperature for 6 h and brain samples were left at room temperature for 24 h (Fig. 2B). Per cent DNA methylation measured in these tissues decreased with increasing time at room temperature. This change was significant after 12 h for liver tissue and 48 h for brain tissue ($P < 0.001$; Fig. 2C).

Our data suggest a strong negative association between time spent at room temperature and per cent methylation assessed by LUMA. This finding is likely an artefact of the LUMA method itself and not due to demethylation of DNA. Methyl groups are unlikely to spontaneously separate from cytosine residues as they are covalently bound. Deamination of 5-methylcytosine (5mC) to thymine can occur, but this is a slow process and would not take place in a timeframe of hours or days (Lindahl 1993). In practice, CpG methylation assessed by

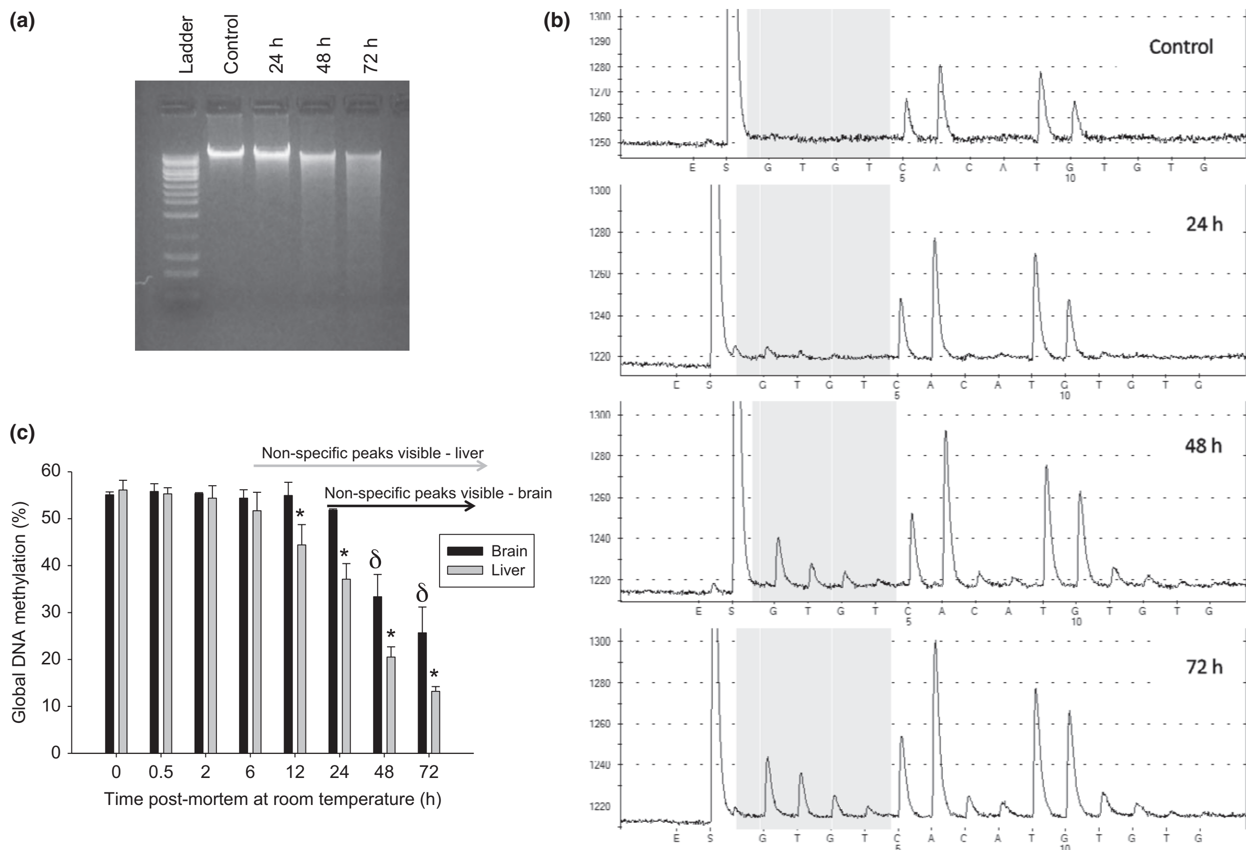

Fig. 2 Effect of storing tissue at room temperature (21 °C) for prolonged periods (0–72 h) on (A) DNA integrity, (B) Luminometric Methylation Assay (LUMA) pyrograms and (C) Global DNA methylation as assessed by LUMA. Brain and liver tissue from day 19 chicken embryos was left at room temperature for various amounts of time and then frozen at −80 °C. Global methylation of DNA isolated from these tissues was assessed using LUMA. (A) Representative agarose gel showing DNA isolated from chicken brain tissue left at room temperature for 0, 24, 48 or 72 h prior to freezing (other timepoints not shown). Each well contained 350 ng of DNA. (B) Representative LUMA pyrograms for DNA samples shown on the gel in Fig. 1A. Nonspecific peaks are first visible in the 24 h-sample (previous timepoints not shown). In liver tissue, nonspecific peaks were first visible after 6 h at room temperature (data not shown). (C) DNA methylation levels decrease with increasing amount of time at room temperature for both tissue types. Each bar represents methylation levels in DNA isolated from three separate individuals. Asterisks (*) and delta symbols (δ) indicate a significant decrease in DNA methylation compared to control (0 h-sample) for liver and brain, respectively (ANOVA, $P < 0.001$). The first timepoint at which nonspecific peaks were visible on the pyrogram is also indicated.

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a different method (bisulphite sequencing) was not affected by delays of processing of up to 48 h postmortem in brain tissue (Ferrer *et al.* 2008). In a more extreme example, methylation was preserved in DNA isolated from ancient bison remains from the late Pleistocene (Llamas *et al.* 2012).

As others have noted (Ammerpohl *et al.* 2009), the LUMA method is more sensitive to poor DNA quality than other methods for determining CpG methylation rates (such as HPLC or bisulphite sequencing) because the presence of single-stranded overhangs in degraded DNA can contribute to the LUMA signal. In degraded DNA, nonspecific 'C' overhangs would sometimes be included in the HpaII/MspI quantification peaks (nucleotide incorporation 10 in Fig. 1). On average, these extra cytosine residues would be counted equally in the HpaII sample ('unmethylated' peak) and the MspI sample ('total' peak). Adding extra counts to both peaks would have the effect of artificially depressing per cent methylation values. The problem of fragmented DNA was addressed by Bjornsson *et al.* (2008) by modifying the original LUMA method. In the modified method, the first eight nucleotide incorporations (two of each nucleotide) are designed to fill in nonspecific overhangs, theoretically reducing their impact on peaks 9 and 10 which are used for quantification (Fig. 1). In practice, we have found that the presence of nonspecific peaks is associated with reduced DNA methylation values regardless of the number of incorporations prior to the data peaks. We attempted to 'rescue' DNA methylation values in degraded samples by increasing the number of nucleotide incorporations prior to the data peaks (four of each nucleotide) to no effect. DNA methylation values remained depressed, even when the nonspecific peaks were dramatically reduced or gone. We also tried decreasing the amount of DNA loaded into each well to the point where nonspecific peaks were no longer visible (i.e. below the detection limit), thinking that this would eliminate the contribution of nonspecific peaks to the signal. However, this also did not rescue DNA methylation values (Figs S2 and S3, Supporting information). Another approach would be to polish the DNA prior to analysis by enzymatically digesting the overhangs (Ammerpohl *et al.* 2009).

Although attempts to restore DNA methylation values by reducing the height of nonspecific peaks failed, our data still point to a nonspecific signal as being the source of the problem with degraded DNA. Of note, in cases where even small nonspecific peaks were observed, a strong and significant relationship between the size of nonspecific peaks and DNA methylation was consistently apparent. For example, in one preliminary dataset comprised of 15 *Xenopus* liver samples, nonspecific peak height was significantly correlated with DNA methylation values (e.g. $r^2 = 0.97$, $P < 0.001$ for nucleotide incorporation 3, data not shown). The significance of this correlation was maintained even when samples with nonspecific peaks that were >5 relative light units were rejected from the dataset ($n = 9$, $r^2 = 0.75$, $P < 0.01$ for nucleotide incorporation 3).

Our findings are particularly important for applications of LUMA to ecological samples because tissues collected from wild animals are not always preserved in an ideal manner. For example, field conditions may not be conducive to immediate freezing of dissected tissue. Samples may be stored in household freezers (e.g. −20 °C) for prolonged periods in remote field stations and subjected to multiple freeze–thaw cycles during transit. Additionally, field-collected tissues are often difficult to obtain and therefore shared among researchers or archived for future use. In these cases, the tissue collection and handling conditions cannot always be strictly controlled. By clarifying sample-handling requirements, we hope to establish Standard Operating Procedures for tissues destined for LUMA and facilitate opportunistic use of rare samples.

The issue of sample quality is clearly important for ecological studies, but perhaps more surprisingly we suspect that our findings relating to sample quality are also very relevant to laboratory-based research. In our work, we have sometimes observed nonspecific peaks in samples that were collected in what was considered to be an ideal manner. Samples that were flash frozen in liquid nitrogen or immediately placed on dry ice were generally clean, but have occasionally produced pyrograms with nonspecific peaks. We have noticed that nonspecific peaks occur more often with liver tissue than with brain tissue, an observation that corresponds well with our sample quality test; nonspecific peaks were observed in liver tissue after 6 h at room temperature and in brain tissue only after 24 h (Fig. 2C). Other LUMA users may also observe nonspecific peaks in their samples, although this is not often reported in the literature. In one case, Wu *et al.* (2011) describe small nonspecific peaks that were disregarded if the peak height was <3 relative light units.

The source of nonspecific peaks in samples originating from flash-frozen tissues is unclear, but may be related to the DNA isolation process. It has been reported that DNA extraction methods can significantly impact LUMA values. For example, a literature search revealed that DNA methylation values reported in human blood varied by as much as 20% between studies and that this variability was related to the DNA isolation method that was used (Soriano-Tárraga *et al.* 2013). The authors suggest that oxidizing conditions in DNA isolation protocols (two precipitation methods and a magnetic bead method) may chemically eliminate

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methylated CpG sites. Another possibility is that the extent of fragmentation of the DNA sample is influenced by the isolation method. As we have shown, even slightly fragmented samples can have depressed DNA methylation values when analysed by LUMA.

Our findings reinforce that samples producing pyrograms with nonspecific peaks are not suitable for LUMA analysis. Our current practice is to reject any samples where nonspecific peaks are present, regardless of size or whether the peaks decrease to zero over the first eight nucleotide incorporations. In both liver and brain tissue, the timepoint at which nonspecific peaks were first visible in the pyrogram preceded the timepoint at which the first significant reduction in per cent DNA methylation values was observed (Fig. 2). In both tissues, there was a slight reduction in DNA methylation (2.5–3%) at the timepoint where nonspecific peaks were first visible (6 and 24 h at room temperature for liver and brain, respectively), just not a statistically significant one. This suggests that nonspecific peaks are a sensitive indicator for problems with sample integrity that will impact DNA methylation values. An additional measure we have taken to ensure good data quality is to load enough DNA to produce a minimum peak height of at least 10 relative light units, enabling the visualization of nonspecific peaks should they be present. In line with this recommendation, we have found that loading more DNA generally produces data with less variance between individuals. This may be because nonspecific peaks become detectable with higher input DNA and thus samples with nonspecific peaks (and potentially lower DNA methylation values) are rejected and not included in the average.

Inter-species comparisons

The second goal of this project was to characterize DNA methylation levels across a variety of species. By comparing our data with previously reported values for 5mC methylation in vertebrates, we hope to validate the LUMA method and increase our understanding of levels of DNA methylation across animal classes.

We detected and quantified LUMA methylation levels in liver and/or brain tissue of all 12 vertebrate species analysed. The number of species surveyed in each animal class was small, but we saw a general pattern of high methylation levels in fish (84–87%), and intermediate levels in mammals (68–72%) and birds (52–71%; Table 2). Others have seen similar patterns of variability among species using different methods for quantifying DNA methylation. For example, Feng *et al.* (2010) used shotgun bisulphite sequencing to assess cytosine methylation in eight different species including zebrafish (*Danio rerio*) and mouse (*Mus musculus*) and found higher levels in the fish than the mammal (the six other species included two invertebrates and four plants). Jabbari *et al.* (1997) used HPLC to tabulate 5mC levels of 87 species of vertebrates and consistently found higher levels in fish, and lower levels in mammals and birds. It is important to note that 5mC values are not directly comparable with LUMA data as they measure different things; LUMA measures the percentage of CpG sites within the recognition sequence 5′-CCGG-3′ that are methylated, whereas 5mC (expressed in mol%) indicates the number of methylated cytosines as a percentage of the total number of nucleotides in the genome. Nonetheless, these two different measures can be compared in relative terms, for example, as general indicators of differences in the extent of DNA methylation among species.

The large range of global DNA methylation levels previously reported in animals is evident in the LUMA data generated in our study. What is the reason behind this inter-species variation? Jabbari *et al.* (1997) speculated that variability in 5mC levels observed across vertebrate genomes is related to average body temperature. Over an evolutionary timescale, higher body temperature in warm-blooded animals may be associated with a greater deamination rate of 5mC to thymine and lower 5mC levels. This hypothesis is supported by the observation that 5mC levels are inversely proportional to body temperature in polar and temperate/tropical species of fish (Varriale & Bernardi 2006a). We report similar levels of global DNA methylation in three different species of fish; DNA methylation values in goldfish, yellow perch and lake trout varied by < 4%. Nevertheless, our results agree with those of Varriale and Bernardi (2006a). The rank order of per cent methylation in brain tissue (lake trout > yellow perch > goldfish) corresponded inversely to the water temperature that each of these species prefers. Likewise, warm-blooded animals in our study all had per cent methylation values significantly below those of fish.

The relationship between body temperature and DNA methylation is more complex for reptiles, which have unique methods of thermoregulation. Anole lizard, the one reptilian species analysed in our study, had a high level of DNA methylation (86% and 89% in liver and brain, respectively), similar to fish. Reptilian DNA methylation assessed as 5mC levels have previously been shown to range widely, spanning the levels found in fish and mammals/birds (Varriale & Bernardi 2006b).

Both our study and Jabbari *et al.* (1997) measured DNA methylation in the amphibian *X. laevis*. We observed a high level of DNA methylation as assessed by LUMA (91%, Table 2), significantly more increased than the highest fish value. In contrast, they observed an intermediate level of 5mC, lower than most fish but higher than birds and mammals. The reason for this

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Table 2 Global DNA methylation in animals

Animal class	Latin name	Common name	Tissue	DNA methylation* Mean% (stdev)	Sample size†
Amphibian	*Xenopus laevis*‡	African clawed frog	Brain	90.7	1
			Liver	91.3	1
	Xenopus tropicalis§	Western clawed frog	Brain	76.0	1
			Liver	78.4	1
Reptile	*Anolis carolinensis*	Green anole lizard	Brain	88.9 (0.26)	3
			Liver	86.2 (2.5)	2
Fish	*Perca flavescens*	Yellow perch	Brain (tel)	84.3 (0.55)¶	3
			Liver	87.4 (0.22)	3
	Carassius auratus	Goldfish	Brain (tel)	83.9 (1.8)	3
			Liver	84.9 (0.67)	2
	Salvelinus namaycush	Lake trout	Brain (tel)	86.7 (0.43)	3
Mammal	*Neovison vison*	American mink	Brain (cort)	70.1 (1.9)¶	12
	Ursus maritimus	Polar bear	Liver	68.2 (4.0)	5
	Delphinus delphis	Short-beaked common dolphin	Brain (cer)	69.7 (2.3)	3
	Lagenorhynchus acutus	Atlantic white-sided dolphin	Brain (cer)	71.7 (2.7)	3
Bird	*Gallus gallus*	Domestic chicken	Brain (cort)	56.0 (0.92)¶	8
			Liver	52.1 (2.8)	9
	Coturnix japonica	Japanese quail	Brain (cort)	71.4 (1.0)	5
			Liver	70.4 (1.2)	5

Stdev, Standard deviation; tel, telencephalon; cort, cortex; cer, cerebellum.
*Per cent DNA methylation as assessed by LUMA. This refers to the percentage of CpG sites within the HpaII/MspI recognition site (CCGG) that are methylated within the genome.
†Sample size indicates the number of individuals of each species that were analysed.
‡Tetraploid.
§Diploid.
¶Values for mink, chicken and yellow perch are also reported in Basu *et al.* (2013).

discrepancy is not known, but it may be related to differences in measurement type. Interestingly, we found a striking difference in DNA methylation between two closely related species of frog. Substantially, higher levels of DNA methylation were observed in *X. laevis* (91% in both brain and liver) than in *X. tropicalis* (76% and 78% in brain and liver, respectively). This difference may be related to ploidy as *X. laevis* is tetraploid and *X. tropicalis* is diploid. Methylation of ribosomal genes has previously been observed to be increased in tetraploid as compared to diploid frogs (Ruiz & Brison 1989). A similar pattern has been observed in plants (Ochogavía *et al.* 2009).

We report DNA methylation in brain tissue of three mammalian species (mink, 70.1%; short-beaked common dolphin, 69.7%; Atlantic white-sided dolphin, 71.7%) and liver of one species (polar bear, 68.2%). These values fall within the range of what has previously been reported for LUMA in mammals. For example, a review of eight studies that used LUMA to assess DNA methylation in human blood reports values ranging from 52.4 to 78.0% (Soriano-Tárraga *et al.* 2013). The large variability in reported values may be due to differences in DNA isolation method between studies, but could also be related to problems with DNA quality. For example, in a previous study (Pilsner *et al.* 2010), we found that DNA methylation in the brain stem of male polar bears was 57.8%, a value that is 10% lower than what we report for polar bear liver here. It is possible that this discrepancy is related to tissue type; different tissues have previously been shown to exhibit different levels of DNA methylation (Gama-Sosa *et al.* 1983). Another possibility is that there was a difference in sample quality between the present study and the previous one. The brain tissues used in Pilsner *et al.* (2010) were obtained opportunistically and were not preserved in an ideal manner. Nonspecific peaks were observed in some of the pyrograms from these samples indicating that the DNA may have been partially degraded. As discussed earlier, this could have the effect of artificially depressing DNA methylation values as measured by LUMA. The polar bear liver samples for the present study were taken with tissue preservation in mind, and nonspecific peaks were completely absent from the pyrograms.

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Concluding remark

This study demonstrates that LUMA is an effective method for assessing differences in global DNA methylation in multiple species and has applications to ecological samples. Our data suggest that LUMA requires intact DNA and that ensuring that DNA is not fragmented may be dependent on tissue processing as well as initial storage conditions. Surprisingly, DNA isolated from brain tissue stored for up to 12 h at room temperature was found to be acceptable for LUMA. This, along with the finding that multiple freeze–thaw cycles did not affect LUMA data, is encouraging for the application of LUMA to ecological samples collected in the field. Regardless of tissue storage conditions, the presence of nonspecific peaks in LUMA pyrograms was clearly related to reduced DNA methylation values. This finding reinforces that the presence of any nonspecific peaks in LUMA pyrograms precludes the use of the associated DNA methylation data. Beyond method validation, our study shows that LUMA is useful for assessing DNA methylation across a range of taxa and that, in relative terms, the data are consistent with values attained via HPLC.

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J.H. designed the research, performed the research, analysed the data and wrote the paper. K.M. performed the research and analysed the data. N.B. designed the research and edited the paper.

Data accessibility

Individual values for the mean LUMA data reported here can be found in the Supporting Information (Tables S1, S2, and S3, Supporting information).

Supporting Information

Additional Supporting Information may be found in the online version of this article:

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Fig. S1. Lambda standard curve.

Fig. S2. Modifications to the LUMA protocol designed to 'rescue' DNA methylation values.

Fig. S3. Representative pyrograms for 48-h samples shown in Fig. S2.

Table S1. Global DNA methylation values for individuals, as measured by the LUminometric Methylation Assay (LUMA).

Table S2. Global DNA methylation values for quality control samples, as measured by the LUminometric Methylation Assay (LUMA).

Table S3. Global DNA methylation values in suboptimally sampled tissues as measured by LUMA.

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