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Rapid Neural DNA Methylation Responses to Predation Stress in Trinidadian Guppies

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ABSTRACT

DNA methylation (DNAm) is a well-studied epigenetic mechanism implicated in environmentally induced phenotypes and phenotypic plasticity. However, few studies investigate the timescale of DNAm shifts. Thus, it is uncertain whether DNAm can change on timescales relevant for rapid phenotypic shifts, such as during the expression of short-term behavioural plasticity. DNAm could be especially reactive in the brain, potentially increasing its relevance for behavioural plasticity. Most research investigating neural changes in methylation has been conducted in mammalian systems, on isolated individuals, and using stressors that are less ecologically relevant, reducing their generalisability to other natural systems. We exposed pairs of male and female Trinidadian guppies (*Poecilia reticulata*) to alarm cue, conspecific skin extract that reliably induces anti-predator behaviour, or a control cue. Whole-genome bisulphite sequencing on whole brains at various time points following cue exposure (0.5, 1, 4, 24, and 72 h) allowed us to uncover the timescale of neural DNAm responses. Males and females both showed rapid shifts in DNAm in as little as 0.5 h. However, males and females differed in the time course of their responses: both sexes showed a peak in the number of loci showing significant responses at 4 h, but males showed an additional peak at 72 h. We suggest that this finding could be due to the differing longer-term plastic responses between the sexes. This study shows that DNAm can be rapidly induced by an ecologically relevant stressor in fish and suggests that DNAm could be involved in short-term behavioural plasticity.

1 | Introduction

Adaptive phenotypic plasticity allows organisms to shift their phenotype across varying environments, thereby maintaining a higher fitness. Behavioural plasticity may be especially important for success in variable environments, given that behavioural traits are reactive to environmental conditions on relatively short timescales. Two broad categories of behavioural plasticity can be defined. The first type, developmental plasticity, has a slower response time but allows for organisms to respond to environmental conditions by triggering different developmental trajectories that can lead to the integration of behavioural traits with other phenotypes (Mery and Burns 2010; Snell-Rood 2013; Stamps 2016). Alternatively, contextual or activational plasticity is the most rapid form of behavioural plasticity and allows organisms to respond to changes in their immediate environment, such as predator cues or increased foraging opportunities, by expressing particular behavioural patterns (Mery and Burns 2010; Snell-Rood 2013; Stamps 2016). Developmental plasticity is often irreversible, while contextual plasticity is not. Despite the importance of both types of behavioural plasticity for success, the molecular mechanisms underlying them have not been thoroughly investigated.

Epigenetics, gene regulatory mechanisms that alter gene expression without altering the genetic code itself, can be sensitive to environmental shifts, thereby offering a direct link between the environment and the genome (Feil and Fraga 2012). The most well-studied epigenetic mechanism is DNA methylation (DNAm), the addition of a methyl group on a cytosine typically in cytosine-guanine dinucleotides (CpG) but also found in different contexts (e.g., CHH and CHG where H is every base except G) (Jones 2012). DNAm is broadly found across the tree of life from bacteria to fungi, plants, and animals; however, there are some specific examples of organisms that do not have DNAm, for example, *Drosophila melanogaster* (Nasrullah et al. 2022). DNAm plays a major role in gene expression (Maunakea et al. 2010; Jones 2012) and cell-fate decisions (Wilson et al. 2005; Koh and Rao 2013), and has been implicated in phenotypic variation and local adaptive responses (Dolinoy 2008; Kooke et al. 2015; Taff et al. 2019). For example, DNAm is associated with breast plumage and stress resilience in female tree swallows (*Tachycineta bicolor*) (Taff et al. 2019) and with the colonisation of new habitats in brown anole lizards (*Anolis sagrei*) (Hu et al. 2020). Additionally, DNAm has been shown to be environmentally responsive in a number of species (Rubenstein et al. 2016; Heckwolf et al. 2020; Caizergues et al. 2022) and is suggested to play an important role in regulating phenotypic plasticity (Dolinoy 2008; Bossdorf et al. 2010; Putnam et al. 2016). In stickleback (*Gasterosteus aculeatus*), DNAm was shown to be associated with salinity tolerance and inducible by environmental shifts in salinity (Heckwolf et al. 2020). However, despite being proposed as a mechanism for rapid acclimation to environmental change, the speed at which methylation can be modified remains unclear.

For DNAm to underlie phenotypic plasticity, it must be able to shift on ecologically relevant timescales. Although DNAm was previously thought to be relatively stable, changing only during cell division, there is increasing evidence that some methylated sites are reactive on shorter timescales. Marine and freshwater three-spined stickleback reciprocally transplanted across salinity environments showed changes in methylation after four days (Artemov et al. 2017), and Atlantic salmon (*Salmo salar*) exposed to thermal stress showed methylation changes in three days (Beemelmanns et al. 2021). Many of the methylation differences then disappeared after several weeks (Artemov et al. 2017; Beemelmanns et al. 2021). Even quicker still, an invasive model ascidian sea squirt, *Ciona savignyi*, exhibited DNAm responses after only one hour of high-temperature exposure and after three hours of low-salinity exposure; responses returned to control levels after 48 h (Huang et al. 2017). Studies in human cell culture models have also shown rapid DNAm; for example, DNAm shifts were uncovered in as little as two hours in dendritic cells responding to infection (Pacis et al. 2019). These studies suggest that methylation levels can react within a few days or even as rapidly as a few hours, providing a path for DNAm to be involved in more rapid forms of phenotypic plasticity such as contextual behavioural plasticity.

Evidence suggests that DNAm may be especially reactive in the brain. Mature human neurons have been shown to have high levels of DNA (cytosine-5′) methyltransferases (DNMTs), the enzyme that catalyses the transfer of the methyl group to the cytosine (Goto et al. 1994). Hydroxymethylcytosines—which are considered to be an intermediate step in DNA demethylation—are most common in human brain tissue, suggesting that rapid demethylation could also commonly occur there (Guo, Ma, et al. 2011). In adult mice, neuronal activation resulted in changes in the CpG methylation landscape of dentate granule neurons in as little as four hours, with some changes stable at the 24 h mark (Guo, Su, et al. 2011), while stress conditioning induced methylation changes in the brain in as little as one hour, which then reverted to the previous state after 24 h (Miller and Sweatt 2007). DNAm has also been implicated in synaptic plasticity (Miller et al. 2008; Feng et al. 2010), learning and memory (Day and Sweatt 2010; Miller et al. 2010), and adult neurogenesis (Ma et al. 2009), further supporting its potential role in behavioural plasticity. This work suggests that DNAm is dynamically regulated in response to experience in the adult central nervous system and could possibly play a role in stress responses and behavioural plasticity. However, few of these studies use ecologically relevant environmental cues, often using instead, for example, electroconvulsive stimulation or electric shock training—although see work done in insects (e.g., Lyko et al. 2010; Burrows et al. 2011). While such stressors provide a strong cue for studying epigenetic responses, it is difficult to extrapolate the importance of the identified epigenetic mechanisms for behavioural plasticity in the wild. Additionally, much of this work has centred around mammalian study systems, and few studies have investigated these processes in other taxonomic groups. Therefore, our general understanding of time-related patterns in DNAm remains limited.

This study leverages a tractable study system, the Trinidadian guppy (*Poecilia reticulata*), to study the timescale of DNAm responses in the brain to an ecologically relevant stressor, predation stress. Trinidadian guppies, hereafter guppies, are small, tropical fish native to freshwater rivers throughout Trinidad that are frequently used in evolutionary studies due to their ability to quickly adapt to varying environments (Reznick and Endler 1982; Endler 1995; Magurran 2005). Guppies encounter a spectrum of predation pressure, with meta-populations often divided by waterfalls that act as physical barriers to many predator species, which has led to much of their adaptive variation (Endler 1995). Low and high predation populations differ in demographic characteristics as well as a variety of traits, such as life history (Reznick and Endler 1982; Rodd and Reznick 1997), morphology (Johansson et al. 2004; Burns et al. 2009; Evans et al. 2011), colouration patterns (Endler 1980; Schwartz and Hendry 2007), and behaviour (Seghers 1974; Brown et al. 2013; Elvidge et al. 2016; Fox et al. 2024). Guppies, like many fish, are known to respond strongly, with immediate changes in behaviour, to an "alarm cue" that is released from fish skin damaged during a predation event (Brown and Godin 1999; Brown et al. 2009, 2010). This cue can be used to induce predation stress in the laboratory (Brown 2003). Short-term shifts in behaviour exhibited by fish exposed to alarm cue include lowered position in the water column, avoidance of areas containing the cue, and decreased activity (Brown 2003; Speedie and Gerlai 2008; Fan et al. 2022). Alarm cue exposure can induce longer-term behavioural shifts in guppies as well. Female guppies chronically exposed to alarm cue were bolder and showed graded responses to threats as opposed to unexposed female guppies (Elvidge et al. 2014). Female guppies also rapidly learn about threats that are paired with alarm cues (Fan et al. 2022). Males and females

are both responsive to predation threat, but females have been found to have stronger anti-predator responses than males, with males continuing mating attempts even under threat (Magurran and Nowak 1997). Studies on stickleback, a species with a similar sex chromosome system, have identified sex-specific methylation patterns (Metzger and Schulte 2018), but few epigenetic studies have been done in guppies and thus it is currently unclear if sex-specific methylation may underlie sex differences in behaviour.

We hypothesised that DNAm underlies the expression of contextual plasticity in response to alarm cue exposure. We predicted that exposure to alarm cue would induce effects on behaviour and DNAm in the brain of both male and female guppies, but that the timing of these methylation differences would differ between the sexes. We exposed pairs of guppies to alarm cue and measured behavioural responses for five minutes before and after cue exposure. Then, we dissected brains at several time points following alarm cue exposure (0.5, 1, 4, 24, and 72 h) and carried out whole-genome bisulphite sequencing (WGBS) to investigate the timescale of DNAm responses. This work provides important information regarding the timescale of DNAm responses in the brain in response to an ecologically relevant stressor and in an understudied taxonomic group.

2 | Materials and Methods

2.1 | Study Subjects

We used 60 guppies (30 males and 30 females) from a population that was collected from the low predation upper Aripo tributary in Trinidad in 2013 and have since been outbred in laboratory conditions in our laboratories at McGill University. They were housed in 150-L stock tanks fitted with a heater, filters, gravel substrate, and artificial aquarium plants and maintained at 25°C ± 1°C and a 12:12 light–dark cycle. Weekly 30% water changes and water testing (pH, hardness, nitrites, nitrates, and ammonia) were conducted. We fed fish daily with tropical fish flakes (TetraMin, Tetra, Germany) and gave supplemental decapsulated brine shrimp eggs (*Artemia* sp., Brine Shrimp Direct, USA) three times a week. Fish had no prior experience with alarm cue and had not previously been used in any other study. This population of guppies is known to react strongly to alarm cue (Brown et al. 2010).

All procedures followed McGill University Animal Care and Use Committee Protocols (Protocol #7133/7708) and the guidelines from the Canadian Council on Animal Care and the Animal Behavior Society/Association for the Study of Animal Behaviour (ABS/ASAB).

2.2 | Alarm Cue Exposure

Exposures were carried out in three batches, with each batch containing one of every treatment (alarm cue or control cue) and time point (0.5, 1, 4, 24, and 72 h) combination, for a total of 10 tanks per batch and 30 tanks in the whole study. Batch one began on 27 April 2021; Batch two on 21 May 2021; and Batch three on 23 June 2021. One week prior to cue exposures, we moved one male and one female to 9 L tanks that were fitted with a heater, filter, gravel, and artificial plants and maintained under the same conditions as the stock tanks. Tanks had a back board that visually divided the tank into three equal horizontal sections so that fish position could be recorded as upper, middle, and bottom of tank. Opaque barriers on the sides of the tanks meant that fish could not observe neighbouring tanks. We made fresh alarm cue on each exposure day following standard procedures (Brown and Godin 1999; Brown et al. 2009, 2010) and kept it on ice until used. Briefly, skin extracts were taken from an equal ratio of male and female conspecifics from the same population stock tanks and then homogenised and diluted with ddH_2O to a concentration of 0.1 cm^2 tissue/ml. Control cue consisted of ddH_2O also kept on ice until used. After the one week acclimation period, we exposed fish to 3.5 mL, a similar dose to previous work (Brown and Godin 1999), of either alarm cue or control cue distributed to the top of the tank using a clean syringe, taking care not to disturb the fish in the tank. Exposures were carried out between 12:00 and 16:00. For five minutes before and five minutes after cue exposure we recorded fish behaviour using a GoPro Hero4 (GoPro, San Mateo, USA) placed 30 cm away from the side of the tank.

2.3 | DNA Extraction and Whole Genome Bisulphite Sequencing

After the assigned time point, we euthanized fish in ice water as this is considered the most ethical method (Blessing et al. 2010). Immediately after euthanasia, we removed brains. Brains were preserved in RNAlater (Thermofisher Scientific, Waltham, USA) and then frozen at −80°C within 24 h until DNA extraction. We extracted DNA from whole brains using AllPrep DNA/RNA Kits (Qiagen, Hilden, Germany) following the manufacturer's protocol. WGBS library preparation and sequencing was performed at the McGill Genome Center (Montréal, Canada). Paired end libraries of 150-bp long reads were prepared for each fish and sequenced on two lanes of the Illumina NovaSeq6000 S4 (Illumina, San Diego, United States) along with guppy samples from a different project, with 69 individuals pooled per lane.

2.4 | Behavioural Data Collection and Analysis

All behavioural responses to cue exposure were scored by a single observer that was blind to the treatment using BORIS v7.12.2 (Friard and Gamba 2016). The observer recorded the time spent in each section of the tank, time spent frozen (an indicator of stress; Brown and Godin 1999), and time spent foraging. Foraging was defined as active pecking at the substrate (Dussault and Kramer 1981) and ended when the fish was no longer oriented towards the substrate and had not pecked for two seconds. The main behaviour of interest, proportion of time spent at the bottom of the tank without foraging (hereafter substrate use), was calculated by subtracting time spent foraging from the time spent in the bottom section of the tank and dividing by the total trial time (Fan et al. 2022). We excluded foraging at the bottom of the tank from our measure of substrate use in order to focus on defensive behaviours (Wisenden et al. 2004). We then calculated the change in proportion of substrate use by subtracting the before-cue exposure value

from the after-cue exposure value such that a positive value represents an increase in time spent near substrate after cue exposure and a negative value represents a decrease. Freezing instances were rare and therefore not informative, so they were not analysed further. Due to a recording error, one alarm cue tank did not have data for after the cue exposure, so the two individuals in this tank were removed from the analysis. Therefore, our sample size for all behavioural analyses was 58 fish (30 control and 28 alarm cue exposed). Behavioural data were analysed in R v4.3.1 (R Core Team 2022). We ran a linear mixed model to test for a difference in change in proportion of substrate use between cue treatments using the R package *lme4* (Bates et al. 2015). Sex was added as a fixed effect, and tank was added as a random effect with varying intercepts. The model was fit using restricted maximum likelihood. Model assumptions were verified by checking the homogeneity of variance and the independence and normality of the model residuals. We tested the significance of cue and sex with type 2 Chi-square tests using the *car* package (Fox and Weisberg 2019) and the significance of tank using likelihood ratio tests implemented in the *lmerTest* R package (Kuznetsova et al. 2017). We also recorded the time males spent pursuing females and the time males spent engaging in sigmoidal mating displays, a courtship display where males curve their bodies into an S-shape (Magurran and Seghers 1990). We added these two measurements together as a total measurement of mating behaviour and divided by total trial time to obtain the proportion of time males spent performing mating behaviour. To test for shifts in mating behaviour due to alarm cue, we calculated the change in proportion of mating behaviour by subtracting the before-cue exposure value from the after-cue exposure value. We used a *t* test to test for a difference in change in proportion of mating behaviour (total mating behaviour and courtships) between cue treatments. Additionally, mating behaviour could have an impact on female behaviour as it is known to impact foraging rates (Magurran and Seghers 1994a) and habitat use (Darden and Croft 2008). Therefore, we compared male mating behaviour between treatments by using a *t* test to test for a difference in the total proportion of mating behaviour between cue treatments.

2.5 | WGBS Data Processing

We processed sequence reads using the nf-core/methylseq pipeline v1.6.1 (Ewels et al. 2019, 2020), which uses FASTQC v0.11.9 (Andrews 2019) to quality check raw reads and Trim Galore! v0.6.5 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) to trim adaptor sequences and low-quality reads. We used the Bismark v0.22.3 (Krueger and Andrews 2011) pathway in the pipeline to align trimmed reads to the guppy reference genome (GenBank assembly accession GCA_000633615.2) with BowTie2 v2.5.0 (Langmead and Salzberg 2012) and extract methylation data. The average mapping efficiency was 67.27% ± 1.19%, similar to other studies on guppies (Hu et al. 2018) (Table S1). The pipeline uses MultiQC (Ewels et al. 2016) to generate alignment reports, which were assessed for quality. Only CpG context methylation was analysed; however, we also quantified methylation at non-CpG sites and found that an average of 0.83% ± 0.05% of CHG cytosines and 0.94% ± 0.06% of CHH cytosines were methylated, which is similar to previous findings in guppies (Hu et al. 2018), suggesting a highly efficient bisulphite conversion. The average coverage per CpG was 9.1X ± 1X.

Before methylation analysis, we merged the coverage and methylation level from both strands using a custom python script (https://github.com/rcristofari/penguin-tools/blob/master/merge_CpG.py). We filtered CpG sites to a minimum of five reads in all fish per group and removed sites that were in the 99.9th percentile of coverage to control for PCR bias and sites that had low variation defined as a percent methylation standard deviation < 2%. Single nucleotide polymorphisms (SNPs) can result in incorrect methylation calls if C-to-T or G-to-A SNPs are falsely interpreted as unmethylated cytosines and, therefore, should be corrected for. We identified SNPs across all samples using BS-SNPer (Gao et al. 2015) with the following quality filters: minimum base quality of 15, minimum coverage 10, maximum coverage of 1000, minimum read mapping value of 20, minimum mutation rate of 0.02, minimum mutation reads number of 2, threshold of frequency for calling heterozygous SNP of 0.1, and threshold of frequency for calling homozygous SNP of 0.85. Then, we isolated C-to-T SNPs and used the *GenomicRanges* package (Lawrence et al. 2013) to remove the SNPs from further analysis. We uncovered 3,474,289 SNPS, of which 481,090 were C-to-T SNPs. Our filtering resulted in an average of 4,705,834 ± 1,332,385 CpG sites for each comparison made, meaning that we retained ~5% of all CpG sites after alignment (Table S2). All individuals were retained in the differential methylation analysis, resulting in a sample size of 60 fish total: 3 fish for each sex, treatment, and time point combination.

2.6 | Differential Methylation Analysis

Differential methylation was analysed using the *MethylKit* R package v1.18.0 (Akalin et al. 2012) in R v4.3.2 (R Core Team 2022). We analysed differential methylation in two ways. First, we pooled all time points and identified differentially methylated sites (DMSs) and regions (DMRs) between alarm cue and control fish. For this analysis, tank was included as a covariate. Next, we performed DMS and DMR analysis between control and alarm cue fish at each time point. We ran these two analyses for males and females separately. We identified DMSs and DMRs by running logistic regressions for each CpG site. To assess significance, a chi-square test and the SLIM (sliding linear model) method were used to calculate *q*-values, which corrects for multiple testing. We considered sites and regions to be significant if they showed at least 20% differential methylation between alarm cue exposed and control cue exposed fish and *q*-values < 0.0125. We used these cut-off values based on power simulation results for WGBS studies (Lea et al. 2017). DMRs were identified using a tiling method with a sliding window size of 100 bases and a step size of 100 bases, and CpGs filtered to a minimum of three reads with each region then being filtered to a minimum of five reads after tiling. This tiling approach ensures that the same regions were tested for each time point and sex. We clustered samples within each time point and for each sex based on percent methylation across all DMRs with Euclidean distance and Ward's linkage using the *cluster* v2.1.4 package

(Maechler et al. 2022). We used Chi-square tests to determine if DMSs and DMRs were more frequently hypomethylated or hypermethylated. We also used Chi-square tests to check if the proportion of significant sites (DMSs) to total CpGs was significantly different between males and females at the two visually identified peak time points (4 h and 72 h). The differential methylation analysis method we employed can sometimes lead to false positives (Wreczycka et al. 2017). Therefore, we validated the time patterns we found in DMSs and DMRs by rerunning the analysis for each sex and time point in *methylKit*, but with overdispersion correction added and in *DSS* v2.54.0 (Feng and Wu 2019). Both methods greatly reduce sensitivity but increase specificity (Wreczycka et al. 2017). For both validation analyses, we used the same CpG filtering methods and differential methylation settings outlined above (coverage thresholds, q-values <0.0125, 20% differential methylation, 100 bp DMR length, etc.).

2.7 | Functional Annotation and Gene Ontology Enrichment Analysis

We ran functional annotation and gene ontology (GO) enrichment analysis for each time point in males and females. We used the ENSEMBL guppy database (release 108; accessed February 2023) and the *genomation* R package v1.35.0 (Akalin et al. 2015) for functional annotation. The genomic feature was identified for each DMS, DMR, and CpG that passed the filtering steps outlined above. If features overlapped, we gave precedence to promoters > exons > introns > intergenic regions and defined the promoter region as 1500-bp upstream and 500-bp downstream from the transcription start site (TSS). We used the distribution of all CpG sites to build a null distribution that we used to assess if there were shifts in the distributions of DMSs and DMRs. To do this, we compared the distribution of DMSs and DMRs to the null distribution of all CpG sites using a G test. If the distributions were significantly different, we ran post hoc G tests for each genomic feature to determine which features differed significantly from the null distribution. We adjusted for multiple testing using the Hommel method (Hommel 1988). Additionally, we assessed the average methylation rate of CpGs across each genomic feature to see if methylation levels matched expectations found from other methylation studies on fish species (Klughammer et al. 2023).

We used the *GenomicRanges* R package (Lawrence et al. 2013) to identify the nearest TSS to a DMS or DMR and considered a gene to be differentially methylated if a DMS or DMR was located no further than 10 kb away from the TSS. The R packages *GOstats* (Falcon and Gentleman 2007) and *GSEABase* (Morgan et al. 2023) were used to identify overrepresented biological processes, molecular functions, and cellular components for hypermethylated and hypomethylated genes separately and together at each time point. Then, we applied a conditional hypergeometric GO term enrichment analysis with all genes that were associated with any CpG site retained after filtering used as the gene background set. Lastly, we corrected p-values for multiple testing using a false discovery rate and used false discovery rate-corrected $p \leq 0.05$ for the significance cut off.

3 | Results

3.1 | Behavioural Response to Cue Exposure

Alarm cue (AC) and control (C) guppies had a similar mean proportion of substrate use (i.e., time in the bottom third of the tank, excluding foraging behaviour) before cue exposure (AC = 0.35, C = 0.39) but the means diverged after cue exposure, with alarm-cue exposed fish increasing substrate use (AC = 0.75, C = 0.36). This change in substrate use significantly differed between alarm-cue and control exposed fish (Figure 1; estimate: control = −0.42, 95% CI = −0.55 to −0.28, Chi-Sq = 35.47, df = 1, $p < 0.0001$). However, sex did not have a significant impact (estimate: male = −0.04, 95% CI = −0.01 to 0.09, Chi-Sq = 2.36, df = 1, $p = 0.12$). Only a very small proportion of time was spent foraging for both alarm cue and control fish before and after cue exposure (before: AC = 0.07, C = 0.05; after: AC = 0.09, C = 0.06). The average proportion of time males spent performing mating behaviour was similar across treatments before (AC = 0.30, C = 0.30) and after (AC = 0.25, C = 0.29) cue exposure. Accordingly, there was no significant difference in the total proportion of males devoted to mating behaviour between cue treatments ($t = -0.36$, df = 26.41, $p = 0.72$), indicating that females experienced similar levels of male mating behaviour across treatments. Further, there was no significant difference in the change in the proportion of time devoted to mating behaviour between cue treatments ($t = -0.44$, df = 24.8, $p = 0.66$) or to courting specifically ($t = -0.88$, df = 26.85, $p = 0.39$) indicating that despite being exposed to alarm cue males did not significantly reduce mating behaviour.

3.2 | General Patterns of Differential Methylation in Alarm Cue Versus Control Cue Exposed Fish

After pooling across time points, we identified 1846 DMS and 15 DMRs in females and 3907 DMS and 36 DMRs in males between alarm cue and control cue exposed fish. In females, there were significantly more hypomethylated than hypermethylated DMSs and DMRs (DMSs: 1131 hypomethylated and 715 hypermethylated, $\chi^2 = 93.75$, df = 1, $p < 0.0001$; DMRs: 13 hypomethylated and 2 hypermethylated, $\chi^2 = 8.07$, df = 1, $p = 0.005$). In males, there were also more hypomethylated than hypermethylated DMSs and DMRs; however, this difference was only significant for DMSs (DMSs: 2102 hypomethylated and 1805 hypermethylated, $\chi^2 = 22.58$, df = 1, $p < 0.0001$; DMRs: 21 hypomethylated and 15 hypermethylated, $\chi^2 = 1$, df = 1, $p = 0.32$). There were 58 overlapping DMSs between males and females but no overlapping DMRs. The average methylation at CpGs across genomic features was similar between the sexes (Figure S1) and matched genome-wide levels of methylation seen in other fish species (Klughammer et al. 2023).

3.3 | Patterns of Differential Methylation Between Time Points

In females, there were significant DMSs at the 0.5 h time point, with a peak in the number of DMSs at the 4 h time point that then drastically decreased by the 24 h time point (Figure 2A).

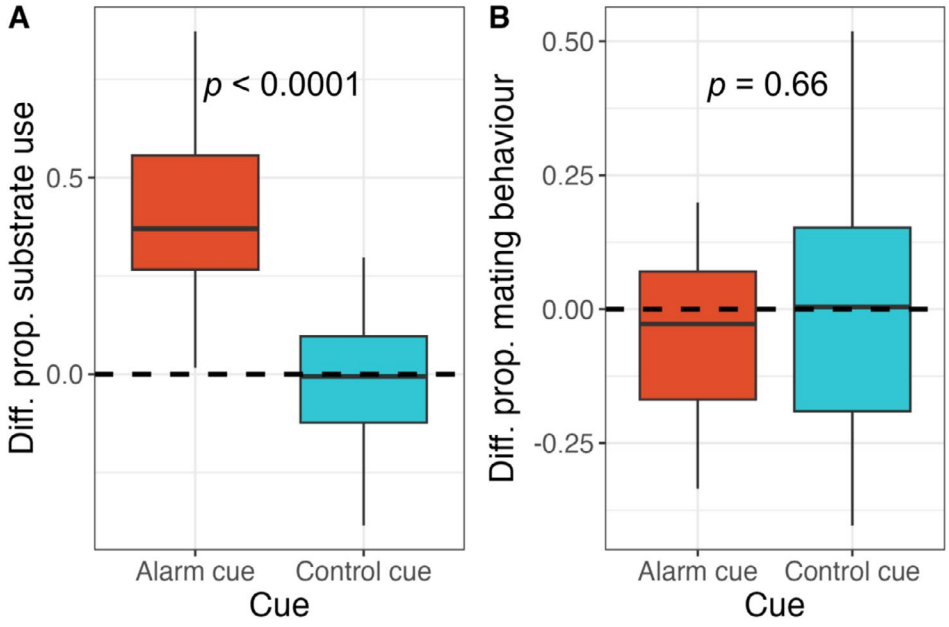

FIGURE 1 | Change in proportion of (A) substrate use and (B) mating behaviour after cue exposure. Substrate use was measured as the amount of time fish spent in the lower third of the tank minus the time spent foraging. Mating behaviour was measured as the amount of time males spent pursuing females and performing sigmoidal displays. Change in proportion of substrate use and mating effort were calculated by subtracting the proportion before cue exposure from the proportion after cue exposure such that a positive number indicates an increase after cue exposure and a negative number indicates a decrease. Boxplots show the interquartile range with the median indicated, and lines show the maximum and minimum values.

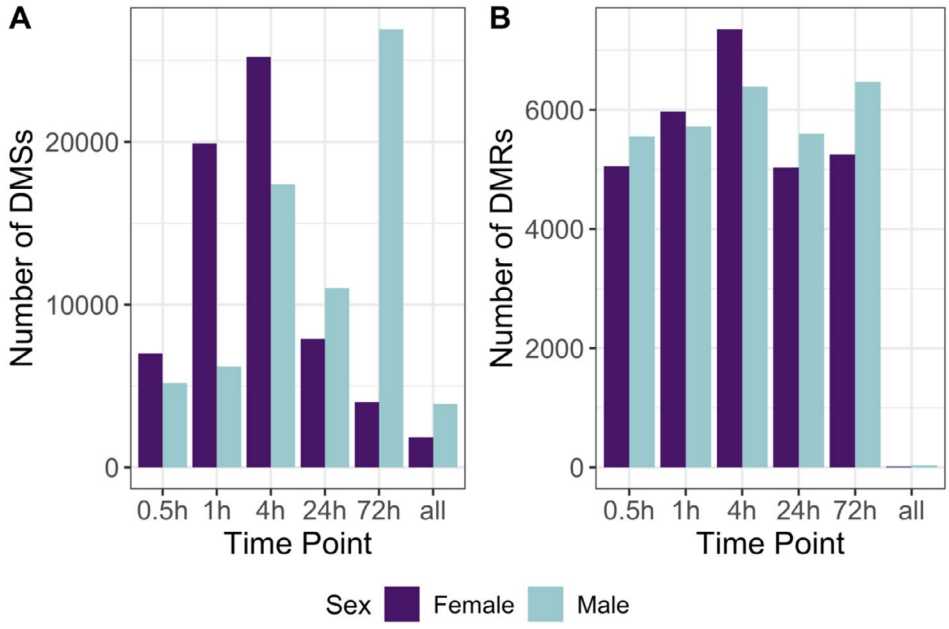

FIGURE 2 | Number of identified differentially methylated sites (DMSs) and regions (DMRs) at each time point comparison.

In males, significant DMSs were also identified at the 0.5 h time point, but this was followed by two peaks in DMSs: a smaller peak at the 4 h time point and then a larger peak at the 72 h time point (Figure 2A). The proportions of significant DMSs to all CpGs tested were significantly different between males and females at both identified peaks: females had more DMSs than males at 4 h ($\chi^2 = 3813.1$, df = 1, $p < 0.0001$), but males had more DMSs than females at 72 h ($\chi^2 = 16{,}336$, df = 1, $p < 0.0001$). DMRs showed similar peaks to DMSs for females, while for males, the peaks were less pronounced (Figure 2B). While we identified fewer DMSs and DMRs in both of our validation analyses using overdispersion in *methylKit* and *DSS*, as expected given that these techniques reduce sensitivity, there were similar patterns of differential methylation across time points since in both analyses. For DMSs and DMRs, females showed a peak at 4 h, whereas males showed a peak at 72 h (Figures S2 and S3). Patterns were more similar between the two *methylKit* analyses, with the time patterns of DMR peaks being less pronounced, while the *DSS* analysis showed a more pronounced time pattern in DMRs that was highly similar to that seen in the DMSs.

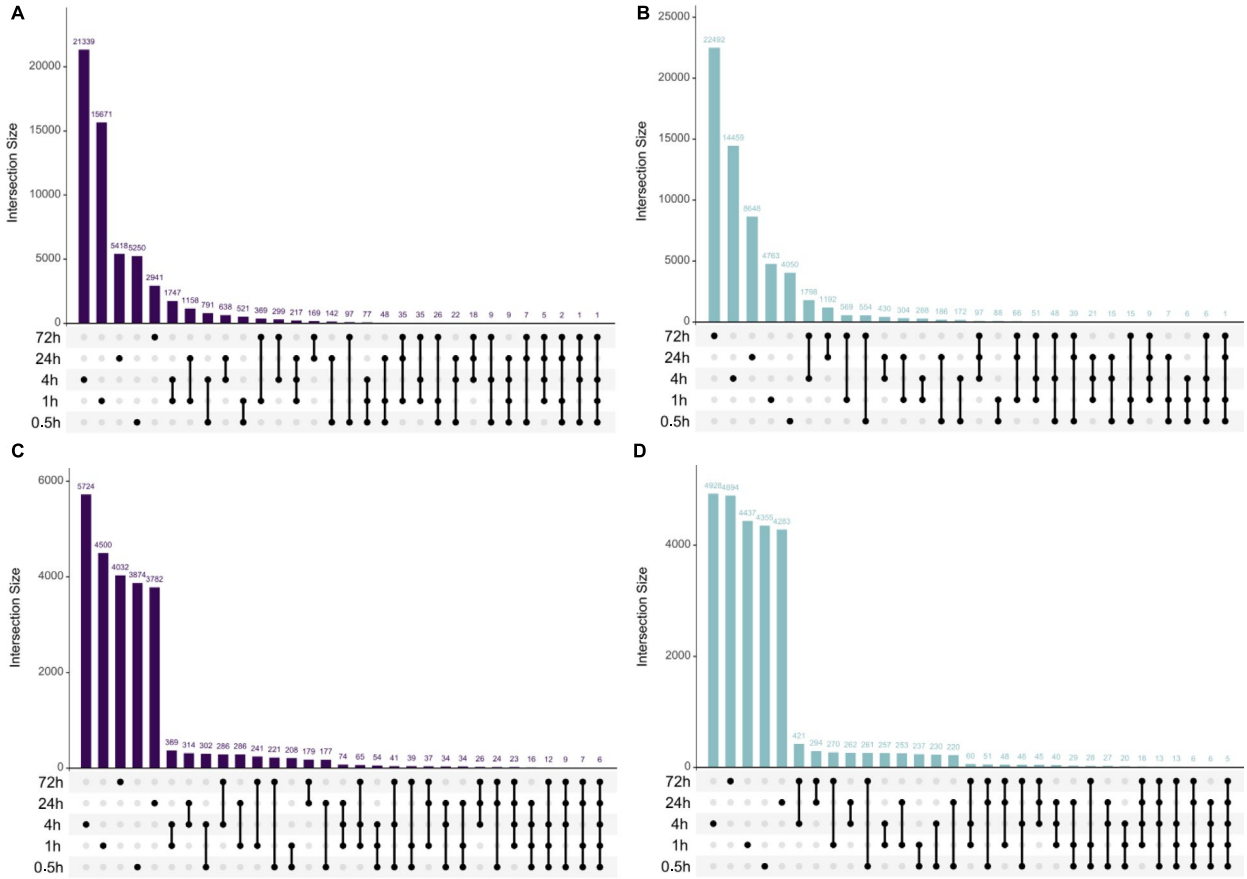

FIGURE 3 | Upset plots showing overlap between time points in (A and B) differentially methylated sites (DMSs) and (C and D) regions (DMRs) for females (dark purple) and males (light blue). (A) DMSs in females, (B) DMSs in males, (C) DMRs in females, and (D) DMRs in males. The bottom section of each plot indicates the intersection being shown for each bar with points indicating the time points involved in the overlap. Bars in the top portion of the plots show the size of overlap for each overlap. Bars are ordered from left to right by degree of overlap, thus note the order of time overlaps differs between figures A through D.

Across all time points, significant differences in methylation in DMRs ranged from 20% to 81% in females and from 20% to 92% in males (20% is the lowest possible value due to the cut-off employed). DMSs and DMRs were not consistently hypermethylated or hypomethylated in males or females across time points (Table S3). In females, the highest overlaps in DMSs were between 1 and 4 h and 1 and 24 h (Figure 3A). In males, the highest overlaps in DMSs were between 4 and 72 h and 24 and 72 h (Figure 3B). These patterns show that some DMSs may briefly return to normal methylation levels and then become significantly changed again. No DMS overlapped between all time points for males and females. For DMRs, the highest overlaps were between 1 and 4 h, and 4 and 24 h for females. In males, the highest overlaps were in 4 and 72 h, and 24 and 72 h. However, there were six DMRs in females and five DMRs in males that overlapped in all time points (Figure 3C,D). Notably, none of these overlapping DMRs were the same for males and females. Individuals clustered by treatment for all time points in both sexes (Figure 4 for females; Figure S4 for males).

The distribution of DMSs and DMRs differed from the null distribution for every time point in both sexes (Figure 5; see Table S4 for all G Test results). For DMSs, this difference was driven by a significant increase in DMSs in promoters and a decrease in DMSs in exons at all time points. At the 1 h time point, there was also a significant increase in intergenic DMSs for both males and females. This increase in intergenic DMSs remained significant up until the 24 h time point for females and remained significant for all following time points in males. For DMRs, this difference was driven by an increase in DMRs in promoters and exons and a decrease in DMRs in introns or intergenic regions; however, the magnitude of these changes was not always consistent across time points. While the increase in DMRs in exons was significant across all time points for both sexes, the increase in DMRs in promoters was significant at all time points except the 72 h time point in females and the 1 h time point in males. Similarly, the decrease in DMRs in introns was significant across all time points in both sexes, while the decrease in DMRs in intergenic regions was not significant at the 1 and 4 h time points for both males and females.

3.4 | GO Enrichment Analysis

In both males and females, genes associated with hypomethylated DMSs and DMRs included genes involved in diverse metabolic pathways, responses to stimuli and chemotaxis, regulation of transporter and neurotransmitter activity, and behavioural regulation (Figures S5–S8). For both males and females, genes associated with hypermethylated DMSs and DMRs were involved

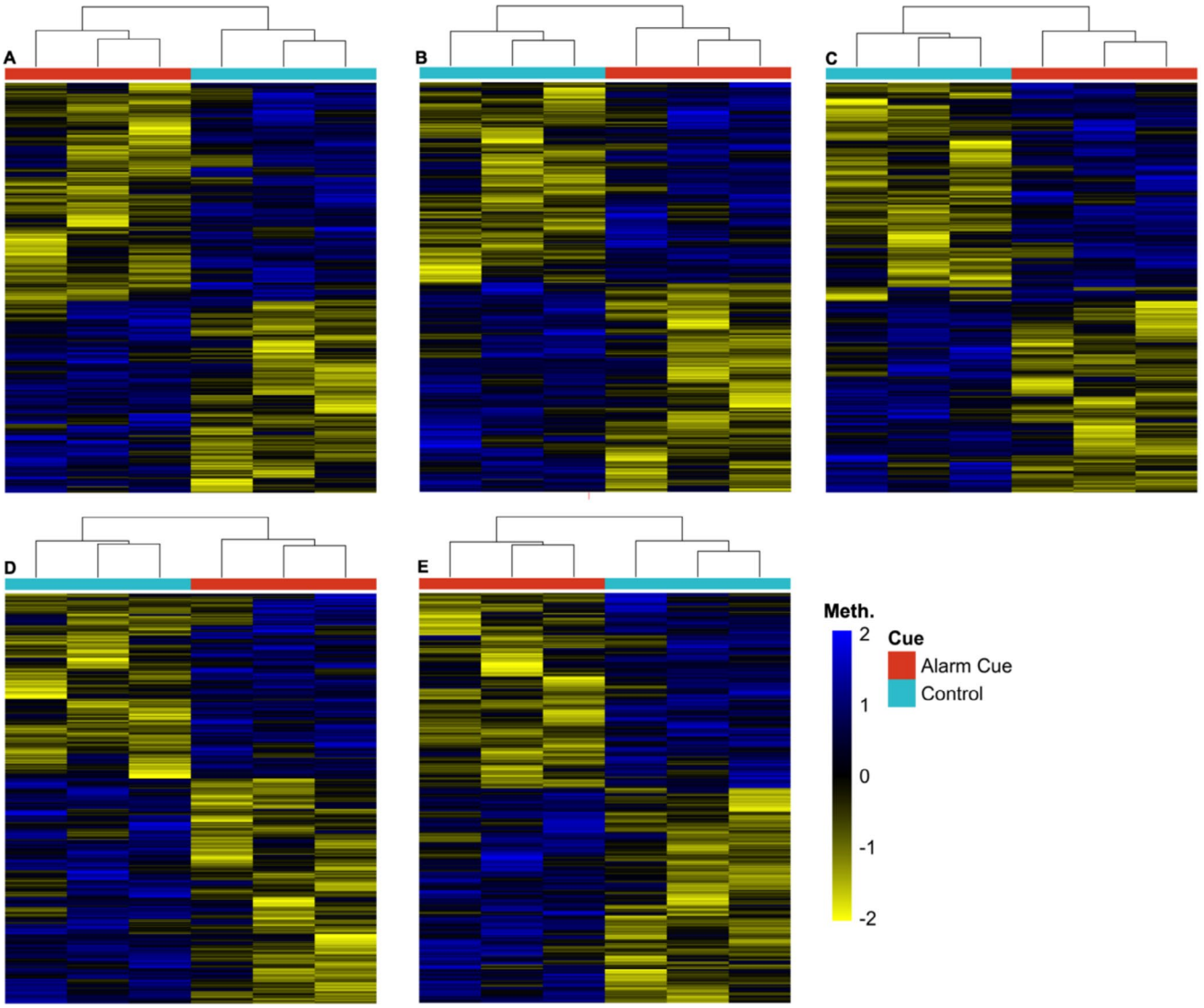

FIGURE 4 | Heatmaps with cluster results for differentially methylated regions (DMRs) identified at each time point for females. Each row shows the relative methylation of a DMR identified at (A) 0.5 h, (B) 1 h, (C) 4 h, (D) 24 h, and (E) 72 h. Thus, each row represents a different DMR in figures A throughE. Each column is an individual fish. Colour scale indicates the scaled percent methylation levels from high (blue) to low (yellow). Hierarchical clustering with Euclidean distance and Ward's linkage was run on samples, as shown above each heatmap.

in cerebellar neuron development and morphogenesis, cell differentiation, regulation of neurotransmitter secretions, and other metabolic pathways (Figures S9–S12). Full lists of over enriched GO terms across all categories (biological processes, molecular function, and cellular components) and all comparisons (hypermethylated, hypomethylated, and pooled) are given in the File S1.

4 | Discussion

While many studies have described environmentally induced shifts in DNAm, few have investigated the time course of these shifts, limiting our understanding of whether these changes might underpin contextual or developmental behavioural plasticity. Additionally, few studies investigate the impact of ecologically relevant stressors on DNAm in the brain or study animals in the group settings that are often typical in nature. We exposed pairs of guppies to alarm cue, which rapidly induced anti-predator behaviour; however, males did not reduce mating behaviour. Changes in DNAm in the brain were induced in response to alarm cue in as little as 0.5 h, with some methylation shifts emerging or being maintained 72 h later. We also found that males and females differed in their patterns of DNAm responses, with both females and males having a peak in differential methylation at 4 h, but males showing an additional peak at 72 h. This difference in methylation response could underpin sex differences in long-term plastic responses.

4.1 | Guppies Show Rapid Neural DNA Methylation Shifts

Both males and females exhibited shifts in DNAm beginning as early as the 0.5 h time point. Rapid shifts in neural DNAm have been previously observed. In mice, shifts in neural DNAm

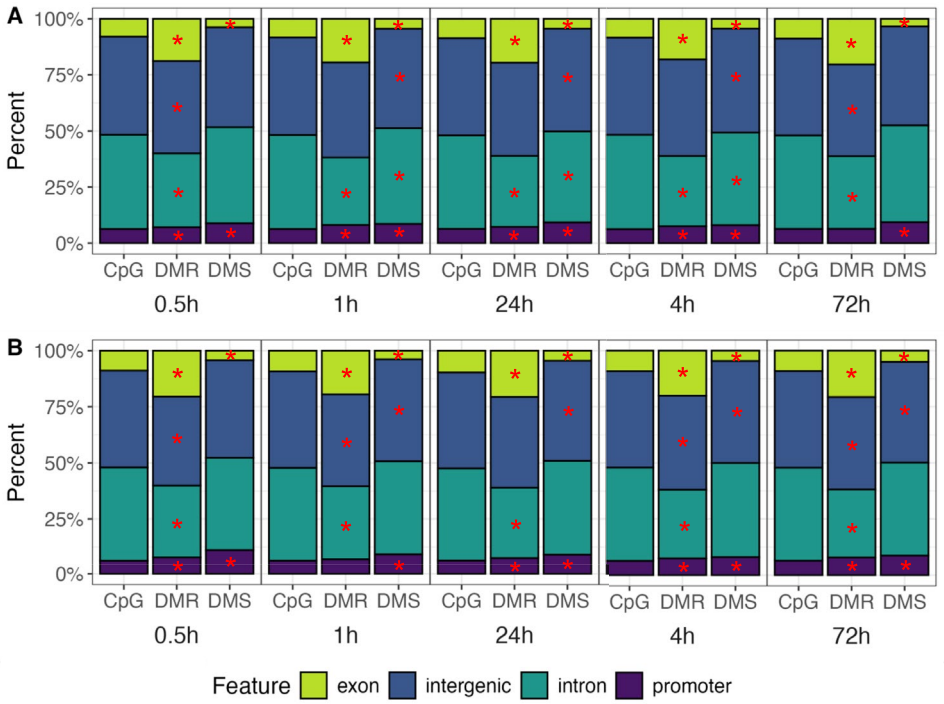

FIGURE 5 | Distribution of differentially methylated sites (DMSs) and regions (DMRs) identified compared to a null distribution of all CpGs at each time point for females (A) and males (B). Asterisks denote significant differences from the null distribution as tested using G tests.

were observed as early as 4 h after neuronal activation (Guo, Ma, et al. 2011) and 1 h in response to fear training (Miller et al. 2010). In fish, DNAm shifts in other tissue types have been shown in three days in salmon and four days in stickleback (Artemov et al. 2017; Beemelmanns et al. 2021), although neither of these studies investigated earlier time points. Our study is the first to show rapid shifts in neural DNAm in a fish species and documents the most rapid responses observed in any study that we are aware of. In mammals, studies suggest that DNAm may be especially dynamically regulated in the brain (Goto et al. 1994); however, it is uncertain whether these characteristics hold true for fish species or specifically for guppies. Additionally, few studies on animals have investigated the timeline of DNAm responses to ecologically relevant stressors, instead choosing to focus on perhaps unrealistically strong stressors that could be more likely to induce a shift in methylation (e.g., Miller et al. 2010), making extrapolation to the wild challenging. We used predation stress, a stressor that is widely encountered in nature. Therefore, our results are consistent with a large body of literature suggesting that shifts in DNAm in response to environmental cues are prevalent in nature (Rubenstein et al. 2016; Heckwolf et al. 2020; Hu et al. 2020; Caizergues et al. 2022), but we add novel information on the timeline of epigenetic responses. Further studies should use ecologically relevant stressors to assess the importance of rapid DNAm shifts in nature. We also studied guppies reared in the absence of predators for several generations and captured from an upstream low-predation site that is known to originate from downstream guppies that are exposed to high predation (Alexander et al. 2006). Domesticated guppies also maintain behavioural responses to alarm cue (Swaney et al. 2015). Our results suggest that the underlying genetic architecture to respond to alarm cue is maintained. Investigating differences in DNAm responses between high- and low-predation populations and the adaptive significance of these changes could help uncover the impact of evolution on the time course of DNAm responses.

The rapid timescales shown in this study suggest that DNAm can react quickly enough to be involved in the expression of contextual behavioural plasticity. There is already correlative evidence to suggest that DNAm is involved in behavioural plasticity in fish. For example, shifts in neural DNAm were associated with social status shifts that cause fast behavioural modifications in a cichlid fish species, *Astatotilapia burtoni* (Hilliard et al. 2019). Additionally, differing environmental enrichment, which has previously been shown to impact behavioural flexibility and cognition, induced shifts in DNAm in inbred populations of a mangrove killifish, *Kryptolebias marmoratus* (Berbel-Filho et al. 2019). However, most of these studies have focused on developmental timescales, with none that we know of investigating potential epigenetic mechanisms of contextual behavioural plasticity. Fish may have especially rapid DNAm shifts in the brain due to their ability to carry out adult neurogenesis and exhibit plastic morphological changes in the brain (Fong et al. 2019). Further work investigating shifts in DNAm and their potential ties to brain plasticity could be of interest.

We also found that at later time points (24 and 72 h), differential methylation was still detectable between the control and alarm cue fish for both males and females (at much higher rates for males, discussed below). While we only examined behaviour for 5 min after cue exposure, studies of other fish show that alarm responses typically last for 30–60 min after the alarm cue is released (Chivers et al. 2013; Wagner et al. 2022). Bisulphite sequencing is not able to distinguish between methylation and hydroxymethylation, which is an intermediate step in active demethylation (Huang et al. 2010). Therefore, some shifts

in DNAm detected at later time points could be intermediate steps prior to demethylation. However, many DMSs detected at later time points were at different sites from the earlier time points, which could suggest involvement in memory formation or longer-term plasticity. DNAm has been shown to play a role in memory formation in several studies, specifically in the processing and formation of stress-related memories in mice (Miller et al. 2008, 2010). In these studies, differential methylation was observed for several days following learning experiences about stress. As previously mentioned, DNAm has also been implicated in developmental behavioural plasticity. Exposure to alarm cue has been shown to impact guppy behaviour even long after the cue is removed. A 3-day exposure to alarm cue caused guppies to change their exploratory behaviour the following day without current alarm cue exposure (Crane et al. 2022). Guppies are also able to learn to fear novel stimuli that are paired with alarm cue exposures (Fan et al. 2022). DNAm changes observed at these later time points could be involved in a longer-lasting behavioural response to alarm cue exposure, such as learning or developmental plasticity; however, future studies would need to be done to confirm this.

Very few DMRs and no DMSs overlapped between all time points, indicating a somewhat ephemeral contribution of each site or region to the overall methylation response. Different sites or regions may be involved in responses at different timescales. Alternatively, the lack of depth in our sequencing could result in some smaller shifts in DNAm going undetected, meaning that some sites or regions could be stable for longer time points but at a level that we could not detect. Additionally, using whole brain tissue means varying cell types and brain regions contribute to the DNAm results. Therefore, it is possible that differences in cell-type heterogeneity could have impacted our results. However, specific brain regions in guppies are <10 mg (Marhounová et al. 2019) and thus pose a considerable challenge for dissection and obtaining enough tissue for WGBS. Future research could apply single-cell sequencing or laser capture microdissection techniques to assess DNAm responses in specific brain regions or cell types (Guo et al. 2023). Studying how neural DNAm responses differ depending on the timescale of stressor exposure would also be of interest.

Our findings in the GO term enrichment analysis suggest that genes that were hypomethylated are involved in responses to stimuli and behavioural regulation, while hypermethylated genes were involved in neuron development and regulation of neurotransmitters. These findings provide further evidence that the DNAm we uncovered could be involved in behavioural plasticity. Typically, hypomethylation indicates an increase in expression, while hypermethylation indicates reduced expression; however, this is not always the case, and sometimes the reverse occurs, or DNAm impacts expression in different ways, such as altering splicing patterns or does not impact gene expression at all (Ehrlich and Lacey 2013). The impact of DNAm on expression likely depends on the genomic context it is found in. For example, recent evidence indicates that gene body methylation can activate transcription (Jjingo et al. 2012). Therefore, these results must be interpreted carefully. Future studies could use a DNMT inhibitor and test for an effect on the expression of contextual behavioural plasticity to further elucidate the role of DNAm.

4.2 | Males and Females Differ in DNA Methylation Landscapes in Response to Alarm Cue

We found important differences in responses to alarm cue between males and females. Individuals of both sexes exposed to alarm cue increased their substrate use. This aligns with other literature showing that alarm cue can rapidly induce anti-predator behaviour in guppies (Brown and Godin 1999; Brown et al. 2009, 2010). However, since males and females were tested together, their behaviour may have influenced one another. Males continued mating attempts during alarm cue exposure, as has been previously described (Kelly and Godin 2001; Evans et al. 2002), so males may have followed females as females moved lower in the water column. Male guppies have been found to be less behaviourally responsive to acute predation stress than female guppies (Magurran and Seghers 1994b; Brusseau et al. 2023), further suggesting male behavioural responses could have been in response to female behaviour, not cue exposure. We also found that males and females differed in the timeline of their DNAm responses to alarm cue. Females showed a peak at the 4 h time point and then a steady decrease, whereas males showed a smaller peak at 4 h and then a second, larger peak at 72 h. Our validation analyses using overdispersion correction in *methylKit* and *DSS* confirm these findings. It is possible that females have a larger peak in DNAm response earlier than males due to stronger anti-predator responses than males. However, it is surprising that males have a second peak at 72 h that is not present in females. This delayed peak could indicate that longer-term processes such as learning or developmental plasticity are being triggered. Since only males are showing this delayed peak, they may be learning about the predation environment differently than females. Males have been observed to alter both anti-predator behaviour and mating tactics in response to predation risk, with changed mating behaviour at least partly the result of changes in female behaviour (Magurran and Seghers 1990; Godin 1995; Dill et al. 1999; Evans et al. 2002) (but see: Chuard et al. 2020). Therefore, male methylation responses may include changes related to both mating and anti-predator behaviour. This emphasises how social settings modulate the costs and benefits of predation and also potential epigenetic responses.

4.3 | Conclusion

DNAm is known to be environmentally sensitive and is suggested to play a role in phenotypic plasticity; however, few studies investigate the time course of DNAm responses. For DNAm to be involved in short-term plastic responses, it must respond on relevant timescales. In this study, we show that Trinidadian guppies exhibit neural DNAm shifts in response to alarm cue exposure on remarkably quick timescales. These results indicate that DNAm can shift on timescales relevant to short-term behavioural responses. However, DNAm differences were present between alarm and control cue exposed individuals even 72 h after exposure, suggesting potential involvement in longer-term behavioural responses as well. Studies showing the impact of environmental cues on DNAm remain useful but should be expanded to obtain information on the timescale and stability of these responses as well as potential sex differences. By further investigating these aspects of DNAm responses, we will get closer to understanding the precise role that DNAm plays in phenotypic plasticity.

Author Contributions

J.A.F. conceptualised the study design, carried out laboratory work, analysed data, and wrote the manuscript. S.M.R. and R.D.H.B. gave feedback on the design and paper and provided funding/facilities.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The WGBS data presented in this study is deposited in the NCBI Sequence Read Archive (SRA) repository (BioProject ID: PRJNA1220975). Behavioural data and code are accessible on Dryad (https://doi.org/10.5061/dryad.x0k6djhw6). Analysis code can also be accessed on Github (https://github.com/janayfox/Guppy-Cue-Exposure).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.