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Transcriptome Analysis in a Scleractinian Coral, *Acropora tenuis*, During the Spawning Season With Reference to the Gonadal Condition

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Synchronous spawning is a striking feature of coral. Although it is important for reproductive success, corals reallocate energy for reproduction to growth when they are damaged by external stimuli. To assess the transcriptome before and after spawning in the scleractinian coral *Acropora tenuis*, we tagged three colonies (one bleached and two unbleached) in the field around Sesoko Island (Okinawa, Japan) in November 2016, sampled them monthly from May to July 2017, and performed RNA sequencing (RNA-Seq) analysis. Histological analysis revealed that the previously bleached colony possessed gametes in June, by which time the other two colonies had already spawned. In RNA-Seq analyses, multi-dimensional scaling based on gene expression similarity among the samples reflected the differences between colonies and between months except for the sample of a non-spawned colony in May, which was similar to the samples in June. The similarity of the non-spawned colony sample in May to the samples in June was also shown in hierarchical clustering based on the expression patterns of the genes that were differentially expressed between months in the spawned colonies. These results suggest that non-spawning was already decided in May, and that the physiological condition in a non-spawned colony in May was advanced to June. RNA-Seq analysis also showed that genes related to gametogenesis and those related to apoptosis were upregulated before and after spawning, respectively.

Key words: coral reproduction, coral spawning, coral bleaching, *Acropora tenuis*, whole transcriptome analysis, RNA-seq, vitellogenin, apoptosis

INTRODUCTION

Synchronous spawning is a striking feature of corals. The timing of their reproduction is generally fixed within a specific season, lunar phase, and time of day, and reproductive events are highly synchronized within a given species. Synchronous spawning has been documented in many coral reefs from tropical to temperate regions (Harrison et al., 1984; Babcock et al., 1986, 1994; Hayashibara et al., 1993; Nozawa et al., 2006). This tight time window for reproduction is considered to ensure opportunities for fertilization, increase the abundance of outcrosses, and avoid predation, e.g., by planktivorous fish species (Babcock et al., 1986; Westneat and Resing, 1988; Domeier and Colin, 1997; Carlon, 1999).

Although synchronous spawning is important for reproductive success, corals reallocate energy for reproduction to growth upon sustaining damage by external stimuli. For

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example, when *Acropora formosa* (= *Acropora muricata*) was divided into fragments artificially and transplanted in the field, the oocytes in small fragments were resorbed, while those in large fragments were not, and the fragments that had resorbed their oocytes showed a higher growth rate than those that had spawned (Okubo et al., 2005, 2007). Therefore, when investigating coral reproduction in the field, it is important to check the corals' gonadal conditions and confirm their spawning. Coral bleaching refers to the expulsion of dinoflagellate symbionts, commonly known as zooxanthellae, from host corals. It is induced by environmental extremes, such as high temperature and irradiance (Brown, 1997; Douglas, 2003). Bleaching also impairs coral reproduction, and several coral species show decreased abundance and/or quality of gametes after bleaching (Szmant and Gassman, 1990; Michalek-Wagner and Willis, 2001; Johnston et al., 2020). The reallocation of energy from reproduction to growth is important for determining whether to prioritize growth or reproductive success, but little is known about when and how corals make this decision.

Acropora is the most diverse genus of scleractinian coral, and includes the greatest number of species (Wallace, 1999). *Acropora* corals are hermaphroditic species that produce and release "bundles" (sacs containing eggs and sperms) in each polyp during spawning. In the Ryukyu Archipelago, most *Acropora* corals spawn within several days around the full moon in May and/or June (Isomura and Fukami, 2018; Baird et al., 2021a). The molecular mechanisms of reproduction in *Acropora* corals have been investigated in *Acropora digitifera* by transcriptomics analysis (RNA sequencing; RNA-Seq) mainly focusing on spawning with reference to lunar and diurnal time points (Rosenberg et al., 2017, 2019). To gain further insight into the molecular mechanisms underlying *Acropora* coral reproduction, we performed RNA-Seq analysis focusing on gonadal development prior to spawning in another *Acropora* coral species, *Acropora tenuis*. Within *Acropora*, *A. tenuis* is considered to have evolved in a more basal clade than *A. digitifera* (van Oppen et al., 2001; Shinzato et al., 2021) and its gonadal development in the Ryukyu Archipelago was documented previously (Tan et al., 2020). That previous study showed that small oocytes for the next spawning can be observed in July, 1 month after spawning, develop gradually until 1 month before the next spawning, and undergo marked maturation in the last month (Tan et al., 2020). Based on these observations, we performed RNA-Seq analysis in *A. tenuis* between 1 month before and 1 month after spawning, when the most drastic changes in the gonad could be observed.

In 2016, we tagged three colonies of *A. tenuis* in the field around Sesoko Island (Okinawa, Japan), an island in the Ryukyu Archipelago, and sampled them every month from May to July 2017. In the field observations before sampling, we found that one of three colonies experienced bleaching that occurred in 2016 (Kayanne et al., 2017). Histological observations revealed that the previously bleached colony still possessed gametes in June 2017, by which time the other two colonies had already spawned. Therefore, we could compare gene expression between the non-spawned and spawned colonies. RNA-Seq analyses revealed that differences between colonies and between months were reflected by multidimensional scaling (MDS) based on similarities in gene expression among the samples, except for the sample of a non-spawned colony in May, which was similar to that in June. These observations suggested that non-spawning was decided in May, and that the physiological condition in a non-spawned colony in May was advanced to June. The results of RNA-Seq analysis also showed that the genes related to gametogenesis and those related to apoptosis were upregulated before and after spawning, respectively.

MATERIALS AND METHODS

Coral collection

In November 2016, we tagged one bleached (colony 1, C1) and two unbleached colonies (C2 and C3), at reefs around Sesoko Island, Okinawa Prefecture, Japan (26°63′11″N, 127°86′33″E) at a depth of about 1.5–2.0 m during low tide by skin diving. Their condition was observed every 1–2 months from the time of attaching tags until commencement of sampling. From May to July 2017, the branches (3–4 cm in length) around the outer edge of each colony were collected monthly using pliers. The collected branches were subjected to histological observation of the gonads and RNA extraction for RNA-Seq analysis and qPCR validation. For additional qPCR validation in another year, a further two colonies were tagged in August 2019 in addition to C1, C2, and C3, and we collected branches from a total of five colonies from April to September 2020. Sampling was conducted on a day around the full moon (see Supplementary Table S1) and a day around the new moon in May 2020. Immediately after collection, the branches were transferred to Sesoko Station of Tropical Biosphere Research Center, University of the Ryukyus. The collection of corals was approved by the Okinawa Prefectural Government (Approval nos. 28-84 [2017] and 2-1 [2020]).

Histological observation

To check the reproductive condition of the collected corals, their gonads were examined by hematoxylin and eosin (HE) staining, as described previously (Tan et al., 2020). After collection, some branches were fixed in Bouin's solution overnight at 4°C. Then they were decalcified in Morse's solution for 3–5 days. Following dehydration in a graded ethanol series and clearing with xylene, portions of the coral tissue were embedded in paraffin, cut into serial sections at 4 μm, and stained with HE for microscopic observation. The sizes of randomly selected oocytes with visible nuclei were measured using ImageJ64 software (National Institutes of Health, Bethesda, MD, USA). The diameter of each oocyte was expressed as the geometric mean of the maximum diameter and the widest diameter perpendicular to the maximum diameter. For statistical analysis of the size of oocytes, parametric or non-parametric methods were applied based on the outcomes of Bartlett's homogeneity and Shapiro–Wilk normality tests. For comparison among colonies in May, the non-parametric Kruskal–Wallis rank sum test and Steel–Dwass test were used. The parametric *t* test was applied for comparisons between May and June in C1.

RNA extraction

Coral branches not used for histological observation were fixed in RNAlater solution in 2017 and flash-frozen in liquid nitrogen in 2020. The samples were stored at –80°C for further processing. After completion of annual sampling, the branches were crushed to a fine powder using a liquid nitrogen-cooled mill. The branches stored in RNAlater solution were thawed at 4°C overnight, placed into the mill immediately after removing RNAlater solution, and flash-frozen in liquid nitrogen. The crushed branches were mixed with TriPure isolation reagent (Sigma-Aldrich, St. Louis, MO, USA) on ice, and total RNA was extracted according to the manufacturer's instructions. RNA was purified using the SV total RNA isolation

system (Promega, Madison, WI, USA).

RNA-Seq analysis

RNA samples were transported to Genewiz Japan Corp. (Saitama, Japan) for cDNA library preparation and HiSeq 2500 2 × 150 bp paired-end read sequencing (Illumina, San Diego, CA, USA). The sequence datasets generated in this study are available in GenBank/EMBL/DDBJ with the accession number PRJDB11485. The reference sequences, the genome of *A. tenuis* (*Acropora tenuis* genome v. 0.11, http://aten.reefgenomics.org/download/, last accessed on 9 May 2022), symbiotic zooxanthella *Symbiodinium goreaui*, a major symbiont of scleractinian corals in the Ryukyu Archipelago (Baker, 2003; LaJeunesse et al., 2004), and *Symbiodinium kawagutii* (Symbiodinium genomes v. 1.0, http://symbs.reefgenomics.org/download/, last accessed on 9 May 2022), were downloaded from Reefgenomics, a repository for marine genomics data (ReFuGe 2020 Consortium, 2015; Liew et al., 2016). After adapter sequences were trimmed with a minimum length of 100 bp using Trimmomatic (Bolger et al., 2014), the raw reads were aligned against these reference genomes using HISAT2 v. 2.1.0 (Kim et al., 2019) with the default settings. Read mapping to *S. kawagutii* was performed to confirm that the *S. goreaui* genome was a better reference than the *S. kawagutii* genome in this experiment. Although *S. goreaui* and *S. kawagutii* were revised as *Cladocopium goreaui* and *Fugacium kawagutii*, respectively (LaJeunesse et al., 2018), the old species names are used in this paper for consistency with the names of the reference genome datasets. The reads aligned to each genome were sorted and counted using SAMtools v. 1.5 (Li et al., 2009; Danecek et al., 2021) and HTSeq v. 0.11.2. (Anders et al., 2015) with the default settings. There were 15,995 and 20,480 putative transcripts to which at least one read was mapped in all samples in *A. tenuis* and *S. goreaui*, respectively, and they were used in further analyses. Due to the small number of mapped reads, no further analysis was performed on *S. kawagutii*.

The similarity of gene expression profiles among samples was examined in *A. tenuis* and *S. goreaui* by MDS based on Spearman's rank correlation coefficient (1 – *rho*), calculated from the number of reads mapped to the transcripts. The differentially expressed genes (DEGs) in *A. tenuis* between colonies and months were detected using the TCC v. 1.26.0 (Sun et al., 2013) and edgeR v. 3.28.1 (Robinson et al., 2010) packages in R (v. 3.3.0; R Development Core Team, Vienna, Austria) with trimmed mean of M-values (TMM) normalization. Genes showing significantly different expression levels in multiple comparisons between colonies and months, according to analysis of variance ($q < 0.05$), were defined as colony- and month-DEGs, respectively. Next, month-DEGs in spawned colonies were screened. Hierarchical clustering and heatmaps of DEGs were performed based on transcripts per million (TPM) using the hclust and heatmap.2 functions of the R package gplots v. 3.1.1 (Warnes et al., 2020). Gene annotations were assigned by querying putative transcript sequences against the National Center for Biotechnology Information (NCBI) GenBank non-redundant protein database using blastx (e-value < $1e^{-5}$).

qPCR validation

To validate the RNA-Seq results, the mRNA levels of representative genes were determined by qPCR. We selected representative genes related to gametogenesis and apoptosis based on gene annotations and RNA-Seq expression analyses (see Supplementary Table S2), because these two physiological functions have been investigated in coral species (Shikina et al., 2012, 2013, 2020; Moya et al., 2016; Tan et al., 2020, 2021). In 2017, RNA samples extracted from one branch other than those used for RNA-Seq were analyzed, and two to four replicates could be prepared for each colony in each month. In 2020, the RNA extracted from one branch of each colony was considered one replicate, and five replicates were prepared each month. cDNA was synthesized from each RNA sample using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). For representative genes not cloned in previous studies, the fragments were amplified using GoTaq DNA polymerase (Promega), cloned into the pGEM-T Easy vector (Promega), and sequenced to confirm their presence and sequence before qPCR. qPCR was performed using a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) and a TB Green Premix PCR Kit (TaKaRa Bio, Kusatsu, Japan) in duplicate. Melting-curve analysis was performed to ensure amplification of single amplicons. The mRNA levels of target genes were normalized relative to those of *β-actin* and *ef1α*

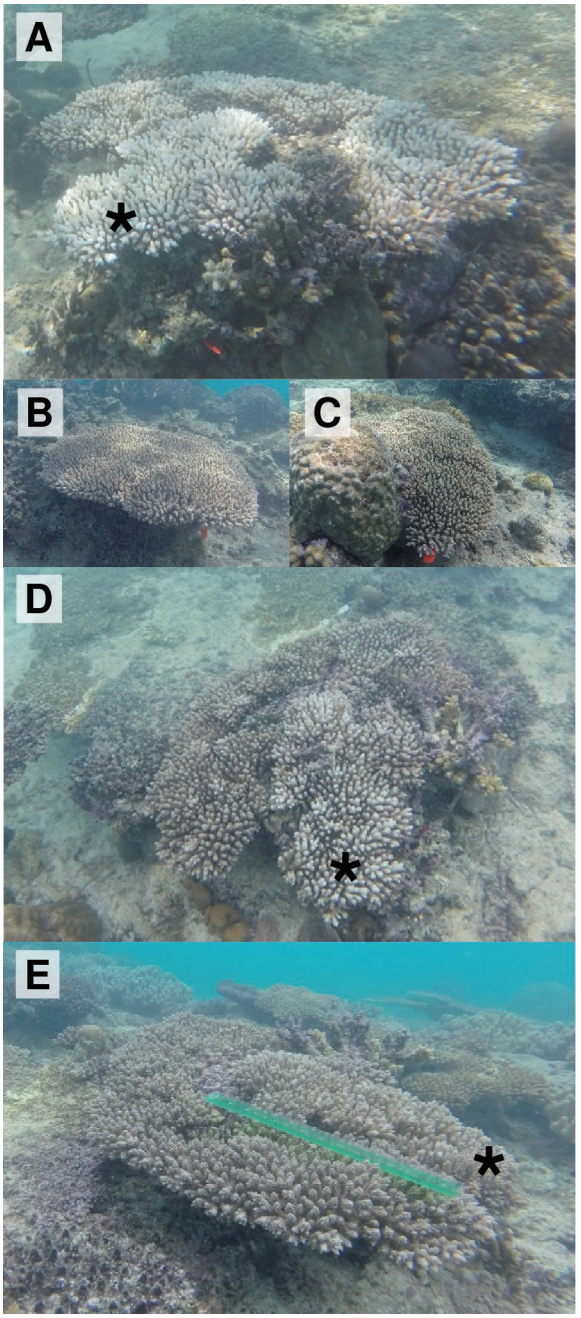

Fig. 1. Colonies of *Acropora tenuis* in November **(A–C)** and December **(D)** 2016, and January 2017 **(E)**. Three colonies, C1 (Colony 1, **[A, D, E]**), C2 **(B)**, and C3 **(C)**, were sampled in 2017. The asterisks in the photographs of C1 **(A, D, E)** indicate the same part of the colony.

(internal controls). The primers used in this study are shown in Supplementary Table S3. For statistical analysis, the non-parametric Kruskal–Wallis rank sum test was applied based on the outcomes of Bartlett's homogeneity and Shapiro–Wilk normality tests. For multiple pairwise comparisons, Dunn's test was used to compare the means among groups.

RESULTS

Field observations

In November 2016, when we tagged three colonies (C1–C3), bleaching was observed in C1, with most branches paling in color (Fig. 1A). In the summer of 2016, coral reefs in Ryukyu Archipelago suffered from extensive bleaching (Kayanne et al., 2017), and C1 seemed not to have recovered from it. In C2 and C3, no bleaching was observed, at least in our field observations (Fig. 1B, C). After 1 month, C1 showed signs of recovery, as the paling regions had decreased (Fig. 1D) and then by January 2017 no white regions were observed (Fig. 1E). From 2019 to 2020, no bleaching was observed in any of the five colonies examined, including two that were newly tagged.

Historical observation of gonads

In 2017, vitellogenic oocytes were observed in all three colonies in May (Fig. 2A–C). Although we did not find marked differences in gonad structure between colonies, the average diameter of oocytes in C1 was 280.64 ± 4.75 μm (mean ± SE; n = 34), which was significantly smaller than that in C2 (314.63 ± 7.21 μm; n = 39) and C3 (306.98 ± 7.15; n = 31) (P < 0.05, Steel–Dwass test). In June, the month of spawning, no mature gametes were observed in C2 or C3 (Fig. 2E, F), but oocytes and sperm were observed in C1 (Fig. 2D). There was no marked difference in gonad structure from May onward in C1, and the diameter of oocytes (288.54 ± 7.41 μm; n = 12) was not significantly different

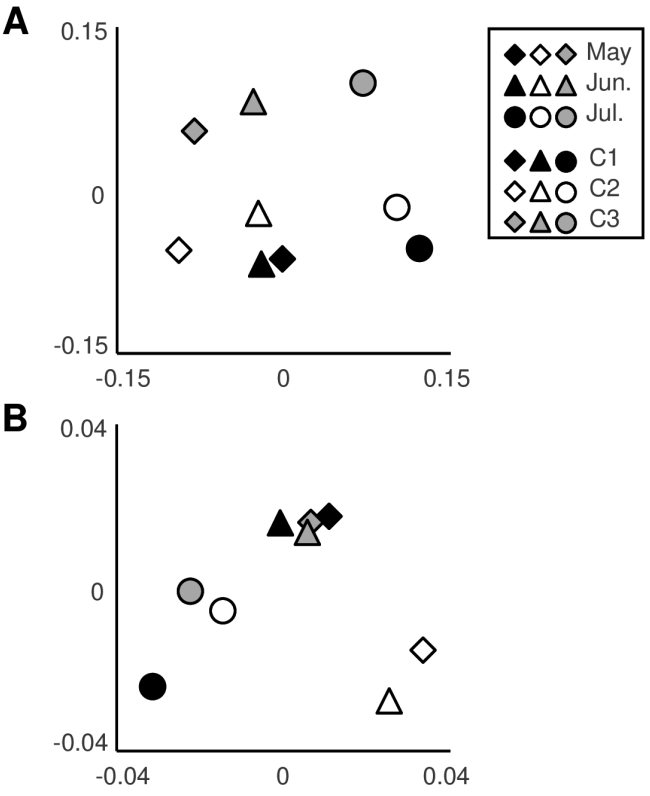

Fig. 3. Multi-dimensional scaling plots for assessing the similarity of samples from three colonies (C1–C3) in May, June, and July. The distance between samples was based on Spearman's rank correlation coefficient (1 – *rho*), calculated from the number of reads mapped to the transcripts of a coral *Acropora tenuis* **(A)** and its symbiotic zooxanthellae *Symbiodinium goreaui* **(B)** from the Reefgenomics database (ReFuGe 2020 Consortium, 2015; Liew et al., 2016). Symbol colors and shapes indicate colonies and months, respectively.

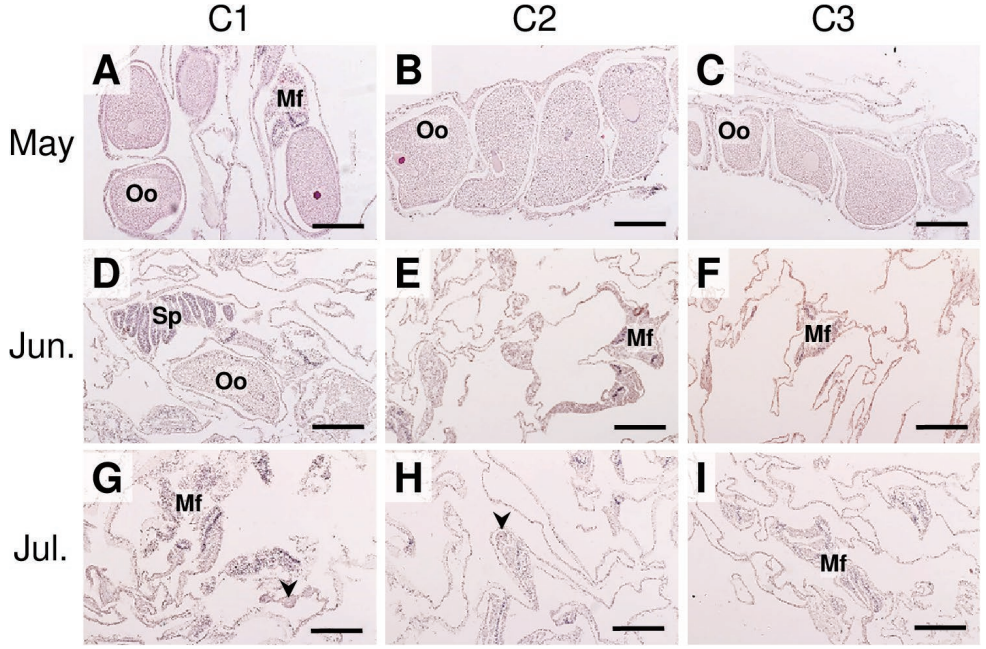

Fig. 2. Representative sections of a gonad of *Acropora tenuis* in May **(A–C)**, June **(D–F)**, and July **(G–I)** 2017 stained with haematoxylin-eosin. Photographs of the colonies tagged as C1 (Colony 1): **(A, D, G)**, C2 **(B, E, H)**, and C3 **(C, F, I)** and used for RNA-seq are shown. Arrowheads in **(G)** and **(H)** indicate immature oocytes. Oo, oocyte; Mf, mesentery filament; Sp, spermatozoa. Scale bar, 200 μm.

from that in May ($P > 0.05$, *t* test). In July, no mature gametes were observed in the three colonies, and a few immature oocytes were observed (Fig. 2G–I). In 2020, mature oocytes were observed in the samples collected from April to June, but not after July for any of the five colonies.

RNA-Seq analysis

After trimming of adapter sequences, 9–22 million reads were obtained for each sample, and approximately 80% were mapped to the *A. tenuis* or *S. goreaui* genome, although the proportions of reads mapped to *A. tenuis* and *S. goreaui* differed between samples (see Supplementary Figure S1 and Table S4). As the number of reads that mapped to both *A. tenuis* and *S. goreaui* was very small compared to the total reads (approximately 0.031% of total reads; see Supplementary Table S4), they were included in further analyses for both *A. tenuis* and *S. goreaui*. Compared to *A. tenuis* and *S. goreaui*, fewer reads were mapped to the *S. kawagutii* genome (approximately 0.344% of total reads) (see Supplementary Table S4). The results implied that *S. goreaui* was a major symbiont in the present study.

MDS plots based on gene expression similarity revealed that month and colony relationships could be explained in part by changes in gene expression in *A. tenuis* (Fig. 3A). In the MDS plot of *A. tenuis*, the differences in gene expression between colonies are shown on the vertical axis, and the differences between months are shown on the horizontal axis, except for a sample of C1 in May (C1-May), which was located closer to the June samples than C2-May and C3-May (Fig. 3A), indicating its transcriptomic similarity to

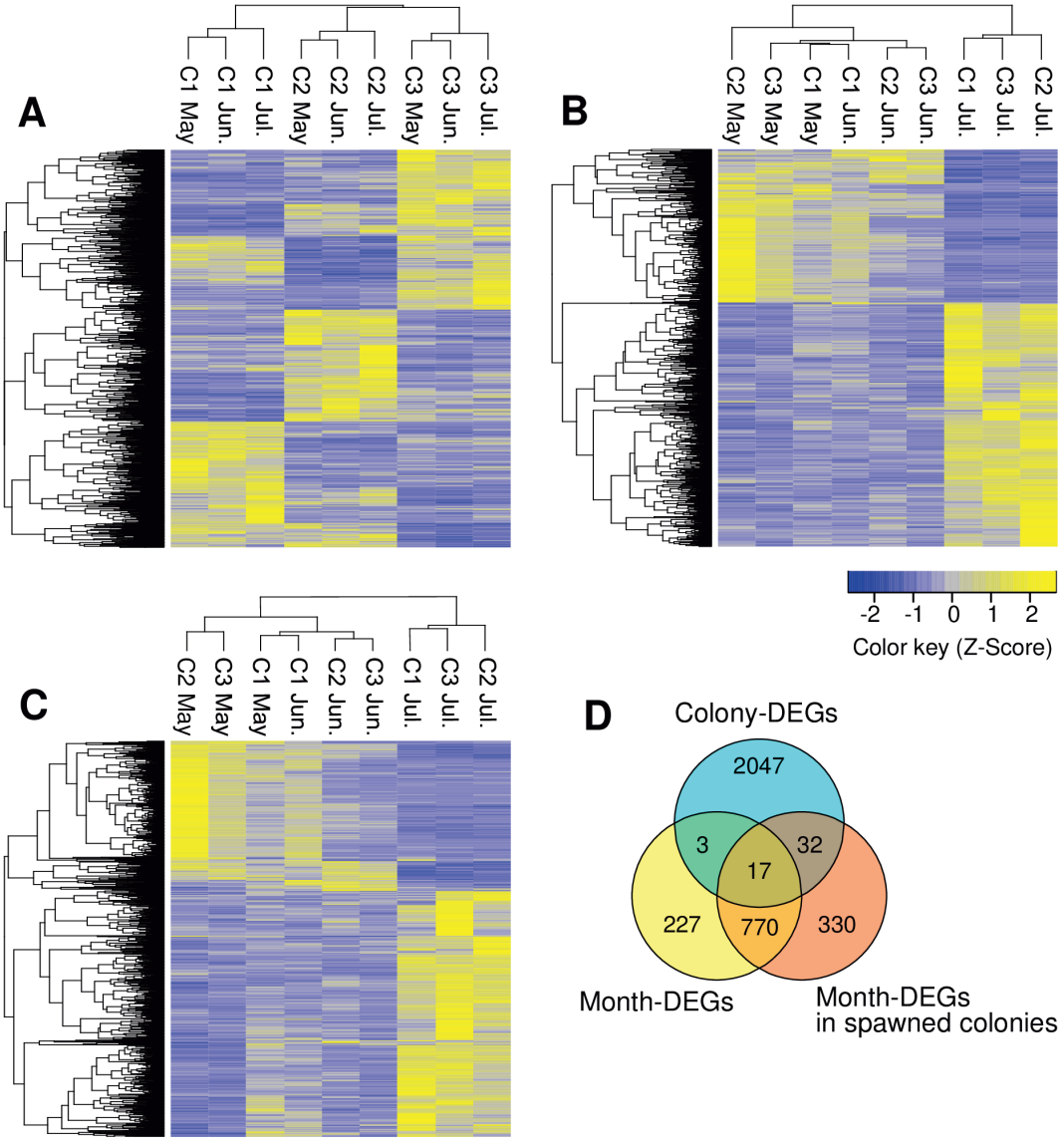

Fig. 4. Expression patterns of differentially expressed genes (DEGs) in a non-spawned colony (C1) and two spawned colonies (C2 and C3) of *Acropora tenuis* from May to July in 2017. DEGs in the comparison of colonies **(A)** Colony-DEGs, in the comparison of months in all three colonies **(B)** Month-DEGs, and in the comparison of months only in spawned colonies **(C)** Month-DEGs in spawned colonies, are shown. **(A–C)** Heatmaps of DEG expression and hierarchical clustering of gene expression data for the DEGs and samples. **(D)** Venn diagram of the number of DEGs between colonies, months in all colonies, and months in spawned colonies.

the June samples. For the reads mapped to the *S. goreaui* genome, there was no clear trend in the relationships according to colonies or months (Fig. 3B). Therefore, it appeared that there were no marked changes in transcripts of symbiotic zooxanthellae according to host colony or sampling month during this experimental period.

Expression patterns of DEGs

Expression analysis of *A. tenuis* revealed 2099 and 1017 colony- and month-DEGs, respectively (Fig. 4, see also Supplementary Tables S5 and S6). The colony-DEGs showed varying patterns of up- and downregulation among colonies (Fig. 4A). The numbers of up- and downregulated colony-DEGs were similar among colonies (see Supplementary Figure S2A). By contrast, most month-DEGs were up- or downregulated in July (Fig. 4B, see also Supplementary Figure S2B), suggesting marked changes in gene expression after spawning. On the other hand, the May and June samples were included in one cluster on hierarchical clustering based on month-DEGs (Fig. 4B). As C1-May was located close to the June samples in the MDS plot, the difference between May and June could have been underestimated in the analysis including C1. To examine this possibility, month-DEGs in spawned colonies (C2 and C3) were screened (Fig. 4C). The results detected 1149 genes as month-DEGs in spawned colonies (see Supplementary Table S7), 787 of which were common to month-DEGs in all colonies (Fig. 4D). The heatmap of month-DEGs in spawned colonies showed that, in most cases, their expression levels were markedly changed after spawning (Fig. 4C, and see Supplementary Figure S2C). Therefore, the transcriptomic change after spawning was not an overestimation caused by a non-spawning colony. In hierarchical clustering based on month-DEGs in spawned colonies, the samples of spawned colonies (C2 and C3) formed three clusters that reflected month, and the difference between May and June, which was not reflected in month-DEGs (Fig. 4B), was also reflected (Fig. 4C). For the non-spawned colony, C1-June and C1-July were included in the June and July clusters, respectively. However, C1-May was located in the same cluster as the samples in June (Fig. 4C). This result indicates that the transcriptomic condition in C1-May was not unique but was similar to those in June, even when focusing on the DEGs in the normally spawned colonies.

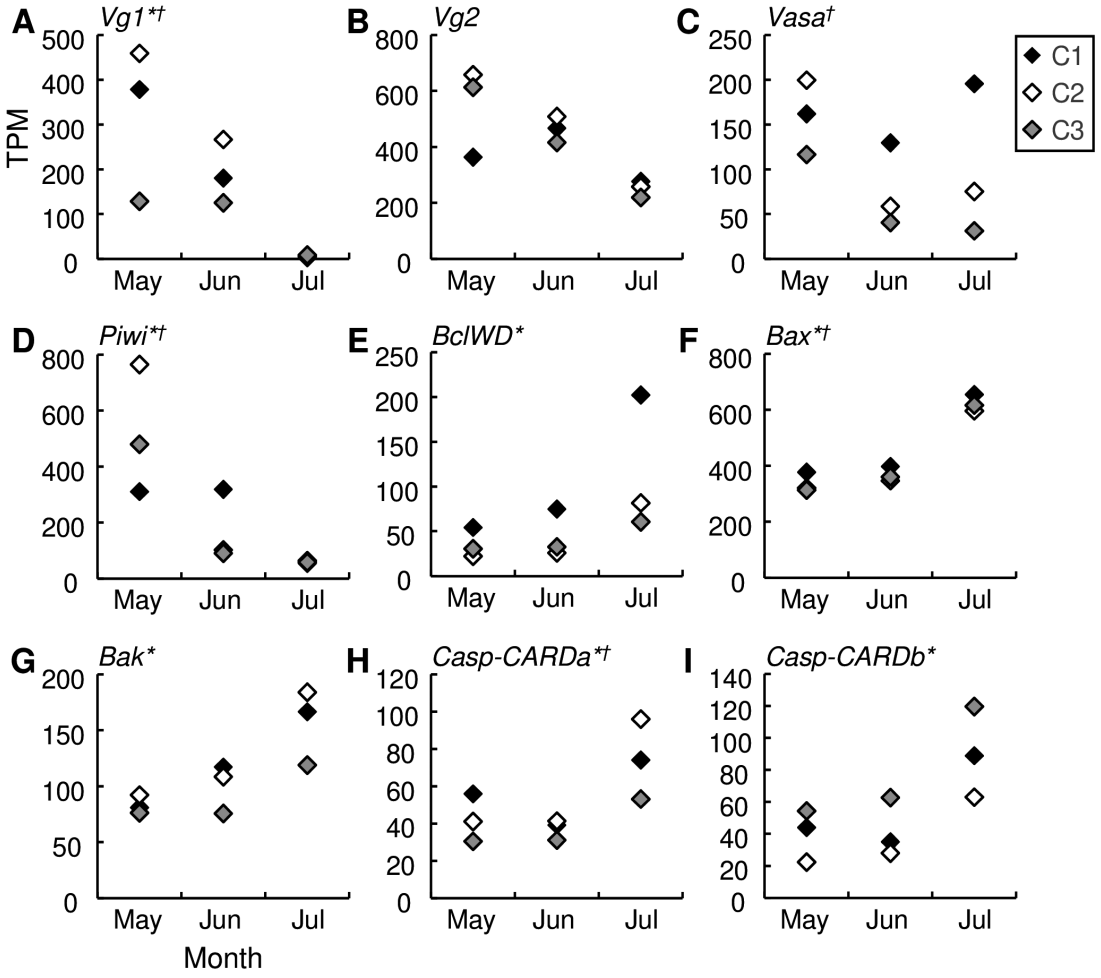

Fig. 5. Expression profiles of *Acropora tenuis* genes related to gametogenesis (*Vg1*, *Vg2*, *Vasa*, *Piwi*), and apoptosis (*BclWD*, *Bax*, *Bak*, *Casp-CARDa*, *Casp-CARDb*) revealed by RNA-seq. The transcripts per kilobase million values of C1 (Colony-1), C2, and C3 are indicated by black, white, and grey squares, respectively. Asterisk (*) and dagger (†) indicate DEGs between months in the comparisons of all three colonies (C1–C3) and two spawned colonies (C2 and C3), respectively.

qPCR validation of representative genes

Based on gene annotations and expression patterns in RNA-Seq, we selected the genes related to gametogenesis (Shikina et al., 2012, 2013; Tan et al., 2020, 2021) and apoptosis (Moya et al., 2016; Shikina et al., 2020) as representatives for qPCR validation. With regard to the genes related to gametogenesis, we performed qPCR validation of *Vg1*, *Vg2*, *Vasa*, and *Piwi* (see Supplementary Table S2). For RNA-Seq, *Vg1* and *Piwi* were included as month-DEGs in the analyses of both all colonies and spawned colonies, while *Vasa* was included as a month-DEG in the analysis of spawned colonies. Although *Vg2* was not a month-DEG, it was included in the analysis because it had a similar expression pattern to the other three genes. RNA-Seq analysis showed that their expression levels tended to be high before spawning (Fig. 5A–D). qPCR validated the RNA-Seq data in 2017 (Fig. 6A–D). In C2 and C3, significant differences were detected for all four genes ($P < 0.05$, Dunn's test). In C1, the expression patterns of these genes were different from those in C2 and C3, particularly for *Vasa* and *Piwi* in June, and there were no significant differences for *Vg1*, *Vg2*, or *Vasa* ($P > 0.05$, Kruskal–Wallis rank sum test). To evaluate the robustness of their expression patterns, we examined two additional colonies in 2020 over a longer period (Fig. 7). The expression of the four genes differed significantly from April to September ($P < 0.05$, Kruskal–Wallis rank sum test), but the expression levels of *Vg2* and *Vasa* were not significantly different among May, June, and July. Therefore, the expression patterns of these two genes were not consistent from May to July (Fig. 7A–D).

We also evaluated the expression of genes related to apoptosis. As its molecular basis has been established in another coral species, *Acropora millepora*, we used the gene names from this species (Moya et al., 2016). We selected *BclWD*, *Bax*, *Bak*, *Casp-CARDa*, and *Casp-CARDb* (see Supplementary Table S2) as representative DEGs, because they were included among the month-DEGs in all colonies. RNA-Seq analysis showed that their expres-

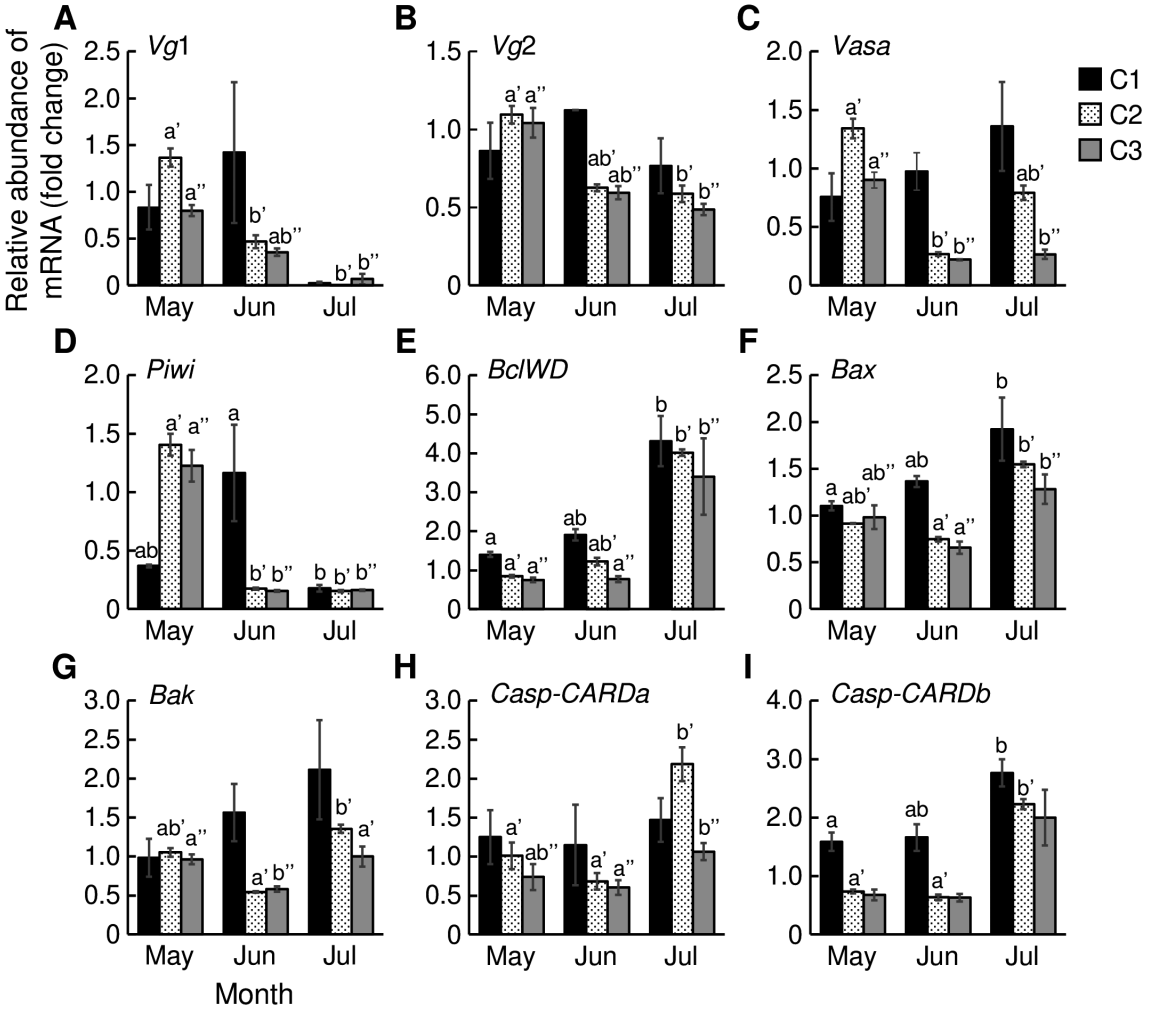

Fig. 6. Relative mRNA levels of genes related to gametogenesis (*Vg1*, *Vg2*, *Vasa*, *Piwi*), and apoptosis (*BclWD*, *Bax*, *Bak*, *Casp-CARDa*, *Casp-CARDb*) in three colonies (C1–C3) of *Acropora tenuis* from May to July in 2017. Total RNA was extracted from a single branch (3–4 cm) of each colony. The qPCR samples were different from those for RNA-Seq. n = two–four. *β-actin* and *ef1α* were used as endogenous controls. The mean values of three colonies in May were set at 1.0 for each gene, and the data are means ± SE. The significance of differences between months was tested for each colony, and letters with no, single, and double quotations indicate the results of C1–C3, respectively. Months with different letters in each column were significantly different (Dunn's test, $P < 0.05$).

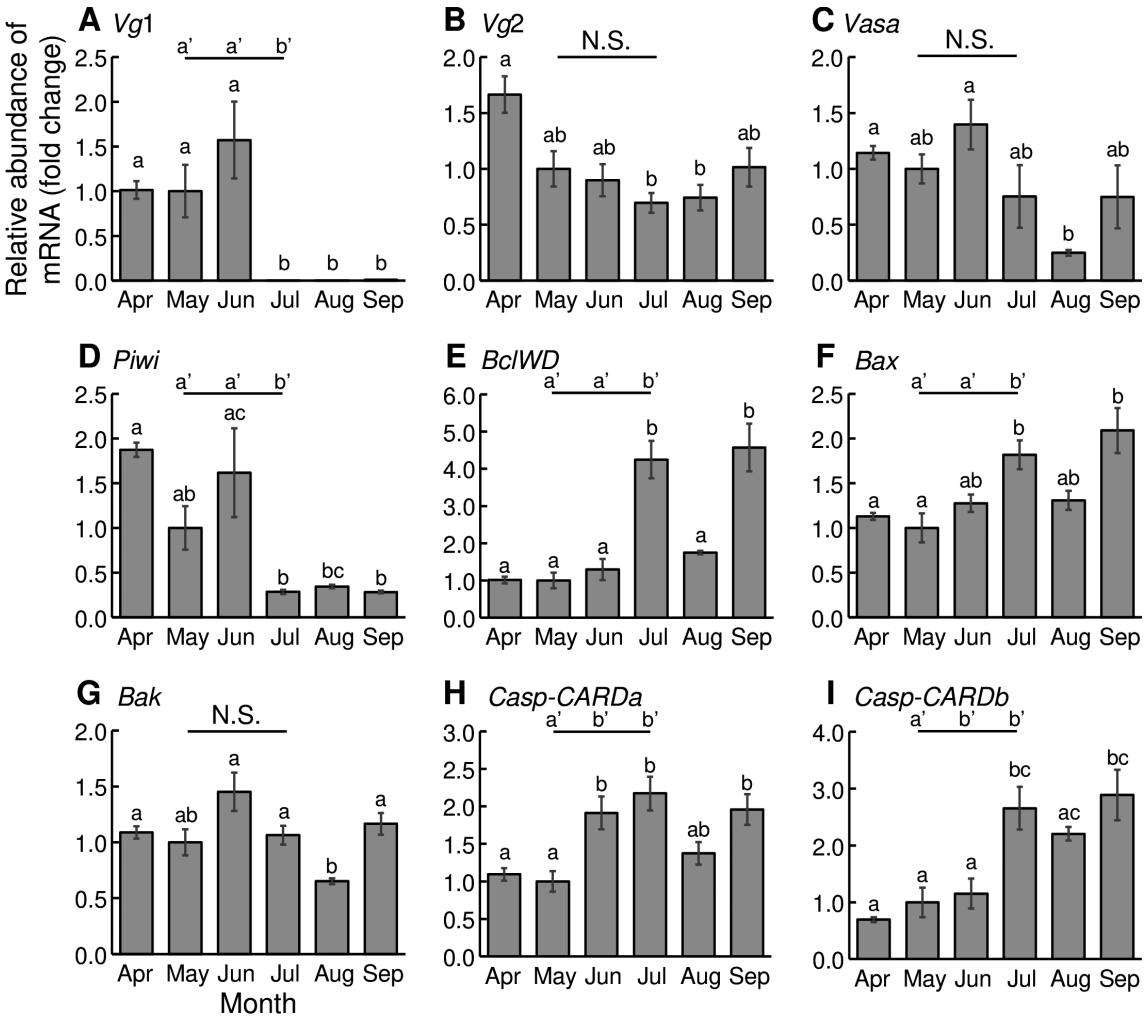

Fig. 7. Relative mRNA levels of genes related to gametogenesis (*Vg1*, *Vg2*, *Vasa*, *Piwi*) and apoptosis (*BclWD*, *Bax*, *Bak*, *Casp-CARDa*, *Casp-CARDb*) in the five colonies of *Acropora tenuis* from April to September 2020. Total RNA was extracted from a single branch (3–4 cm) of each colony. $n = 5$. *β-actin* and *ef1α* were used as endogenous controls. The mean values in May were set at 1.0 for each gene, and the data are means ± SE. Letters with and without quotation marks indicate the results for 3 months (May–July) and 6 months (April–September), respectively. Months with different letters in each column were significantly different (Dunn's test, $P < 0.05$).

sion levels tended to increase after spawning (Fig. 5E–I), which was validated by qPCR. The expression levels of *BclWD* and *Bax* were significantly higher in July than in May and/or June in all three colonies (Fig. 6E, F). *Bak* expression increased from June to July, and differed significantly between June and July in C2 and C3, but not in C1 (Fig. 6G, $P > 0.05$, Kruskal–Wallis rank sum test). *Casp-CARDa* and *Casp-CARDb* expression levels were high in July, although the differences were not significant in one colony (Fig. 6H, I). The expression patterns of the five genes differed significantly from April to September in 2020 (Fig. 7E–I, $P < 0.05$, Kruskal–Wallis rank sum test). *Bak* expression did not differ significantly among May, June, and July ($P > 0.05$, Kruskal–Wallis rank sum test), suggesting that its expression was not consistent during this period (Fig. 7G).

DISCUSSION

Two of three colonies subjected to RNA-Seq analysis, C2 and C3, had no gametes in June 2017, whereas C1 did have gametes. In 2017, the majority of *A. tenuis* around Sesoko Island spawned on June 6th, 2 days before sampling, and a few colonies spawned on June 8th, i.e., on the sampling day (Baird et al., 2021b). Hence, we cannot exclude the possibility that C1 spawned after sampling in June. On the other hand, the size of oocytes increased markedly 1 month before spawning in *Acropora* corals even in the case of irregular spawning (Okubo et al., 2007; Tan et al., 2020). For C1, the size of oocytes did not increase from May to June, suggesting immaturity. Therefore, it is plausible that C1 did not spawn in 2017, resorbed its gametes between June and July, and used the energy to repair the bleaching that occurred in the previous year.

According to RNA-Seq analysis, the gene expression pattern in May in a non-spawning colony was similar to that in June. This suggested that the decision not to spawn had already been made in May, although the oocytes had already developed to some extent. As the oocyte size in the non-spawned colony corresponded to that from late winter to early spring (Tan et al., 2020), maturation may have stopped during this period. There may be a checkpoint determining

spawning from late winter to early spring. Reallocation of energy from reproduction to growth has been investigated in detail in *A. formosa* (Okubo et al., 2005, 2007). When *A. formosa* was divided into fragments in November, the oocytes in the early vitellogenic stage were resorbed within 2 months, whereas those in the late stage continued developing and finally spawned (Okubo et al., 2007). In this study, *A. tenuis* retained immature oocytes after bleaching until the spawning season. We could not determine the reason for this difference, although oocyte resorption cascades may differ according to the type of damage and/or between species.

MDS plots based on gene expression similarity revealed by RNA-Seq analysis showed similarity of gene expression patterns between the non-spawning colony in May and all colonies in June based not only on analysis of all transcripts (Fig. 3A) and month-DEGs in all colonies (Fig. 4B) but also analysis of month-DEGs only in the spawned colonies (Fig. 4C). These observations suggest that the transcriptomic condition in the non-spawned colony in May was advanced to June. However, it was difficult to identify biological processes that were activated or inactivated specifically in the non-spawned colony in May. As corals are sessile organisms, their transcriptome is likely strongly affected by the environment and optimized to the settlement location, as reflected in their environmentally induced morphological plasticity (Todd, 2008; Budd et al., 2012; Kitahara et al., 2016). Therefore, we could not confirm whether the activation (or inactivation) of a biological process in C1 was due to non-spawning, its settlement location, or other factors. In fact, the number of colony-DEGs was approximately two-fold greater than that of month-DEGs (Fig. 4). Further studies are needed to assess the allocation of energy from reproduction to growth.

The expression levels of genes related to gametogenesis and apoptosis were high before and after spawning, respectively, consistent with previous studies of coral species (Shikina et al., 2012, 2013, 2020; Tan et al., 2020, 2021). It appears to be common for *Acropora* corals to possess two vitellogenin genes (Tan et al., 2021). In this study, the fluctuation of *Vg2* expression levels was moderate, particularly from May to September, in contrast to the marked decrease in *Vg1* expression after spawning. In our previous study, *Vg2* expression decreased in October, and was still low in March; however, we lacked data for the winter months (Tan et al., 2021). These seasonal differences in expression pattern suggest that the two vitellogenin genes play different roles in *A. tenuis*.

The relationship between apoptosis and reproduction has been investigated in a gonochoric stony coral, *Fimbriaphyllia ancora* (previously *Euphyllia ancora*). In *F. ancora*, the number of apoptotic cells in the gonadal somatic cell layer increased in the testes during the middle and late phases, and in the ovaries in the early and middle phases, of gonadal development. This was considered to contribute to structural adjustments of the gonads, and to facilitate the enlargement and subsequent release of gametes (Shikina et al., 2020). In this study, the expression levels of genes related to apoptosis were increased after spawning in *A. tenuis*. This may have been a result of apoptosis of gonadal somatic cells, accompanied by early ovary development, although this must be confirmed in future studies. It would be interesting to compare the physiological basis of gonadal development between gonochoric and hermaphroditic scleractinian corals.

In conclusion, the results of RNA-Seq analysis well reflected the temporal changes in the transcriptome in *A. tenuis* as shown in the MDS plot (Fig. 3A), and genes related to gametogenesis and to apoptosis were identified as representative upregulated genes before and after spawning, respectively. The present study demonstrated the utility of assessing coral conditions based on transcriptome analysis. The next step should be to extend the experimental period to a whole year because oogenesis is an annual event in *A. tenuis* (Tan et al., 2020). Continuous sampling of the same colonies in such studies would be ideal, because we found that the results of RNA-Seq analysis also reflected differences between colonies.

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COMPETING INTERESTS

There is no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

HT, NI, and AT designed the research. HT, HH, EST, and RI performed the sampling. HT, HY, and MM performed the transcriptomic analysis. HT and EST performed qPCR. HT, MM, NI and AT wrote and prepared the manuscript. All authors reviewed the manuscript.

SUPPLEMENTARY MATERIALS

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Supplementary Figure S1. Proportions of reads mapped to the genomes of *Acropora tenuis* and *Symbiodinium goreaui*.

Supplementary Figure S2. Proportions of differentially expressed genes (DEGs) showing each expression level.

Supplementary Table S1. Sampling dates of *Acropora tenuis*.

Supplementary Table S2. Genes tested by qPCR.

Supplementary Table S3. Sequences of primers.

Supplementary Table S4. Number of the reads mapped to expected transcripts of *Acropora tenuis*, *Symbiodinium goreaui*, and *Symbiodinium kawagutii*.

Supplementary Table S5. Differentially expressed genes in the comparison between colonies.

Supplementary Table S6. Differentially expressed genes in the comparison between months in all colonies.

Supplementary Table S7. Differentially expressed genes in the comparison between months in spawned colonies.

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