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RESEARCH ARTICLE

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# Seasonal heterochrony of reproductive development and gene expression in a polymorphic salamander

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## Abstract

**Background:** Life cycle evolution includes ecological transitions and shifts in the timing of somatic and reproductive development (heterochrony). However, heterochronic changes can be tissue-specific, ultimately leading to the differential diversification of traits. Salamanders exhibit alternative life cycle polymorphisms involving either an aquatic to terrestrial metamorphosis (biphasic) or retention of aquatic larval traits into adulthood (paedomorphic). In this study, we used gene expression and histology to evaluate how life cycle evolution impacts temporal reproductive patterns in males of a polymorphic salamander.

**Results:** We found that heterochrony shifts the distribution of androgen signaling in the integument, which is correlated with significant differences in seasonal reproductive gland development and pheromone gene expression. In the testes, androgen receptor (*ar*) expression does not significantly vary between morphs or across seasons. We found significant differences in the onset of spermatogenesis, but by peak breeding season the testes were the same with respect to both histology and gene expression.

**Conclusion:** This study provides an example of how seasonal heterochronic shifts in tissue-specific *ar* gene expression can disparately impact seasonal development and expression patterns across tissues, providing a potential mechanism for differential diversification of reproductive traits.

**KEYWORDS**

androgen receptor, hormone signaling, metamorphosis, paedomorphosis, pheromone, spermatogenesis

## 1 | INTRODUCTION

The evolution of developmental timing (heterochrony) can result in life cycle shifts via alterations in ontogenetic events.[1–3] Concurrent shifts in tissue development within an organism indicate a global mechanism, such as hormonal signaling, controls these heterochronic changes.[4,5] However, not all tissues change to the same degree. As a consequence, some traits may have a greater potential to diversify by heterochrony than others, but the mechanisms underlying these disparate patterns are largely unexplored.

Salamanders display shifts in life cycle strategies, which make them an important model for testing how heterochronic transitions influence trait evolution.[6,7] The prototypical salamander is biphasic with an aquatic larval stage that metamorphoses into a terrestrial adult.[6] However, the diversity of salamanders includes many deviations from the ancestral biphasic pattern.[7,8] Some clades have lost metamorphosis, allowing them to reach adulthood in an aquatic "larval" form (paedomorphosis).[1,2] This phenomenon is genetically encoded in some species while in others environmental pressures and shifting

  

ecological variables can influence the propensity to metamorphose.[9,10] Other clades forego a free-living aquatic larval stage and develop into a terrestrial salamander within the egg (direct development).[8] Regardless of life cycle mode, many temperate species of salamanders display seasonal reproductive cycles involving gonadal development,[11–14] production of pheromones in hypertrophied courtship glands,[15–21] and synchronized reproductive behaviors.[22–24]

In salamanders, life cycle differences and reproductive patterns appear to be largely driven by shifts in the distribution and timing of hormone signaling pathways.[5,13,25,26] Heterochrony can alter the degree to which tissues are exposed and responsive to hormones. Seasonal reproductive processes of polymorphic salamanders provide a developmental comparison of two distinct tissue systems: (1) primary sex characteristics: the gonads, including the germline, and (2) secondary sex characteristics: pheromone-producing glands that develop on either metamorphosed or juvenilized (paedomorphic) integument. Both of these tissue systems are under the developmental control of sex hormones produced in the gonads.[12,13,26,27] Therefore, not only can heterochrony alter the environment that tissues develop under, but this process can also change the tissue itself.

In this study we test how heterochrony impacts male reproduction in a polymorphic stream-dwelling salamander (*Eurycea tynerensis* [*E. tynerensis*]). We assay gene expression levels of androgen receptor (*ar*) in the integument and gonads, as this receptor is vital for mediating the local impacts of androgens on tissues. We then test for correlations between seasonal *ar* signaling, morphogenesis of reproductive glands and testes, and expression of a panel of pheromone and select spermatogenic genes, respectively. This study shows how heterochrony can manifest seasonally through shifts in *ar* gene expression, and differential androgen sensitivity across reproductive tissues may provide a basis for reproductive trait diversification.

## 2 | RESULTS

### 2.1 | Differential expression of *ar* and heterochronic evolution of secondary sex characteristics

Androgen signaling is necessary for the development of male reproductive systems including the gonads and secondary sex characteristics.[13,26–33] Peak plasma androgen levels in salamanders have been correlated with the appearance of secondary sex characteristics and androgen exposure has been shown to increase pheromone production.[13,26,27,33] However, it is unclear how these signaling patterns evolve to produce polymorphic reproductive tissues derived from heterochrony. We first utilized quantitative polymerase chain reaction (qPCR) to test how *ar* expression varies seasonally across adult male biphasic and paedomorphic *E. tynerensis* in two pheromone producing glands, the mental gland on the ventral surface of the chin and the caudal gland on the dorsal base of the tail. While *ar* expression may not be directly related to plasma androgen levels, its presence is necessary for action by androgens and can provide insight into tissue sensitivity to androgens.

The seasonal expression of *ar* is highly correlated with the seasonal morphogenesis of the mental and caudal glands, but these patterns differ between the alternative developmental modes. Mental and caudal glands significantly increase in size across the season (mental gland: $F_{4,43} = 4.66$; $p < .01$, caudal gland: $F_{4,43} = 19.31$; $p < .001$), reaching their peak at the onset of the breeding season in August. However, paedomorphs do not develop an externally observable mental gland, while the two morphs do show similar growth in the caudal gland across months (Figure 1A,B). We also found that *ar* expression significantly increases across months in both the mental ($F_{4,34} = 14.60$; $p < .001$) and caudal glands ($F_{4,35} = 9.16$; $p < .001$) with the highest expression levels in July and August (Figure 1C,D). Compared with metamorphs, paedomorphs have lower *ar* expression in the mental gland region across seasons ($F_{1,34} = 49.03$; $p < .001$; Figure 1D). Specifically, metamorphs have significantly higher levels of *ar* expression in the winter (December/January), March, and August (Figure 1C,D). Localized seasonal *ar* expression is positively correlated with the seasonal development of the mental gland in metamorphic *E. tynerensis* ($r = 0.82$ $p < .001$; Figure 1E) and the caudal gland in both morphs (metamorph: $r = 0.69$; $p < .001$, paedomorph: $r = 0.71$, $p < .001$; Figure 1F). In summary, seasonal *ar* expression is correlated with seasonal development of pheromone producing glands. The chins of paedomorphs are an important comparison because they do not develop a mental gland or display up-regulation of *ar* in this region.

We then tested for seasonal shifts in pheromone gene expression in the mental and caudal glands of paedomorphs and metamorphs. Pheromones from two distinct families are expressed in the mental and caudal glands of *Eurycea* species: Sodefrin Precursor-like Factor (*spf*[34–36]), and Plethodon Modulating Factor (*pmf*[37–39]). The expression of three mental gland specific genes (*pmf, spfa1, spfa2*)[36] and five caudal gland specific genes (*spfa3*, *spfβ1*, *spfβ2*, *spfβ3, spfβ4*)[36] were analyzed across seasons and life cycle modes. For all three mental gland specific pheromone genes, metamorphs have significantly higher

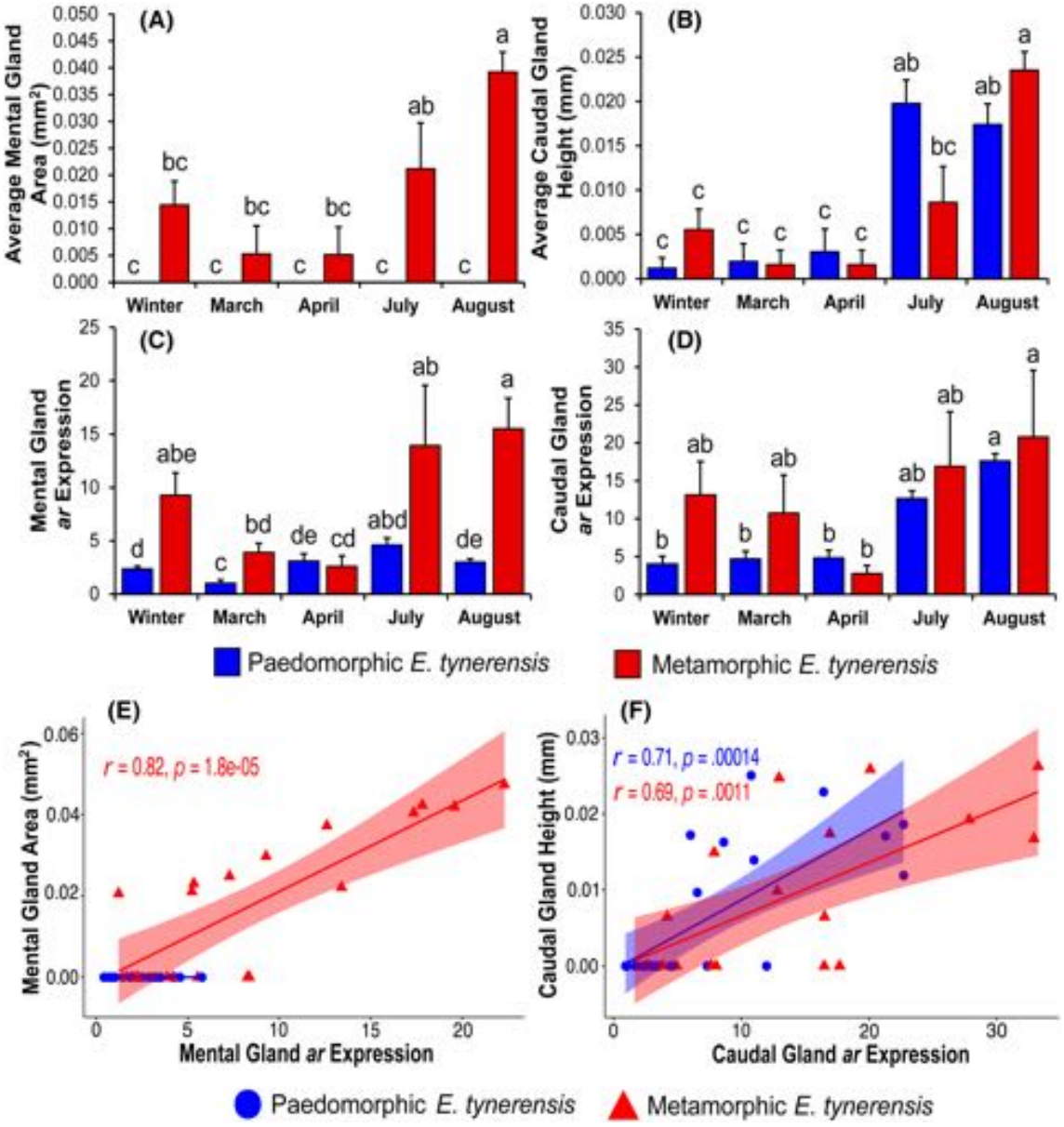


**FIGURE 1** Seasonal changes in average gland size (A, B), *ar* expression (C, D), and their relationship (E, F) in mental glands and caudal glands of metamorphic and paedomorphic *Eurycea tynerensis* (*E. tynerensis*). Gland size was normalized by snout vent length (SVL) and androgen receptor (*ar*) expression, as determined by quantitative PCR, was normalized with ribosomal protein L8 (*rpL8*). Letters above bars (A–D) indicate significantly different groups as determined by Tukey's honest significant difference test. The relationship between gland size and *ar* expression (E, F) was assessed using Pearson's correlation test (*r* is the pearson's correlation coefficient and *p* is the significance value).

overall expression levels (*pmf*: $F_{1,33} = 8.82$, *spfa1*: $F_{1,34} = 10.41$; $p < .01$, *spfa2*: $F_{1,34} = 14.27$; $p < .001$) and there is a significant interaction effect between month and life cycle for *spfa1* and *spfa2* (*spfa1*: $F_{4,34} = 7.17$; $p < .001$, *spfa2*: $F_{4,34} = 4.82$; $p < .01$). Paedomorphic *E. tynerensis* display extremely low expression levels of *pmf, spfa1*, and *spfa2* in the region where a mental gland would develop (Figure 2A–C). In the mental glands of metamorphs, *spfa1* and *spfa2* have significantly different expression levels across seasons (*spfa1*: $F_{4,14} = 6.18$, *spfa2*: $F_{4,14} = 5.18$; $p < .01$). Expression of *spfa1* and *spfa2* are highest in July and August, which is the beginning of the breeding season for this species (Figure 2A–C). Expression of *pmf* is also, on average, highest in July and August but the difference is not significant. The expression patterns of mental gland pheromones show a strong positive correlated with the seasonal expression of *ar* in the mental gland

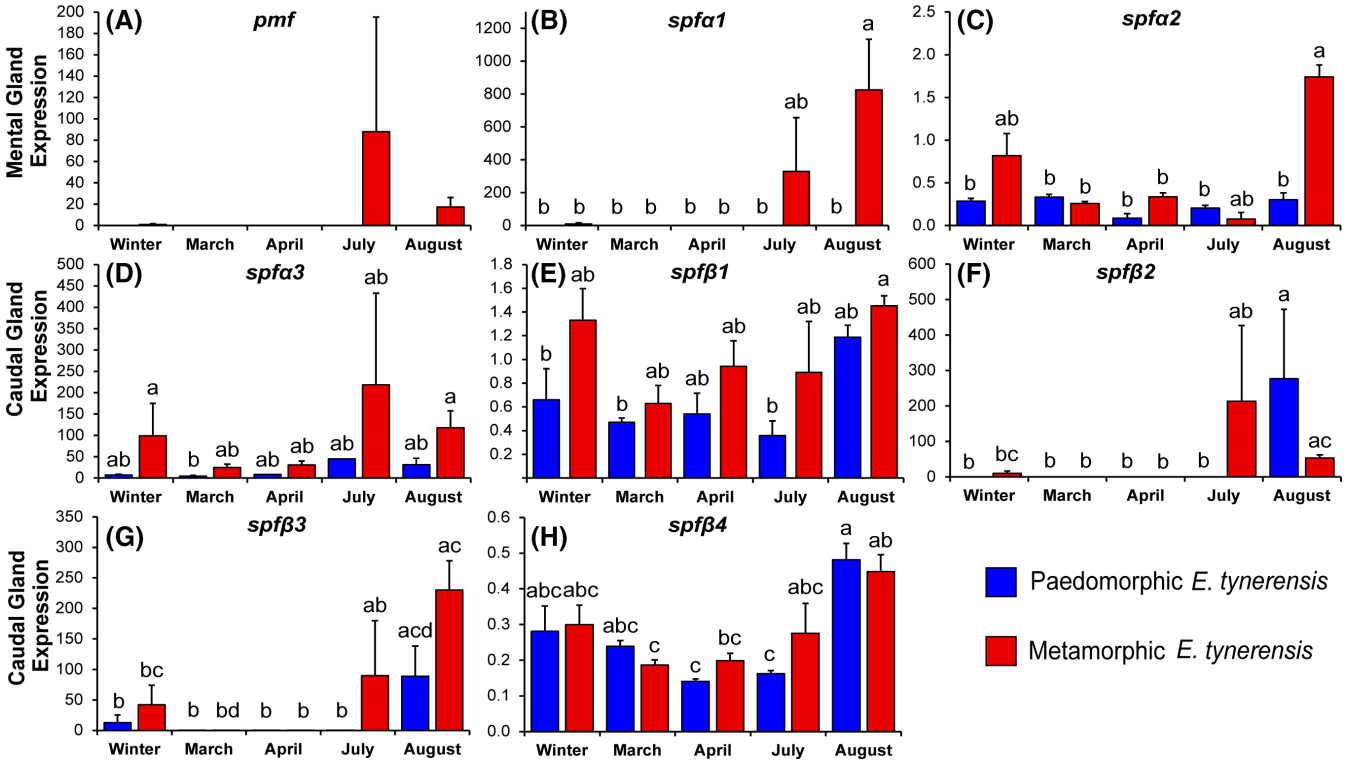


FIGURE 2 Quantitative PCR analysis of the seasonal expression of pheromone genes in the mental (A–C) and caudal (D–H) glands of metamorphic and paedomorphic *Eurycea tynerensis* (*E. tynerensis*). Expression values were normalized with ribosomal protein L8 (*rpL8*). Letters above bars indicate significantly different groups as determined by Tukey's honest significant difference test.

in metamorphs (*pmf*: $r = 0.55$; $p < .05$, *spfα1*: $r = 0.46$; $p = .06$, and *spfα2*: $r = 0.48$; $p = .052$), but not in paedomorphs (Figure 3).

All five of the caudal gland specific genes show significant differences in seasonal expression, with the highest levels in July and August (*spfα3*: $F_{4,29} = 2.60$; $p < .05$, *spfβ1*: $F_{4,33} = 4.27$; $p < .01$, *spfβ2*: $F_{4,35} = 13.77$, *spfβ3*: $F_{4,35} = 11.33$, *spfβ4*: $F_{4,29} = 11.30$; $p < .001$). However, *spfβ1* and *spfβ4* are expressed at much lower levels than the other caudal gland pheromones (Figure 2D–H). Overall, metamorphs have significantly higher expression of *spfα3* ($F_{1,29} = 13.53$; $p < .001$), *spfβ1* ($F_{1,33} = 14.28$; $p < .001$), and *spfβ3* ($F_{1,35} = 4.64$; $p < .05$). In the caudal gland, expression of all the *spfβ* genes are significantly correlated with *ar* expression for at least one life cycle mode (*spfβ1* metamorph: $r = 0.48$; $p < .05$, *spfβ2* paedomorph: $r = 0.54$; $p < .01$, *spfβ3* metamorph: $r = 0.68$; $p < .01$, *spfβ3* paedomorph: $r = 0.43$; $p < .05$, *spfβ4* metamorph: $r = 0.55$; $p < .05$, *spfβ4* paedomorph: $r = 0.49$; $p < .05$; Figure 3). Taken together, *ar* expression is highly correlated with both gland development and pheromone expression. The loss of this pattern in paedomorphic chins shows how heterochrony has the potential to shift tissue hormone sensitivity and divergence of secondary sex characteristics, including the composition of pheromones produced.

## 2.2 | Seasonal patterns of spermatogenesis and *ar* expression are largely conserved between life cycle modes

Spermatogenesis is a complex process involving the sequential development of diverse cell types under the regulatory control of androgens.[29–31,40] Despite the importance of androgens in the regulation of spermatogenesis, seasonally breeding organisms vary in their relationship between androgen levels, gamete production, and sexual behaviors. In some salamanders, peak plasma androgen expression levels are uncoupled from the peak of spermatogenesis but synchronous with the appearance of secondary sexual characteristics.[13] Using qPCR, we found that expression of *ar* in the testes peaks in the winter at the onset of the spermatogenic cycle and then again before the start of the next reproductive season (August), creating a bimodal expression pattern (Figure 4A). However, individual variance in expression levels is extremely high, so although there are seasonal shifts in *ar* expression, these differences are not significant across months or between life cycle modes. Due to the bimodal pattern of *ar* expression in the testes, we divided the *ar* expression data into two subsets (winter through April and April through August) for subsequent analyses to

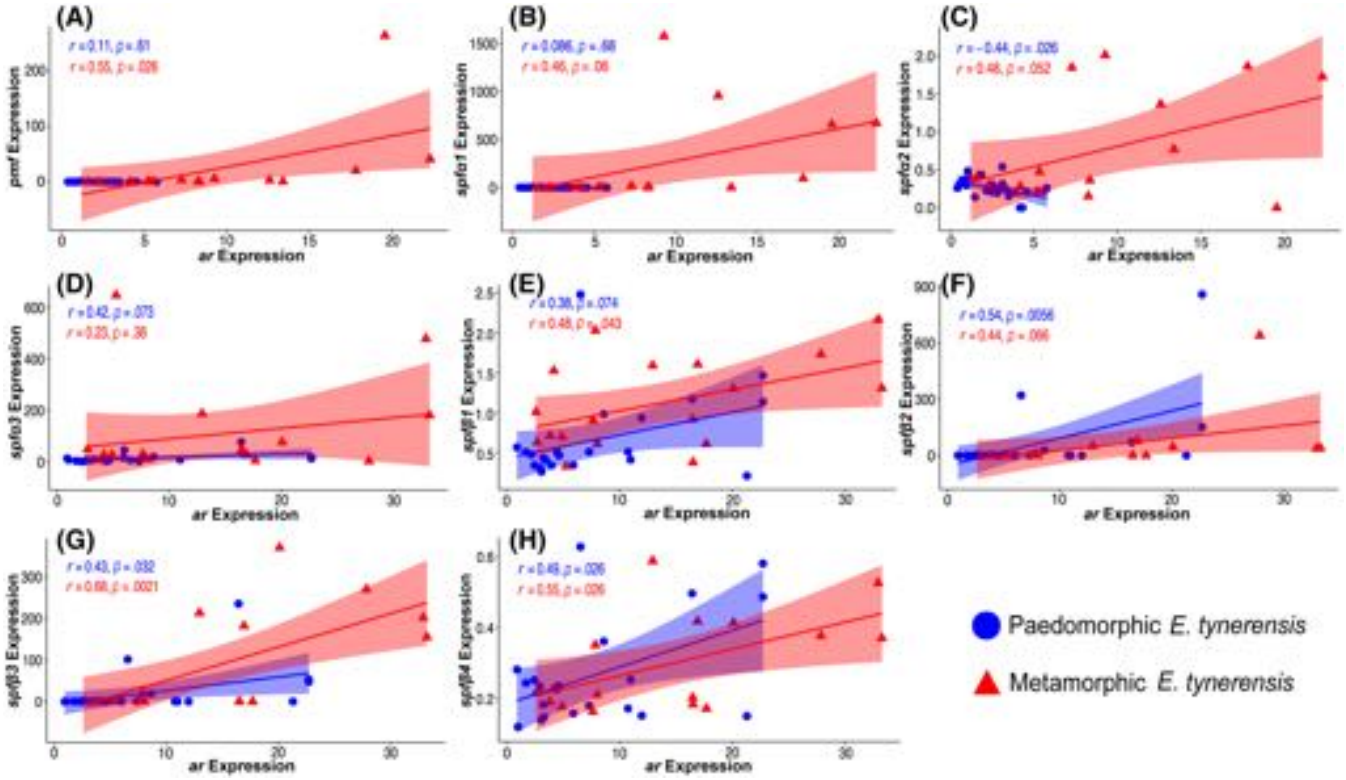


FIGURE 3 Relationship between pheromone expression and androgen receptor (*ar*) expression in the mental (A–C) and caudal (D–H) glands of metamorphic and paedomorphic *Eurycea tynerensis* (*E. tynerensis*). Pearson's correlation coefficient (*r*) was used to test for a significant (*p*) relationship.

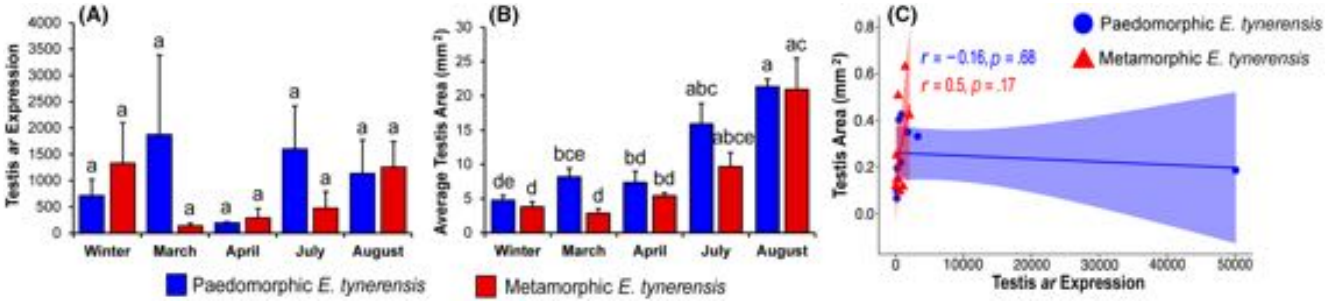


FIGURE 4 Seasonal changes in androgen receptor (*ar*) expression (A), testis size (B), and the correlation between the two (C) in metamorphic and paedomorphic *Eurycea tynerensis* (*E. tynerensis*). Testis size was normalized by snout vent length (SVL) and *ar* expression, as determined by quantitative PCR, was normalized with ribosomal protein L8 (*rpL8*). Letters above bars (A, B) indicate significantly different groups as determined by Tukey's honest significant difference test. The relationship between gland size and *ar* expression (C) was assessed using Pearson's correlation test (*r* is the pearson's correlation coefficient and *p* is the significance value).

evaluate the relationship between *ar* expression in the testes, seasonal testis morphogenesis, and spermatogenesis. Testis area ($mm^2$) significantly increases as the spermatogenic cycle progresses ($F_{4,43} = 36.48$, $p < .001$; Figure 4B). Testes of both metamorphs and paedomorphs follow this conserved growth pattern.[41–43] Expression of *ar* in the testes is not significantly correlated with seasonal shifts in testis area (metamorphs: $r = 0.5$; $p = .17$, paedomorphs: $r = -0.16$; $p = .68$; Figure 4C).

We identified and quantified the four major cell types that define discrete sequential stages of spermatogenesis (spermatogonia, spermatocytes, spermatids, and sperm) in the testes (Figure 5). All four cell types differed significantly in abundance between months (spermatogonia: $F_{4,43} = 8.04$; $p < .001$, spermatocytes: $F_{4,43} = 8.87$; $p < .001$, spermatids: $F_{4,43} = 14.47$; $p < .001$, sperm: $F_{4,43} = 47.06$; $p < .001$), which is consistent with the seasonal nature of the reproduction in this species. During the winter,

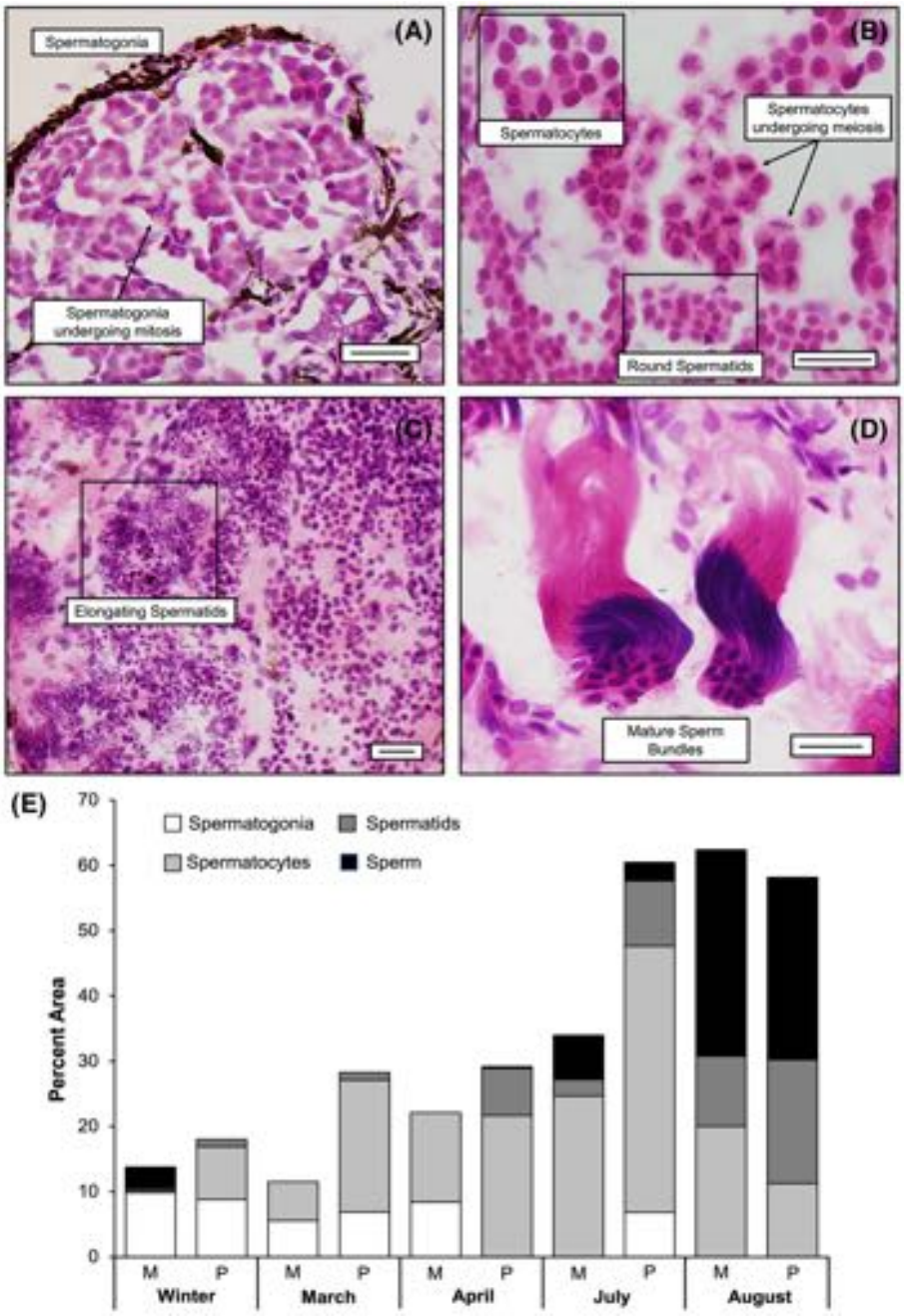


**FIGURE 5** Images from testes cross sections depicting the four major spermatogenic stages (spermatogonia, spermatocytes, spermatids, and sperm) quantified in this study (A–D). Sections were taken at 12 μm and stained with hematoxylin and eosin. Scale bars in each image panel represent 50 μm. (E) Percent area occupied by the four spermatogenic stages within testes between metamorphic (M) and paedomorphic (P) *Eurycea tynerensis* across months. Percent area data was standardized against max testis area ($mm^2$) to show the seasonal increase in total area.

the beginning of the spermatogenic cycle, paedomorphs have significantly more spermatocytes than metamorphs ($F_{1,16} = 9.00$; $p < .01$). In April, metamorphs still have a significant number of spermatogonia while paedomorphs have progressed to the later stages of spermatogenesis ($F_{1,16} = 29.59$; $p < .001$; Figure 5). In metamorphs, spermatogonia and sperm abundance show a positive correlation with *ar* expression (spermatogonia: $r = 0.71$; $p = .073$, sperm: $r = 0.58$; $p = .098$) while spermatocyte ($r = 0.19$; $p = .58$) and spermatid ($r = 0.48$; $p = .19$) abundance are not correlated (Figure 6). In paedomorphs none of the spermatogenic stages show a correlation with *ar* expression (spermatogonia: $r = 0.28$; $p = .54$, spermatocytes: $r = 0.032$; $p = .92$, spermatids: $r = 0.14$; $p = .75$, sperm: $r = 0.17$; $p = .69$; Figure 6). There is a clear trend where paedomorphs begin their spermatogenic cycle earlier than metamorphs with the development of spermatocytes and spermatids prior to metamorphs (Figure 5). However, the spermatogenic cycle of metamorphs developmentally catches up to paedomorphs by the breeding season when these patterns are synchronous between the two morphs.

To further evaluate shifts in seasonal patterns of spermatogenesis between life cycle modes we analyzed the expression of spermatogenic genes across months. Currently very little is known regarding what genes

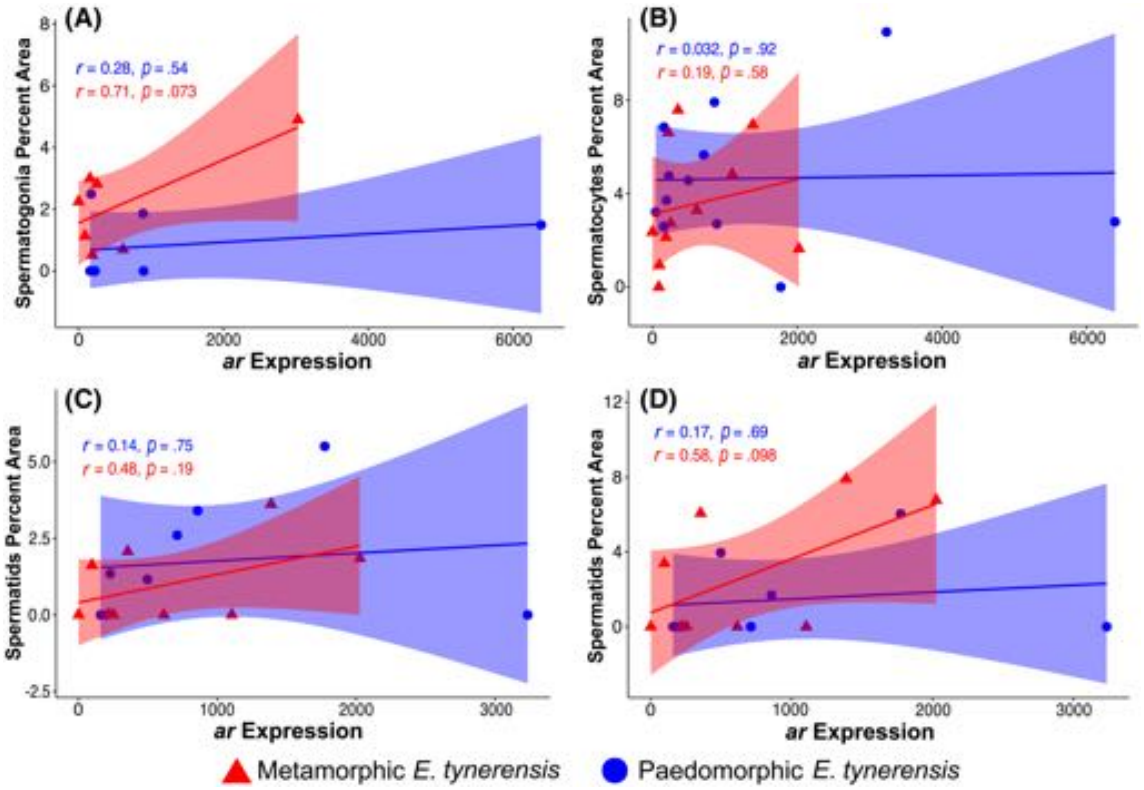


**FIGURE 6** Relationship between spermatogenic development and androgen receptor (*ar*) expression in the testes of metamorphic and paedomorphic *Eurycea tynerensis* (*E. tynerensis*). Pearson's correlation coefficients (*r*) were used to test for significant relationships (*p*) among cell types and *ar* expression. Seasonal shifts in abundance of the different spermatogenic cell types were not strongly correlated with *ar* expression.

are important for the regulation and maintenance of spermatogenesis in amphibians.[44] Therefore, we reviewed published spermatogenic single cell transcriptomic and expression studies to develop a series of candidate genes known to be expressed during specific spermatogenic stages in other vertebrates (Table 1). We found transcripts of 17 of these genes (GenBank accession numbers OR683618-OR683634) in previously annotated *E. tynerensis* testes transcriptomes and these transcripts were then used for qPCR assay development (see methods for more detail on assay design).

Expression patterns of the 17 genes were analyzed across season and life cycle mode using qPCR. Across months, eight genes differed significantly in their expression patterns (*tekt1*: $F_{4,30} = 5.79$, *tssk4*: $F_{4,29} = 5.68$; $p < .01$, *sept7*: $F_{4,34} = 6.27$, *spag6*: $F_{4,33} = 8.87$, *meig1*: $F_{4,34} = 9.51$, *spata7*: $F_{4,28} = 36.71$, *ropn1*: $F_{4,34} = 23.27$, *zpbp1*: $F_{4,34} = 7.52$; $p < .001$; Figure 7) while the other 9 genes assayed did not display significant seasonal shifts in expression. Of the eight genes, *meig1, spata7*, and *sept7* were more broadly expressed across months and displayed a gradual increase in expression up to their peak in July/August (Figure 7A,B,D). Three genes, *spag6, zpbp1*, and *tekt1*, had very low expression levels in winter and early spring, peaked in expression during July, when mature sperm are starting to form in the testes (Figure 5) but decreased expression levels in August (Figure 7C,E,G). There was also very little expression of *tssk4* and *ropn1* in the winter and spring months, but expression levels dramatically increased starting in July, with August having the highest expression levels (Figure 7F,H). This suggests that *tssk4* and *ropn1* may be important for maintenance and function of mature sperm.[45,46] The seasonality of their expression provides support that these genes are associated with specific processes/cell types during the reproductive cycle.

We found few seasonal expression differences between life cycle modes of *E. tynerensis*. Two genes, (*klc3*: $F_{4,34} = 3.78$, *spag6*: $F_{4,33} = 3.62$; $p < .05$), significantly differed in their expression levels across months between life cycle mode, but not within months. In addition, paedomorphs have significantly higher overall expression of *tekt1* ($F_{1,30} = 8.91$; $p < .01$) and *spo11* ($F_{1,26} = 7.26$; $p < .05$). These results indicate little difference in the timing and level of spermatogenic gene expression between metamorphs and paedomorphs for the genes evaluated in this study. However, there are differences between morphs in the correlation between

**TABLE 1** Literature highlighting expression of the 17 target genes utilized in this study across four main spermatogenic cell types (Sg-Spermatogonia, Sct-Spermatocytes, Std-Spermatids, and Sperm). Some genes are more broadly expressed across cell types while others can be used as cell specific markers.

| Gene | Species | Spermatogenic stage | Expression data | Method | Reference |
|---|---|---|---|---|---|
| *dmrt1* | Human | Sg | RNA | Single cell transcriptomics | 87–89 |
| | Mouse | Sg | RNA | Single cell transcriptomics | 88 |
| | Zebrafish | Sg, Sct, Std | RNA | In-situ hybridization | 90 |
| | Lizard | Sg, Sct | RNA | In-situ hybridization | 91 |
| | Frog | Sg | Protein | Immunohistochemistry | 92 |
| *sycp3* | Human | Sg, Sct | RNA | Single cell transcriptomics | 87,88 |
| | | Sct | Protein | Immunohistochemistry | 89 |
| | Mouse | Sgt, Sct | RNA | Single cell transcriptomics | 88 |
| | Zebrafish | Sct | Protein | Immunohistochemistry | 93 |
| *dmc1* | Human | Sct | RNA | Single cell transcriptomics | 88 |
| | | | Protein | Immunohistochemistry | 89 |
| | Mouse | Sct | RNA | Single cell transcriptomics | 89 |
| *spo11* | Human | Sct | RNA | Single cell transcriptomics | 87–89 |
| | Mouse | Sct | RNA | Single cell transcriptomics | 88 |
| | Eel | Sct | Protein | Immunohistochemistry | 94 |
| *spata7* | Rat | Sct | Protein | Immunocytochemistry | 95 |
| *piwil1* | Human | Sct | RNA | Single cell transcriptomics | 88 |
| | | Sct, Std | RNA and protein | qPCR, immunohistochemistry | 96 |
| | Mouse | Sct | RNA | Single cell transcriptomics | 88 |
| | Chicken | Std | RNA | qPCR | 97 |
| *meig1* | Mouse | Sct, Std, and Sperm | Protein | Immunocytochemistry | 98,99 |
| *tekt1* | Mouse | Sct | RNA | In situ hybridization | 100 |
| | | Std | Protein | Indirect immunofluorescence | |
| | | Sperm | Protein | Immunoblotting | 101 |
| | Rat | Sperm | Protein | Immunoblotting | 101 |
| | Bull | Sperm | Protein | Immunoblotting | 101 |
| *spag6* | Mouse | Sct, Std | RNA | qPCR | 102 |
| | | Sct, Std | Protein | Immunohistochemistry | 103 |
| | Canine | Sperm | Protein | Immunohistochemistry | 104 |
| *zpbp1* | Human | Sct, Std | RNA | Single cell transcriptomics | 87 |
| | Mouse | Sct, Std, Sperm | RNA<br>Protein | In-situ hybridization<br>immunohistochemistry | 105 |
| *sept7* | Mouse | Sct, Std and Sperm | Protein | Immunofluorescence assay | 106 |
| *tssk4* | Mouse | Std | RNA and Protein | Real-time PCR, immunohistochemistry | 107 |
| | | Sperm | Protein | Immunohistochemistry<br>Western Blot | 107<br>45 |
| *enkur* | Mouse | Std, Sperm | Protein | Immunohistochemistry | 108 |
| *klc3* | Mouse | Std, Sperm | RNA and Protein | Real-time PCR, immunohistochemistry | 109 |
| *ropn1* | Human | Std | Protein | Immunohistochemistry | 110 |
| | | Sperm | RNA and Protein | Real-time PCR, indirect immunofluorescence, Western Blot | |
| | Mouse | Sperm | Protein | Immunocytochemistry | 111 |
| *tekt2* | Human | Sperm | Protein | Immunofluorescence | 112 |

TABLE 1 (Continued)

| Gene | Species | Spermatogenic stage | Expression data | Method | Reference |
|---|---|---|---|---|---|
| | Rat | Sperm | Protein | Immunocytochemistry | 113 |
| *odf2* | Human | Std | RNA | In-situ hybridization | 114 |
| | | Sperm | Protein | Immunocytochemistry | |
| | Mouse | Std | RNA | In-situ hybridization | 115 |
| | | Sperm | Protein | Immunocytochemistry | |

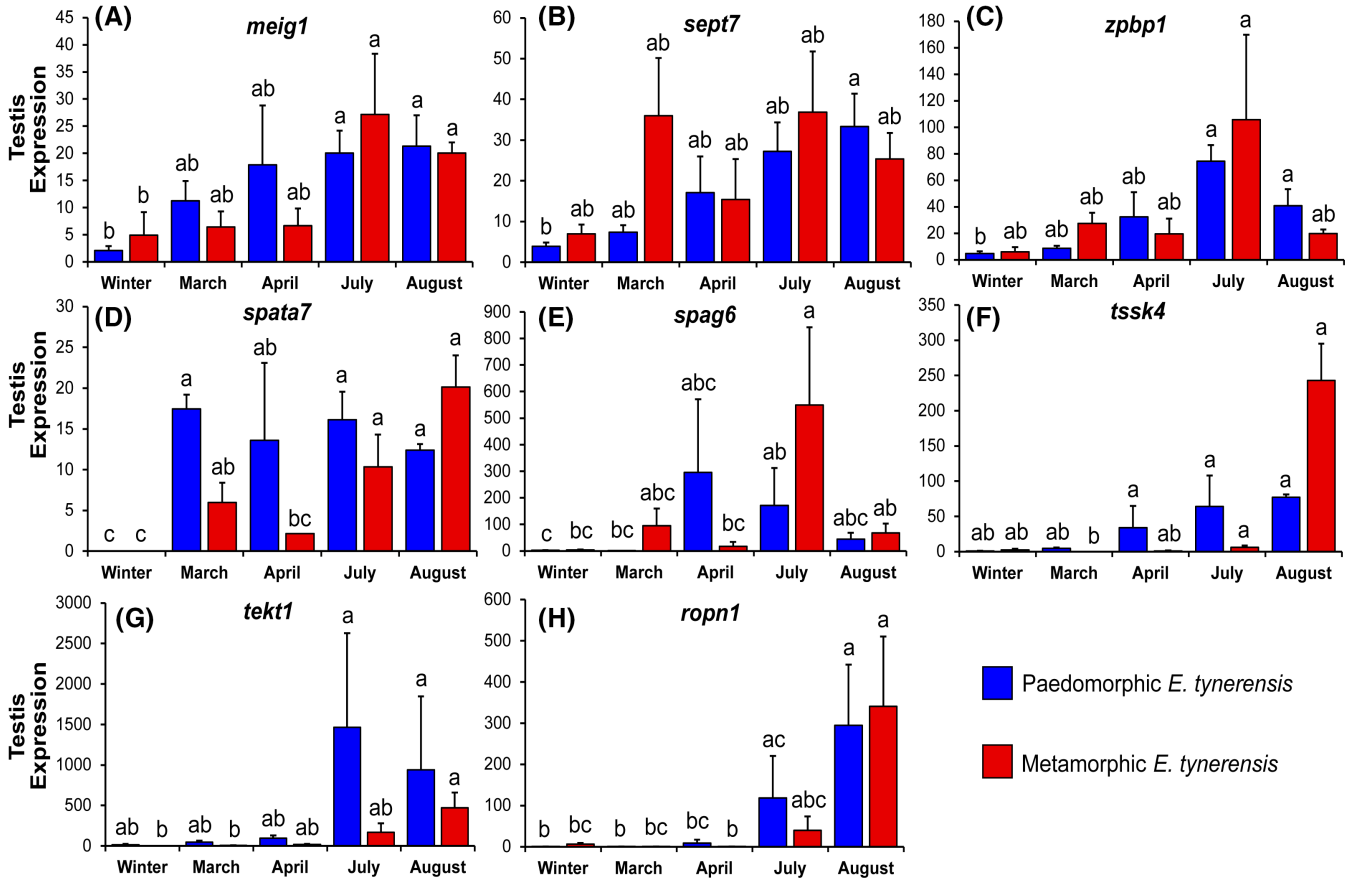


FIGURE 7 Seasonal expression of eight spermatogenic genes (A–H) in the testes of metamorphic and paedomorphic *Eurycea tynerensis* (*E. tynerensis*). Expression levels were determined using quantitative PCR and expression was normalized using ribosomal protein L8 (*rpL8*). Letters above bars indicate significantly different groups as determined by Tukey's honest significant difference test.

spermatogenic gene expression and testes *ar* expression. In metamorphs *spata7* ($r = 0.90$; $p < .001$), *tssk4* ($r = 0.80$; $p < .01$), and *ropn1* ($r = 0.73$; $p < .05$) expression are significantly correlated with *ar* expression while in paedomorphs only expression of *tekt1* ($r = 0.98$; $p < .001$) is correlated with *ar* expression (Figure 8). The remainder of the genes are not significantly correlated with seasonal *ar* expression. Overall, we found few heterochronic shifts in the seasonal timing of spermatogenesis and *ar* expression in the testes. Androgen regulation of spermatogenesis is a conserved, albeit complex, process.[30,31,40,44] Therefore, in contrast to secondary sex characteristics like pheromone-producing glands, heterochrony seems to only have minimal impact on the seasonal timing of spermatogenesis. In other words, the differences in reproductive biology between these phenotypically distinct life cycle modes involve secondary but not primary sex characteristics.

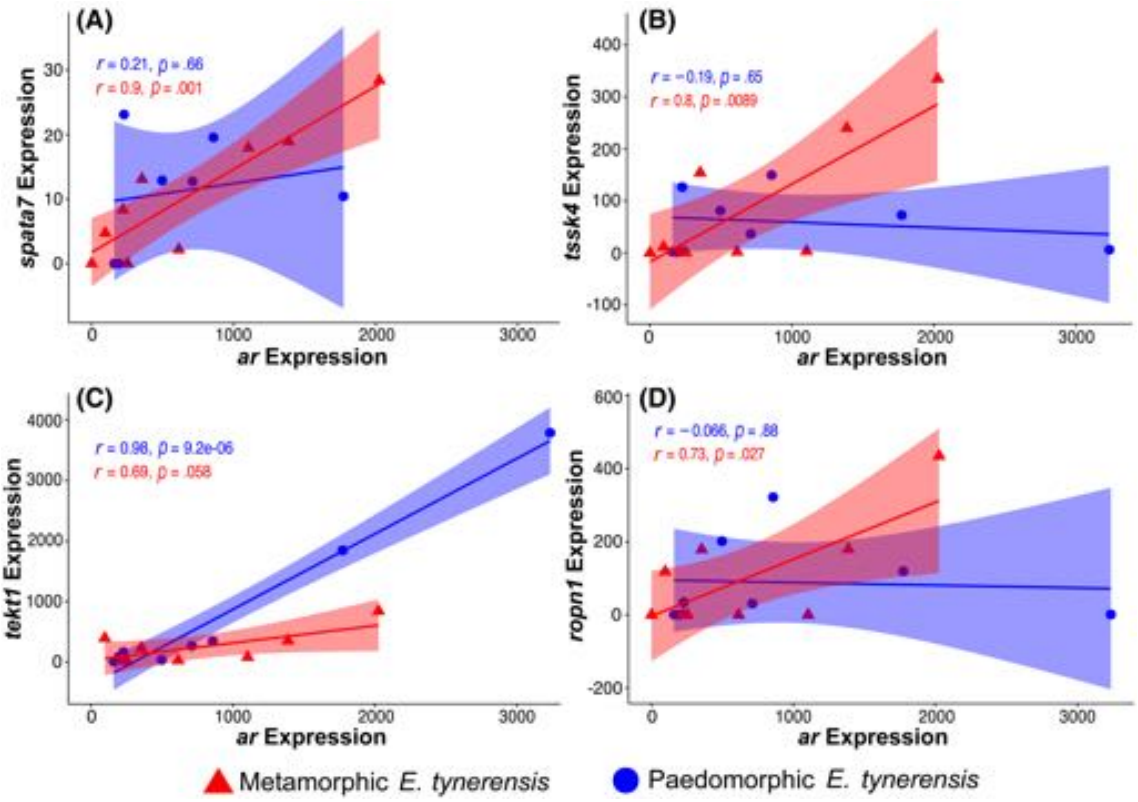


FIGURE 8 Relationship between four of the spermatogenic genes that showed significant changes in seasonal expression patterns and androgen receptor (*ar*) expression in the testes. Significant correlations ($p$) were determined using Pearson's correlation test ($r$ = Pearson's correlation coefficient). *E. tynerensis*, *Eurycea tynerensis*.

## 3 | DISCUSSION

Heterochrony has played a significant role in shaping multicellular life. However, the developmental mechanisms that underlie different phenotypes derived from heterochrony are often understudied.[47–49] One compelling question is what mechanisms allow heterochronic changes to differ across tissues. Developmental timing varies dramatically for some tissues, while it is highly conserved for others. Testing the mechanistic basis for such patterns is critical for understanding how traits evolve to produce diverse organisms. In this study we used a polymorphic salamander, *E. tynerensis*, to test how heterochrony impacts the seasonal development of two distinct tissue types: primary sex characteristics (the gonads) and secondary sex characteristics (pheromone producing courtship glands) that develop from the integument. We show how seasonal heterochrony differentially influences the seasonal development and gene expression between these two systems (Figure 9), which are correlated with shifts in *ar* expression. Differences in the expression of signaling receptor genes can lead to uncoupling of tissues from global endocrine signals during development, providing a mechanism for heterochronic-driven differential trait diversification.

### 3.1 | Heterochronic shifts in hormonal signaling drive secondary sex characteristics

The retention of a multitude of larval traits in adult paedomorphic salamanders has made them a model for explaining heterochrony.[1–3] The simultaneous truncation of diverse traits suggests a global mechanism, such as a change to hormone signaling, is responsible for paedomorphic developmental patterns.[4,5] This can occur through a reduction in circulating hormone or reduced responsiveness of tissues, such as lower levels of hormone receptor expression. For example, thyroid hormone is necessary for the metamorphosis of many amphibian tissues.[50–53] The tissues of some paedomorphic populations of *E. tynerensis* are known to have a reduced sensitivity and responsiveness to thyroid hormone compared with metamorphic populations.[54] This suggests that changes in signaling sensitivity can drive heterochronic shifts in paedomorphic traits. However, hormone sensitivity can vary across tissue types within and among life cycle modes.[54–56] In this study we show differences in heterochronic shifts of *ar* expression across reproductive tissues between metamorphic and paedomorphic *E. tynerensis*,

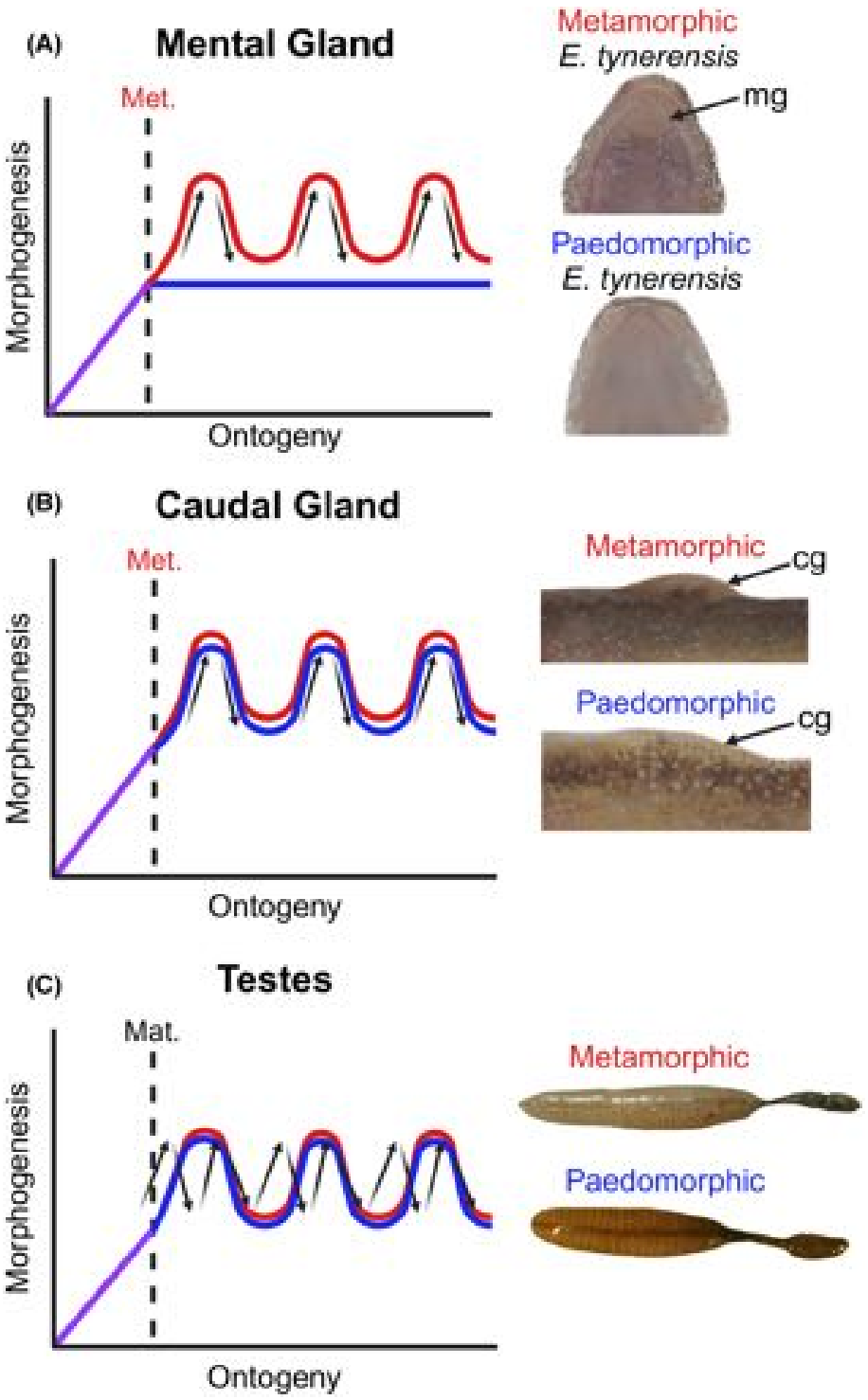


**FIGURE 9** Models of seasonal heterochrony based on the mental gland (A), caudal gland (B), and testes (C) of metamorphic and paedomorphic *Eurycea tynerensis* (*E. tynerensis*) (photos). Plots show patterns of morphogenesis for metamorphs (red), paedomorphs (blue), or both (purple). Arrows in graphs depict the seasonal cycle of a mechanism driving the differences between morphs, such as androgen receptor (*ar*) expression. Paedomorphic *E. tynerensis* never develop a fully functional mental gland and do not up-regulate *ar* in this region (A). In contrast, metamorphic and paedomorphic *E. tynerensis* exhibit similar seasonal cycles of development and ar expression in the caudal gland (B) and testes (C). Graphics were created with BioRender.com. cg, caudal gland; Mat, maturation; Met, metamorphosis; mg, mental gland.

which may be a mechanism for the divergence of secondary sex characteristics independent of gametogenesis.

In salamanders, androgen exposure has been linked to courtship gland hypertrophy,[27] increased production of pheromones,[26] and *ar* is expressed in the dermal glands of reproductive individuals.[57] Additionally, peak plasma androgen levels have been shown to occur during the development of reproductive glands in plethodontids.[13,33] While we did not measure plasma androgens directly in this study, we show that *ar* expression is positively correlated with gland morphogenesis and pheromone gene expression. We demonstrate that a heterochronic shift in *ar* expression is correlated with the loss of glandular development and pheromone expression in the integument below the chin of paedomorphic *E. tynerensis*. Paedomorphic *E. tynerensis* did not display a significant increase in the

expression of *ar* in the mental gland (Figure 1C). The lack of *ar* up-regulation in paedomorphic *E. tynerensis* mental glands indicates a potential heterochronic shift in mental gland sensitivity to androgens, which may determine why paedomorphs do not develop a fully functioning mental gland. The loss of androgen sensitivity in paedomorphic chins would represent a permanent delayed onset in reproductive development (post-displacement[2,3]; Figure 9). It is possible that instead of a reduced sensitivity to androgens, circulating plasma androgen levels are low in paedomorphs. However, paedomorphic *E. tynerensis* still develop a fully functional caudal gland, which is correlated with increased levels of *ar* expression. This is consistent with other species where there is a strong relationship between increased androgens and courtship gland hypertrophy.[13,27] Whole organism or tissue culture androgen exposure and androgen blocking experiments are necessary to disentangle these possible mechanisms.

More direct tests are needed to determine whether androgen simply creates the glandular architecture needed for pheromone production or if it is involved in the increased transcription/translation of pheromones.[20] Furthermore, across salamanders, there have been independent transitions to paedomorphosis as well as the evolution of diverse pheromone-producing glands that function in different environments. For example, male pond-breeding European salamandrids develop enlarged cloacae during the courtship season, but paedomorphic individuals have greatly reduced cloacae compared with their metamorphic counterparts.[58–60] Interestingly, the paedomorphic males also display less to females of both morphs but have similar mating success to metamorphic males.[61] It is unknown whether this decrease in cloacal size is coupled with a decrease in cloacal gland pheromone expression. While this trait shows seasonal plasticity between morphs,[60] how androgen sensitivity may play a role in these differences has not been investigated. How heterochronic shifts in androgen sensitivity drive the diversification of salamander courtship glands across a diversity of species needs to be evaluated.

In contrast to the pheromone-producing glands, *ar* expression in the testes did not vary significantly by season in *E. tynerensis*, although it does display a clear bimodal expression pattern. Seasonal expression of *ar* in the testes is also conserved between metamorphic and paedomorphic individuals of *E. tynerensis*. Regulation of spermatogenesis is a complex process mediated by multiple hormones[29] and is poorly understood in amphibians.[44] In tetrapods, androgens are necessary for the completion of meiosis, specifically with the formation of spermatids.[30,31,62] Consistent with this we find increases in *ar* expression in July and August (Figure 4A) along with increased spermatid presence (Figure 5E), however, there is not a significant correlation between spermatid presence and *ar* gene expression in either morph (Figure 6C). In addition to this, our results do not show a clear relationship between genes expressed in spermatids (Table 1) and *ar* gene expression. Further studies are needed to elucidate the hormonal regulatory control of spermatogenesis in amphibians and the genes involved in this process. Additionally, larger scale phylogenetic comparisons of *ar* expression are needed to understand whether patterns of expression in the testes are more broadly conserved across life cycle mode shifts. While the expression of *ar* in the testes is conserved, the mental gland area in paedomorphic *E. tyenerensis* has become decoupled from the global circulation of androgens. This tissue decoupling allows for traits to evolve along differing trajectories (Figure 9), which could provide variation for phenotypic diversification.

### 3.2 | Cell type diversity allows for heterochronic driven trait diversification

Recently, there has been an appreciation that cell type diversity is a basis for phenotypic novelty.[63] Cell type diversity can be derived through heterochrony[64,65] and some tissue types may have a higher propensity to produce diverse cells than others. Vertebrate integument varies extensively in cell type and anatomy[64,66,67] and can undergo drastic changes during ontogeny.[68–71] In amphibians these changes to the integument are abrupt during metamorphosis[68,71–74] and seasonal reproductive development.[11,13,15,18,33,75] Our analyses revealed that paedomorphic *E. tynerensis* do not display seasonal development of a mental gland, as in metamorphs, which leads to differences in seasonal mental gland pheromone expression between morphs. While paedomorphs do not exhibit expression of pheromone genes in the integument below the chin, they do have similar patterns of pheromone expression to metamorphs in the caudal gland.

Metamorphosed amphibians have cornified integument, and additional integumentary glands (e.g., serous and mucous glands), whereas larval integument includes apical and Leydig cells.[68,71–74] Less is known regarding the ultrastructure of paedomorphic integument, but there is clear variation among paedomorphic species. Some paedomorphic species retain larval structures in their integument, while others have integument that resembles metamorphosed species.[68,76] An additional level of integumentary variation is between the dorsal and ventral components which is largely unexplored.[72] We found that paedomorphs develop a fully functional caudal gland (dorsal), but not a mental gland (ventral), suggesting that there are developmental differences in the propensity for the dorsal and ventral skin cells to diversify. In addition to structural changes,

transcriptional shifts also occur in the integument during thyroid hormone induced metamorphosis.[77] Expression of thyroid hormone receptors, keratin genes, proliferation/differentiation genes (epithelial membrane proteins), and collagen degradation genes (matrix metalloproteinases) are up-regulated following exposure to thyroid hormone.[77] It is unknown how the transcriptional profiles of integument cells differ between metamorphic and paedomorphic adults. This is an essential piece to understanding how ontogenetic differences in cell type, and shifts in developmental timing, provide a basis for the production of diverse courtship glands from the integument of salamanders.

In contrast, the cell type diversity and the process of spermatogenesis are highly conserved across vertebrates.[78–81] We found few differences in the seasonal development of spermatogenesis between morphs. Our histological analysis revealed that paedomorphic *E. tynerensis* begin spermatogenesis before metamorphs, however, by the breeding season (August) both life cycle modes have similar composition of spermatogenic stages in their testes (Figure 5E). However, while we did identify significant seasonal expression patterns for 8 of our 17 candidate genes (Figure 7), spermatogenic gene expression did not reflect the same temporal shift that is seen in the histological data. This may be because the candidate genes that showed significant seasonal expression are associated with the latter stages of spermatogenesis, whereas the histological data revealed that the strongest temporal shift between life cycle modes is at the beginning of spermatogenesis. It is possible that other genes (not evaluated here) would be more reflective of these histological shifts. The process of meiosis is highly conserved, even beyond vertebrates, providing limited opportunity for the evolution of novel cell types. Spermatogenesis, however, could potentially be manipulated by heterochrony to produce diverse sperm shapes[81,82] though this line of inquiry remains to be investigated.

### 3.3 | Conclusions

Heterochrony is an important driver of diversification, but the mechanisms that underlie variation in diversity among tissues are not well understood. In this study we used a polymorphic salamander, *E. tynerensis*, to test how heterochrony impacts male primary and secondary sex characteristics. Our results reveal a seasonal heterochronic shift in the morphogenesis and gene expression of integumentary derived pheromone producing glands (secondary sex characteristics), but not in the conserved testes and cell types of spermatogenesis (primary sex characteristics) (Figure 9). These shifts are correlated with differences in *ar* expression. This study is an example of seasonal heterochrony, where traits have shifted in their seasonal development patterns (Figure 9). Shifts in androgen sensitivity provide a potential mechanism by which heterochrony can differentially act on reproductive tissues, allowing for the diversification of certain traits and the conservation of others. Additional mechanistic and comparative research is needed to: (1) further elucidate the mechanisms that influence gland development and (2) to understand how underlying cellular diversity and developmental timing have contributed to reproductive gland diversification.

## 4 | EXPERIMENTAL PROCEDURES

### 4.1 | Animal processing

*E. tynerensis* (Oklahoma salamander) is a polymorphic species of stream salamander in the family Plethodontidae that is endemic to the Ozark Plateau of Oklahoma, Arkansas, and Missouri.[83] We collected male *E. tynerensis* from two populations (one metamorphic and one paedomorphic) during "winter" (December/January; metamorph: $n = 11$, paedomorph: $n = 14$), March (metamorph: $n = 4$, paedomorph $n = 7$), April (metamorph: $n = 4$, paedomorph: $n = 5$), and "summer" (July; metamorph: $n = 4$, paedomorph $n = 3$, August; metamorph: $n = 4$, paedomorph: $n = 4$). The spermatogenic cycle of fall breeding *Eurycea* generally starts in winter/early spring and male *E. tynerensis* reach peak reproductivity starting around August, which is why these collection periods were chosen. Specimens from the winter, March, and April were processed within 24 h after being brought back to the lab. "Summer" salamanders were collected in May and maintained in the lab until processing (beginning of July and beginning of August) because they are not surface active during the summer (though they may be subsurface active). All salamanders in the lab are maintained at a constant temperature of 20°C(±1) with a 12:12 photoperiod. While it is unknown whether these conditions mimic those experienced by *E. tynerensis* during the summer, our breeding colony of *E. tynerensis* maintains an annual seasonal reproductive cycle that closely mimics their seasonal cycle in the wild when maintained with these constant conditions. A 0.1% solution of tricaine methanesulfonate (MS-222) was used to anesthetize specimens for processing. Images of specimens and testes were taken with a Dino-Lite Edge digital microscope during processing for subsequent area measurements. Testes were excised from specimens, and one was frozen for qPCR while the other was preserved in 5% paraformaldehyde for histological analysis. Pheromone glands were also taken for qPCR analysis. Male salamanders in the family, Plethodontidae, have

two distinct pheromone-producing courtship glands: mental glands under their chin[84] and caudal glands on the base of their tail.[18,36] Testes from an additional 18 specimens (reproductively active; metamorph: $n = 6$, paedomorph: $n = 5$, reproductively inactive; metamorph: $n = 4$, paedomorph: $n = 3$) were utilized for transcriptomic analysis.

## 4.2 | Histology

Histology was used to assess the seasonal cycle of spermatogenesis in *E. tynerensis* and more specifically, what spermatogenic stages the testes were composed of at a given time point. After fixation in paraformaldehyde, testes were rinsed with phosphate buffered saline and submerged in a 30% sucrose solution. Testes were embedded in Tissue Freezing Media, sectioned on a Microm HM550 Cryostat Microtome (Thermo Fisher) at 12 μm, and stained using hematoxylin and eosin.[85]

To determine the seasonal composition of spermatogenic stages in the testes, sections from the anterior, middle, and posterior ends were used for analysis. This is because spermatogenesis in salamanders happens in a wave-like fashion from the caudal to cephalic end of the testis.[41,42] Three sections were measured from each of the three portions of the testis; thus, nine points of measurements were taken per testis. We identified the stages of spermatogenesis (spermatogonia, spermatocytes, spermatids, and sperm) within each section using an Olympus BX53 compound microscope. Images of sections were taken with a Olympus DP72 camera mount and area measurements for each spermatogenic cell type were recorded along with the total area of the section using the program CellSens. Area measurements were specifically restricted to only the actual spermatogenic cells, excluding areas of the testis that were not presently occupied by these cells. The area data of each spermatogenic stage was summed from each histological section of the anterior, middle, and posterior portions of the testis. These values were subsequently summed together to get a total value per testis. The total areas of testes sections were also summed together. For each testis, sums of each cell type were then divided by the corresponding sum of the total section areas to get the percent of each cell type per total section area.[42] This value was multiplied by the total testis area to get how much area each spermatogenic cell type occupied in the testis.[42] Total testis area was calculated from testis length and width, which was found using ImageJ.[86] The spermatogenic stage area for each testis was then log transformed and corrected by the log transformed snout–vent length of the corresponding specimen.

Differences in testis area across months and between life cycle modes was tested using a two-way analyses of variance (ANOVA) with the package *stats* in *R*, along with Tukey's honest significant difference post hoc test with Bonferroni correction. To test for shifts in spermatogenesis between life cycle modes and across the season two-way ANOVA were performed for each spermatogenic cell type, along with Tukey's honest significant difference post hoc tests with Bonferroni correction.

## 4.3 | Seasonal gene expression analyses

To test for seasonal spermatogenic gene expression differences between metamorphs and paedomorphs qPCR was performed for 17 spermatogenic genes (Table 1) along with *ar*. qPCR assays of the 17 spermatogenic genes and *ar* were created utilizing previously annotated *E. tynerensis* testes transcripts of these genes of interest (Table S1). Transcripts of the Taqman (BHQ1a-6FAM) primer and probe assays were made on the exonic boundaries of the 17 spermatogenic genes using the IDT OligoAnalyzer Tool (Table S1). Assays were conserved across both study populations of *E. tynerensis*. We also assessed seasonal pheromone expression using qPCR. We utilized previously synthesized qPCR assays for *pmf* plus seven *spf* genes: *spfa1*, *spfa2*, *spfa3*, *spfβ1*, *spfβ2*, *spfβ3, spfβ4* (see Herrboldt et al.[36] for more details on primer design; Table S1). Pheromone gene qPCR assays were validated in Herrboldt et al.[36] where we show that normalized expression (Qty) values are correlated with normalized read counts from transcriptomic sequencing for each gene and across samples and tissue types. *ar* expression was also quantified in the mental and caudal glands, specifically to test for correlations between *ar* signaling and the loss of the mental gland and associated pheromone genes in paedomorphs.

SuperScript II (Invitrogen) and random hexamer were used to synthesize cDNA and ABI TaqMan Gene Expression Master Mix was used for the qPCR reactions. All the samples for a given gene along with negative controls, negative RT reactions and a six-point standard curve were run simultaneously on an ABI StepOne Plus qPCR machine at the University of Tulsa. The standard curve for each gene was used to interpolate expression values (Qty) from the number of cycles (CT values). Expression values were then normalized against the housekeeping gene ribosomal protein L8 (*rpL8*)[54] and log transformed for statistical analysis.

For each gene, a two-way ANOVA with Tukey's honest significant difference post hoc tests with Bonferroni correction was performed in *R* to test

how expression differed between life histories across the seasonal cycle. Pearson correlation tests were performed in *R* to test for relationships between *ar* expression and pheromone/spermatogenic gene expression in their respective tissues.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

## DATA AVAILABILITY STATEMENT

Gene transcripts are available on GenBank under the accession numbers OR683618-OR683634. Histological slides, specimens, and tissue samples are available from Ronald M. Bonett upon request.

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## SUPPORTING INFORMATION

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