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RESEARCH ARTICLE

Fused Testes in Neotropical Frogs of the Genus *Pristimantis* (Anura, Craugastoridae): New Insights Into a Poorly Known Character in Vertebrates

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ABSTRACT

Amphibian testes vary in shape, from multilobed in caecilians and salamanders to compact, ovoid organs in anurans. Although these variations have been studied extensively in amphibians, there has been little investigation into the structural, copulatory, and reproductive behavioral consequences of unpaired testes, a character shared among some amphibians, cyclostomates, and some teleosts. We analyzed the morphology and structure of unpaired testes in *Pristimantis fetosus* and *Pristimantis permixtus*. We also report a single testis in *P. hernandezi*. Our results suggest that the testis arrangement in these species results from the hypertrophy and fusion of two testes rather than the loss or reduction of one testis. Furthermore, the occurrence of germ cells at different stages of development suggests that spermatogenesis is similar to that described for vertebrates, with spermatogonia undergoing mitosis to form spermatocytes, which then undergo meiosis to form spermatids. Like other brachycephaloid frogs, *Pristimantis* with fused testes exhibit direct development and reproduction on land, but they are the only anurans known to undergo testicular fusion. We propose to recognize the occurrence of fused testes as a unique putative synapomorphy for a new species group distributed in the Colombian Andes, which we refer to as the *P. hernandezi* species group. A comparative survey among vertebrates reveals no apparent variations in testicular organization, sperm development, or copulative and reproductive behavioral characters associated with the fusion of testes, suggesting that its occurrence might not have functional implications for vertebrate testes. The independently evolved occurrence of fused testes in cyclostomates, teleosts, and amphibians raises an exciting perspective on the study of the molecular origin, evolution, and functional significance of testis variation in vertebrate reproduction and biology.

1 | Introduction

In amphibians, testes can be elongated and composed of one or several lobes, as in caecilians and salamanders (Wake 1968; Uribe et al. 2014), or compact, ovoid organs, as in anurans (Propper 2011; Méndez-Tepepa et al. 2023). Amphibian testes are divided into a germinal compartment that comprises the seminiferous tubules, spermatogonia, spermatocytes, spermatids, spermatozoa, and Sertoli cells, and the interstitial compartment composed of collagen fibers, blood vessels, Leydig cells, and connective tissue (Propper 2011; Méndez-Tepepa et al. 2023). Although the composition of the germinal and interstitial compartments appears to be conservative throughout amphibians, there is evidence that germ cell development can occur independently of Sertoli cells (Pudney 1995). Consequently, spermatozoa produced in such testes do not aggregate into bundles but instead exist as individual cells. Testis variation also includes the size and number of lobes in caecilians and salamanders (Uribe et al. 2014; Marlatt 2024) and the size, position relative to the kidneys, and coloration in anurans (Bhaduri 1953; Emerson 1997; Franco-Belussi et al. 2009; Byrne et al. 2002). Moreover, while not mentioned in the main reviews of amphibian testes structure, testes can also be fused.

The occurrence of fused testes in amphibians was first reported in the world's smallest oviparous caecilian, *Idiocranium russeli* Parker, 1936, by Wake (1968). Three decades later, Lynch and Rueda-Almonacid (1998) reported fused testes in the Andean direct-developing frog *Pristimantis fetosus* (Lynch and Rueda-Almonacid 1998). To our knowledge, these are the only known cases of fused testes in tetrapods. Among non-tetrapod vertebrates, unpaired testes have also been reported in cyclostomates, all of which possess fused testes (Pudney 1995; Docker 2024), and some teleost fish. Among teleosts, in addition to the typical paired condition, species have been reported to possess a single testis (e.g., *Tomeurus gracilis* Eigenmann, 1909, Poeciliidae), partial testicular fusion (e.g., *Perca flavescens* Mitchill, 1814, Percidae; *Goodea atripinnis* Jordan, 1880, Goodeidae), and complete testicular fusion (e.g., *Poecilia reticulata* Peters, 1859, Poeciliidae; Billard 1986; Uribe et al. 2014). Although the biological significance of unpaired testes remains unclear, in some (but not all) teleosts this condition is correlated with the formation of sperm bundles and internal fertilization followed by viviparity or oviposition of fertilized eggs (Grier and Parenti 1994; Parenti et al. 2010).

The structure and germ cell development of unpaired testes have been studied in cyclostomates (Nishiyama et al. 2013; Docker 2024) and teleosts (Billard 1986; Grier and Parenti 1994; Parenti 2004; Uribe et al. 2014) but remain unstudied in amphibians. To address this knowledge gap, we present here an analysis of the morphology, structure, and spermatogenesis of the single testis of *Pristimantis fetosus* and *Pristimantis permixtus* (Lynch et al. 1994), the latter being a newly identified species exhibiting this character. We also report the occurrence of a single testis in *P. hernandezi* (Lynch and Ruiz-Carranza 1983). Our objectives in this study are to (1) describe the morphology of the single testis and (2) test if spermatogenesis in unpaired testes differs from the typical anuran pattern.

2 | Materials and Methods

We examined the gross morphology and histology of the unpaired testes of four specimens of *P. fetosus* (ICN 40010, subadult, snout–vent length [SVL] 25.6 mm; ICN 40015, adult, 28.6 mm SVL; ICN 40023, adult, 30.5 mm SVL; ICN 40028, subadult, 26.1 mm SVL) and four specimens *P. permixtus* (ICN 08830, adult, 25.2 mm SVL; ICN 08895, adult, 25.3 mm SVL; ICN 22824, juvenile, 13.9 mm SVL; ICN 23537, juvenile, 15.6 mm SVL) obtained from specimens deposited in the Instituto de Ciencias Naturales, Museo de Historia Natural, Universidad Nacional de Colombia (ICN). We determined age classes (juvenile, subadult, and adult) based on an assessment of both testicular development and SVL reported in the original species descriptions. According to background knowledge, adult male *Pristimantis* typically exhibit larger, swollen testes and generally larger SVL than subadult and juvenile males (Lynch and Duellman 1997). Although the specific preservation history of these specimens is not known, we assume they were initially fixed in formalin and subsequently transferred to 70% ethanol (standard practice at ICN). We removed and measured testes and kidneys to the nearest 0.1 mm with a Vernier caliper and prepared for light microscopy. We analyzed the position of testis, its connection to the kidneys, and the number of fat bodies at its cephalic end to infer if the testicular arrangement in these species resulted from fusion or the loss of one testis. We dehydrated tissues in a gradient series of increasing ethanol solutions (50%–100%), cleared them with xylene, and infiltrated and embedded them in paraffin wax. We then trimmed and serially sectioned the paraffin/tissue blocks into strips 5 µm of thickness using a rotary microtone. We stained tissues with Harris hematoxylin followed by an eosin counterstain. We assessed spermatogenesis by evaluating the presence of different stages of gametocytes (spermatogonia to spermatozoa) within the seminiferous tubules of the testis followed Saidapur (1983) system with the aid of an optical microscope (Nikon SMZ445) at Universidad Industrial de Santander, Colombia. Furthermore, we assessed the presence or absence of ducts or septa in adult testes to determine if the testicular arrangement in these species resulted from fusion or the loss of one. We captured images using an iPhone 14 and processed them for focus-stacking with Adobe Photoshop CS8 and cross-sectional area measurement with ImageJ. We subsequently edited the images using Inkscape software (XQuartz 2.8.1, http://www.inkscape.org/).

The distribution of unpaired testes in other *Pristimantis* species was assessed by examining 122 preserved specimens from 121 species, including representatives of all species groups recognized within the genus (Supporting Information Online Material, Supporting Information S1: Appendix 1).

3 | Results

3.1 | Testicular Structure

Macroscopically, *P. fetosus* and *P. permixtus* possess a single unpigmented testis positioned medially, with bilaterally symmetric fat bodies in the cephalic area (Figures 1–3). The testis is enveloped by the tunica albuginea and is located medially in the abdominal cavity, connected to the anteroventral portion of both kidneys by a narrow mesorchium (Figures 1–3). The connection via the mesorchium extends from the lateral ends of the testis to the medial regions of both kidneys (Figures 1D and 2D). Efferent ducts (vasa efferentia) are not visible due to their short length and the narrow mesorchium. The juvenile and subadult testis of *P. permixtus* and *P. fetosus* is short, extending less than half the length of the kidney, whereas in adults the testis extends posteriad to cover almost the entire kidney (Figures 1–3). The juvenile and subadult testis is also slightly wider than a single kidney, whereas in adults, it is broader than both kidneys combined (Figures 1–3). The juvenile testis of *P. fetosus* measured 1.7 mm in length and 1.3 mm

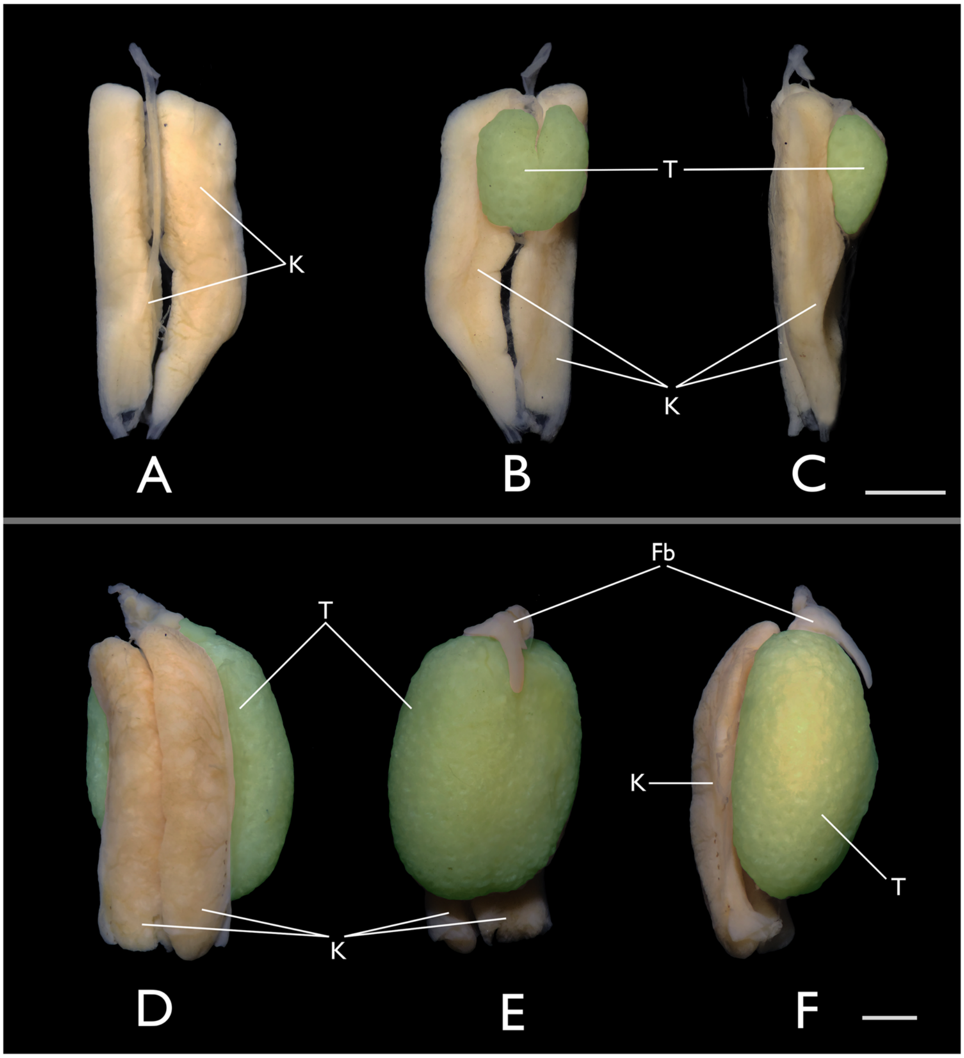

FIGURE 1 | Macroscopic views showing the unpigmented ovoid testis associated with fat bodies and kidneys in subadult (A–C, ICN 40010) and adult (D–F, ICN 40015) male *Pristimantis fetosus*. Fb, Fat Body; K, Kidney; T, Testis. Green denotes the testis. Scale bar = 1 mm.

in width, whereas the adult testis measured 5.0 mm in length and 3.4 mm in width. For *P. permixtus*, the subadult testis measured 1.2 mm in length and 0.8 mm in width, while the adult testis had 3.8 mm in length and 3.0 mm in width. In *P. fetosus* the testis is cephalically bifid (partially divided) in subadults (Figure 1B) but undivided in adults (Figure 1E). In both species, the internal testicular structure is composed of seminiferous tubules with a circular or spherical shape, which give the testis a granular appearance macroscopically (Figures 1–3). Transverse sections show the absence of ducts or septa dividing or separating the testis (Figure 4A,B). In *P. fetosus*, the tunica albuginea is thickened ventrally (Figure 4A) in the region of the medial cleft in subadult testes (Figure 1B).

3.2 | Spermatogenesis

Individual or small groups of primary spermatogonia, which do not form cysts, are found at the base of the peritubular tunic of *P. permixtus* (Figure 4D). Primary spermatogonia are distinguished by their size in *P. permixtus*, being considerably larger than other germ cells. Following the mitotic division of primary spermatogonia, which results in secondary spermatogonia, germ cells become organized within cysts that are supported by Sertoli cells in *P. permixtus* (Figure 4D). Secondary spermatogonia are smaller than primary spermatogonia and have a more pronounced color because their chromatin is slightly more condensed in *P. fetosus* and *P. permixtus* (Figure 4C,D). Primary and secondary spermatocytes, representing a more advanced stage in spermatogenesis with the beginning of the first meiotic division, are also evident. Primary spermatocytes, derived from secondary spermatogonia, are smaller than their predecessors in *P. permixtus*. As a result of the first meiotic division, secondary spermatocytes become visible in both species. These are much smaller cells with more nuclear compaction, housed within cysts in a large population. Spermatids, resulting from the second meiotic division of spermatocytes, are visible with an elongated morphology in both species (Figure 4C,D). Spermatozoa, generated from the differentiation of spermatids, exhibit a head constituted by a highly compacted nucleus. They are more abundant in *P. fetosus* than *P. permixtus* (Figure 4C,D). Developing spermatozoa are well-organized in bundles due to their association with Sertoli cells, where the sperm heads are anchored. Loss of association between the spermatozoa and the

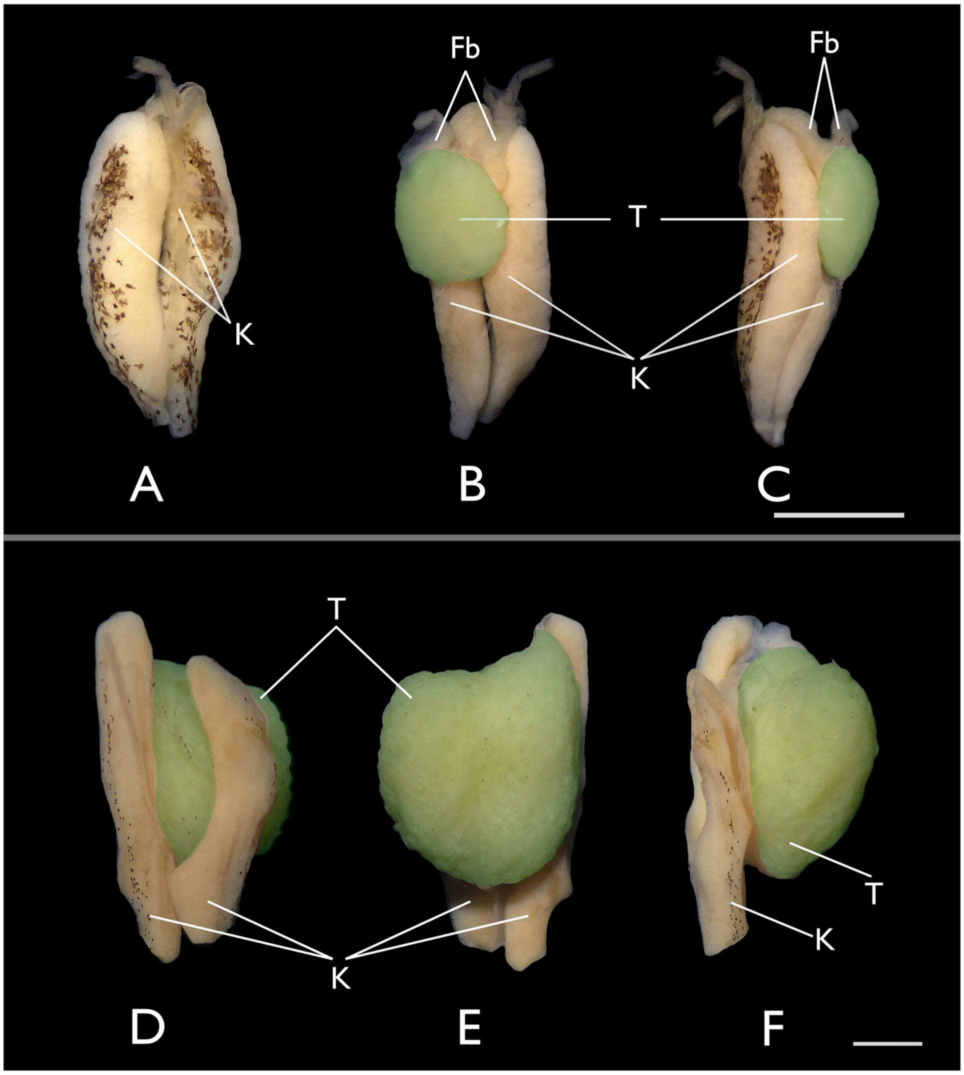

FIGURE 2 | Macroscopic views showing the unpigmented ovoid testis associated with fat bodies and kidneys in juvenile (A–C, ICN 22824) and adult (D–F, ICN 08830) male *Pristimantis permixtus*. Fb, fat body; K, kidney; T, testis. Green denotes the testis. Scale bar = 1 mm.

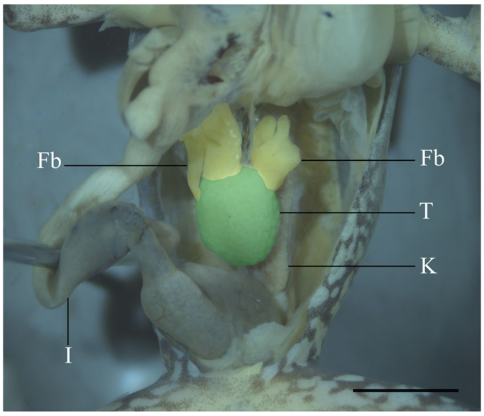

FIGURE 3 | Ventral view of male *Pristimantis permixtus* (ICN 8895, adult) revealing the position of the testis in the abdominal cavity. Fb, fat body; I, Intestine; K, Kidney; T, Testis. Green and yellow denote the testis and fat bodies, respectively. Scale bar = 4 mm.

Sertoli cells was not evident; therefore, spermatozoa are not visible free in the tubular lumen in either species (Figure 4C,D).

4 | Discussion

The evidence gathered from the overall morphology, including medial position, connection to both kidneys, and histological structure, of the unpaired testes of *P. fetosus* and *P. permixtus* suggests fusion rather than the loss or reduction of one testis. In *P. fetosus*, testicular fusion is also supported by differences between subadult and adult testes, with the testis being cephalically divided in the subadult (Figure 1B) and undivided in the adult (Figure 1E). Although signs of fusion are subtle in the ventral region of the adult testis, histological examination reveals ventral emargination and thickening of the tunica albuginea (Figure 4A). These findings, when considered together, corroborate the initial report of fusion by Lynch and Rueda-

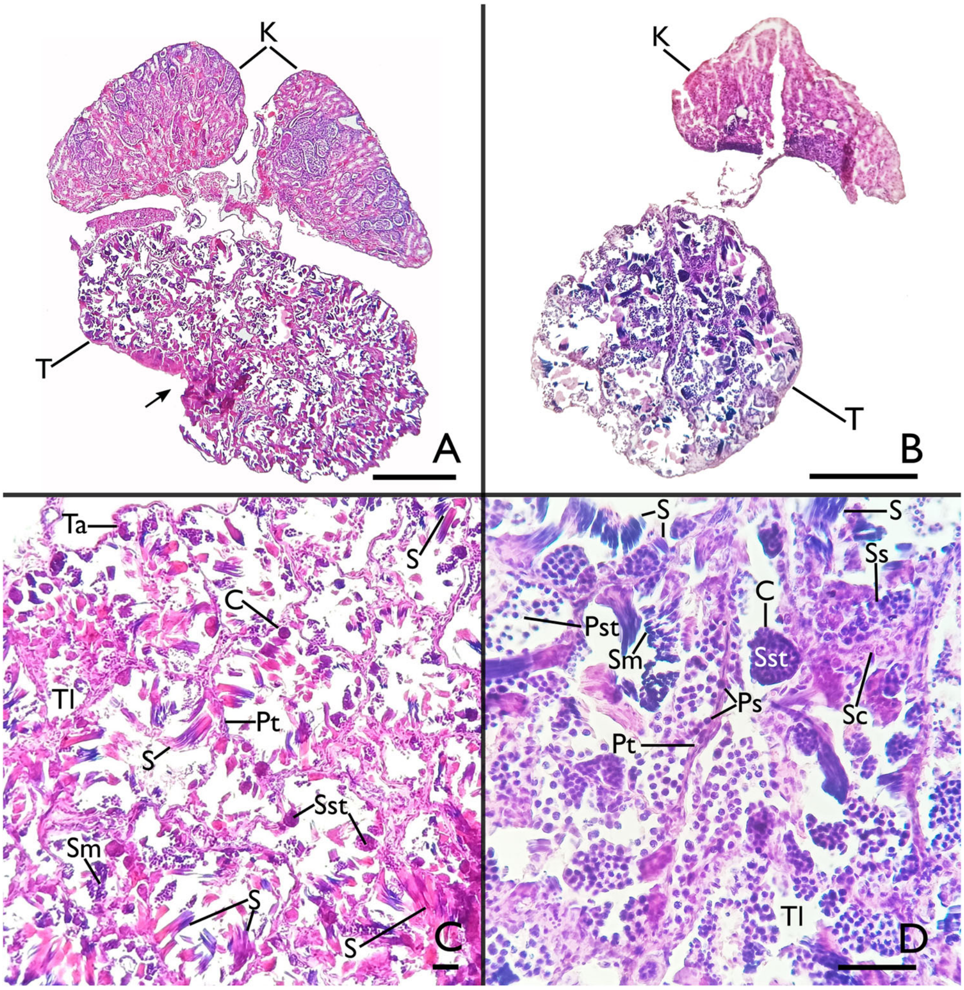

FIGURE 4 | Cross-sectional view of the testis (T) and kidneys (K) of *Pristimantis fetosus* (A, ICN 40015, arrow shows thickening of the tunica albuginea) and *Pristimantis permixtus* (B, ICN 08830, without depiction of the left kidney). Scale bar = 0.5 mm. Overview of the testis of *P. fetosus* (C, ICN 40015) and *P. permixtus* (D, ICN 08830) showing the arrangement of the seminiferous tubules. Cysts (C); Tubule Lumen (Tl); Peritubular tunic (Pt); Spermatozoa (S); Primary spermatogonia (Ps); Primary spermatocyte (Pst); Sertoli cells (Sc); Spermatids (Sm); Secondary spermatogonia (Ss); Secondary spermatocyte (Sst); Tunica albuginea (Ta). Scale bar = 50 µm.

Almonacid (1998) and suggest that the testicular fusion in *P. fetosus* likely proceeds from caudal to cephalic and dorsal to ventral regions of the gonad.

The hypothesis of testicular fusion in *P. permixtus* is further supported by the presence of paired fat bodies at the cephalic end of the testis (Figures 2B,C and 3). In the early stages of development of vertebrates, the gonads form as sexually bipotential structures from the genital ridges, which arise at the ventromedial surface of the embryonic kidneys (mesonephroi; Brambell 1927; Wartenberg et al. 1991). In the case of anuran amphibians, it has been recognized that fat bodies differentiate from the anterior end of this genital ridge, while the posterior part of the ridge, separated by a constriction from its anterior section, become the genital organ (Marshall and Bles 1890; Humphrey 1927a, 1927b). Consequently, if one of the testes had failed to develop, we would expect only a single fat body to occur, with the presence of paired fat bodies at the cephalic end of the testis of *P. permixtus* indicating early fusion of initially paired testes.

Regarding the stage of development at which testicular fusion occurs, the lack of distinct ducts or septa in adults (Figure 4A,B), sub-adults, and juveniles (not shown) of both species suggests that fusion occurs during early testis development. Gonadal development in direct-developing frogs is mediated by thyroid hormone, which initiates stimulation around Townsend-Stewart stage 12 (Townsend and Stewart 1985; Lynn 1936; Callery and Elinson 2000; Laslo et al. 2019). Consequently, testicular fusion in *P. fetosus* and *P. permixtus* likely occurs sometime after Townsend–Stewart stage 12 and before hatching. Further research that includes the study of embryos is needed to understand the ontogeny of fused testes in these species.

In addition to *P. fetosus* and *P. permixtus*, we also observed fused testes in *P. hernandezi*. Together, these three species are the only anurans known to possess fused testes. To date, none of these species has been included in any published phylogenetic analysis, and all three species are currently unassigned to any species group within *Pristimantis* (Padial et al. 2014). On the basis of available evidence, we recognize the occurrence of fused testes as a putative synapomorphy for a new species group distributed in the Colombian Andes between 1800 and 3700 m above sea level, the *P. hernandezi* species group, composed of *P. fetosus*, *P. hernandezi*, and *P. permixtus*. Quantitative phylogenetic analysis of multiple lines of evidence (e.g., morphology and DNA sequences) is required to further test the monophyly of this group and the homology of the testicular fusion in these species.

Concerning the relatively large size of the testes observed in *P. fetosus* (length 17% of SVL) and *P. permixtus* (15% of SVL), the available evidence indicates that reproductively active male *Pristimantis* have larger and more swollen testes than non-reproductive males or immature individuals (Lynch and Duellman 1997). Consequently, a comprehensive study comparing the testis size of adult male *Pristimantis* through estimates of wet testis mass while controlling for body size and phylogenetic relationships and excluding juveniles, non-breeders, and first-year breeding males is needed to identify patterns of testis size variation.

4.1 | Spermatogenesis

Sperm development in *P. fetosus* and *P. permixtus* follows the typical anuran pattern (Pudney 1995; Propper 2011). That is, there is no regional localization of spermatozoa development within the testis, making it possible to find cysts at different stages of spermatogenesis withing a single seminiferous tubule. The occurrence of germ cells at different stages of development allows us to infer that spermatogenesis is similar to that described for vertebrates, beginning with spermatogonia undergoing mitotic divisions to produce spermatocytes, which subsequently undergo meiotic divisions to form spermatids. These spermatids then differentiate into spermatozoa through spermiogenesis (Pudney 1995; Propper 2011).

Regarding differences in sperm abundance observed between *P. fetosus* and *P. permixtus*, it is important to note that such differences can be influenced by the age of the individual and/or the specific time when the specimen was collected. Similarly, although available evidence indicates that anuran species inhabiting tropical regions usually have a continuous production of gametes throughout the year (e.g., van Oordt 1960; Montezol et al. 2018; Marlatt 2024), this is not always the case. For example, Granados-Pérez and Ramirez-Pinilla (2020) reported that spermatogenesis appeared to be discontinuous in *P. merostictus*, *P. miyatai*, and *P. uisae*, as they observed some adult males reinitiating sperm production during breeding events. Consequently, further research on the reproductive phenology of *P. fetosus* and *P. permixtus* is needed. This study should consider additional reproductive parameters such as collection months, the presence of reproductive females, or evidence of reproductive behavior (e.g., calling activity, oviposition) to understand the testicular cycles in these species.

4.2 | Fused Testes in Vertebrates

Among vertebrates, fused testes have been reported in cyclostomates, teleosts, and amphibians (Gymnophiona and Anura, but not Caudata). A comparative survey among vertebrates reveals no apparent variations in testicular organization and sperm development associated with the fusion of testes, suggesting that its occurrence might not have structural implications for vertebrate testes. A similar scenario occurs in the absence of copulative and behavioral reproductive implications associated with the fusion of testes; however, information on this topic is much more limited, especially in hagfishes and amphibians. In some teleosts of the families Goodeidae and Poeciliidae, although fused testes are correlated with sperm bundle formation, internal fertilization via the gonopodium, and subsequent viviparity or oviposition of fertilized eggs (Grier and Parenti 1994; Parenti et al. 2010), a causal relationship remains unknown. Regarding amphibians, there is no correlation between the fusion of testes and copulative or reproductive behavior, although the oviparous caecilian, *Idiocranium russeli*, exhibits internal fertilization and shares the direct development reproductive strategy with *P. fetosus*, *P. permixtus*, and *P. hernandezi* (Lynch and Duellman 1997; Exbrayat 2009). While external fertilization is known (or presumed) to occur via axillary amplexus in *Pristimantis* (Lynch and Duellman 1997), it is important to note that the fertilization mechanisms remain unknown for most *Pristimantis* species, including those that exhibit fusion of testes. Among brachycephaloid frogs, internal fertilization is known to occurs exclusively in *Eleutherodactylus jasperi* Drewry and Jones 1976, and *Craugastor laticeps* (Duméril 1853) (McCraine et al. 2013), neither of which exhibits fused testes (Wake 1978). Given current knowledge, the structural, copulative, and reproductive behavioral consequences of fused testes in vertebrates remain unresolved.

Even though the developmental mechanisms underlying testicular fusion in vertebrates are not known, candidate genes involved in vertebrate testis differentiation have been identified. In particular, analysis of sex-specific gene expression in the developing gonads of vertebrates reveals that the gene networks controlling testis differentiation in amphibians are more similar to those in fish than those in other vertebrates (Roco et al. 2021). Although it is well known that sharing similar sets of genes does not necessarily lead to sharing body forms and structures of long-diverged lineages (Carroll 2008), the occurrence of fused testes in both fish and amphibians suggests that similar genetic mechanisms might be involved in testicular fusion. Fish and amphibians (anamniotes) also share the organization of the testis and the pattern of spermatogenesis, whereby spermatogenesis occurs in cysts that develop within seminiferous tubules for most species. In contrast, amniotes do not have cyst-based spermatogenesis; instead, spermatogenesis occurs directly in seminiferous tubules (Yoshida 2016; Méndez-Tepepa et al. 2023; Marlatt 2024). Moreover, cysts are stationary in clades with fused testes (cyclostomates and anuran and caecilian amphibians). Conversely, in chondrichthyans and salamanders cysts migrate throughout the testis as they mature during spermatogenesis (Pudney 1995; Marlatt 2024).

Although comparative surveys across vertebrate taxa have not been detailed enough to fully describe the differences and similarities of male gonads, the occurrence of fused testes in anamniotes raises an exciting perspective on the evolution of the vertebrate testis. Extensive additional research will be required to document the diversity and functional aspects of fused testes in vertebrates, such as the relationship between sperm production, sperm competition, and of some sperm traits (e.g., sperm morphology). A good starting point for this endeavor could be the current background available on testis size variation, one of the most studied topics in vertebrate testis morphology. Current evidence suggests that sexual selection through sperm competition and/or male mating rate is one of the main selective pressures influencing testis size (including mass) (Parker 1970; Leonard and Córdoba-Aguilar 2010; Vahed and Parker 2012; Baker et al. 2020). This is because larger testes are linked to increased sperm production, which can enhance reproductive success in environments with high sperm competition or an increase in the number of females available for mating (Halliday 1998; Calhim and Birkhead 2007; Vahed and Parker 2012; Ramm and Schärer 2014; Baker et al. 2020).

While some studies of vertebrates have consistently shown a link between sperm competition, male mating rate, and testis size, other research suggests that additional factors might also have an important role in the evolution of testis size. Emerson (1997) conducted a comparative analysis of frog taxa in which one male typically gained exclusive access to a female during mating, perhaps (but not necessarily) with a lowered risk of sperm competition. She found positive correlations between testis mass, egg number, and androgen levels, suggesting that natural selection favors the production of more sperm when there are more eggs to fertilize and/or frog species exhibit high levels of male agonistic behavior (e.g., male–male aggression). In another study on Australian myobatrachoid (Myobatrachidae and Limnodynastidae) and hylid (Pelodryadinae) frogs, Byrne et al. (2002) demonstrated that oviposition location (land vs. water) and female clutch size explained less variation in relative testis size among taxa than did the risk of sperm competition. A contrary situation was reported in reptiles (Uller et al. 2010), in which environmental factors were more important than sperm competition in shaping testis size.

Conclusions from current knowledge of testis size variation demonstrate that its occurrence has been studied primarily to understand reproductive and life history strategies in the context of selection acting on individuals. In particular, current evidence suggests that testis size variation might be explained through a combination of natural and sexual selection, instead of solely attributing it to sexual selection as initially thought. Based on this context, future research exploring the evolutionary implications of fused testes in vertebrates should concentrate on the reproductive and life history strategies of these species, as well as on male and female genital morphology. This is especially important given that there are teleosts in which the fusion of testes and ovaries occurs simultaneously (Grier and Parenti 1994). Finally, considering that the current utility of fused testes might reflect a newly acquired function rather than their evolutionary history, detailed studies on development, tissue differentiation, and genetics are crucial for understanding the evolutionary origin and relevance of fused testes in vertebrate reproduction and biology.

Author Contributions

Jhon Jairo Ospina-Sarria: conceptualization, investigation, writing – original draft, methodology, funding acquisition, validation, visualization, writing – review and editing, formal analysis, software, data curation. **Martha Patricia Ramírez-Pinilla:** validation, visualization, supervision, data curation, investigation, writing – original draft, conceptualization. **Taran Grant:** conceptualization, investigation, funding acquisition, writing – original draft, methodology, validation, visualization, writing – review and editing, formal analysis, project administration, supervision.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that supports the findings of this study are available in the Supporting Information of this article.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.
Appendix S1: List of studied specimens.