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Quantifying variation in female internal genitalia: no evidence for plasticity in response to sexual conflict risk in a seed beetle

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Abstract

Sexually antagonistic coevolution can drive the evolution of male traits that harm females, and female resistance to those traits. While males have been found to vary their harmfulness to females in response to social cues, plasticity in female resistance traits remains to be examined. Here we ask whether female seed beetles *Callosobruchus maculatus* are capable of adjusting their resistance to male harm in response to the social environment. Among seed beetles, male genital spines harm females during copulation and females might resist male harm via thickening of the reproductive tract walls. We develop a novel Micro-CT imaging technique to quantify female reproductive tract thickness in 3-dimensional space, and compared the reproductive tracts of females from populations that had evolved under high and low levels of sexual conflict, and for females reared under a social environment that predicted either high or low levels of sexual conflict. We find little evidence to suggest that females can adjust the thickness of their reproductive tracts in response to the social environment. Neither did evolutionary history affect reproductive tract thickness. Nevertheless, our novel methodology was capable of quantifying fine-scale differences in the internal reproductive tracts of individual females, and will allow future investigations into the internal organs of insects and other animals.

Key Words: female genital evolution, sexual conflict, sexually antagonistic coevolution, phenotypic plasticity, experimental evolution, sex ratio

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1. Introduction

Evolutionary conflict between the sexes can arise over the expression of traits that improve the fitness of one sex but are detrimental to the fitness of their sexual partners [1,2]. Sexual selection favours traits in males that confer greater fertilisation success regardless of the outcomes for females, while antagonistic selection on females can favour morphological and behavioural traits that function to resist harm induced by males [3,4]. Harmful traits in males are diverse in form and include: male harassment during courtship and mating [5,6], infanticide [1,2], and toxic ejaculates [6,7]. One conspicuous form of sexual conflict is the damage imposed to the female's reproductive tract by male genitalia during copulation [8]. Genital damage during copulation is found across a variety of taxa, particularly within arthropod lineages [9–14] and is considered to be a by-product of male traits enhancing fertilisation success, rather than harm directly benefitting males [15–18].

The evolution of harmful traits in one sex is expected to generate selection on the opposite sex favouring the evolution of traits to resist harm, initiating sexually antagonistic coevolution between the sexes [14,19–22]. While comparative evidence suggests that coevolution of harmful male traits and female resistance traits is widespread [22–25], whether these traits can respond plastically to an individual's environment has received less attention. Males of a wide range of taxa have been found to adjust ejaculate size, composition and quality in response to exposure to rival males [26–31], and recent work suggests that male genital morphology can be adjusted in response to the competitive environment experienced during sexual development [32,33]. Thus, males exhibit phenotypic plasticity in sexual traits that can be costly for females. Firman and Simmons [34] found that female house mice exposed to greater levels of sperm competition risk produced ova with lower fertilizability, presumably to mitigate against the threat of polyspermy when males compete for fertilizations. This finding suggests that females too may be capable of responding to the

47 socio-sexual environment, and reduce the costs associated with male competition. Using a
48 model species in which sexual conflict has been widely documented, we test the hypothesis
49 that sexual conflict favours the evolution of phenotypic plasticity in female resistance traits in
50 response to the immediate risk of sexual conflict.

51 Seed beetles (*Callosobruchus maculatus*) are a widely used model species for sexual
52 conflict studies [35]. Research has focused on the role and outcomes of male harmfulness on
53 the persistence of polyandry, the effect of sperm competition on male ejaculate investment,
54 the effects of kinship on male harmfulness, and a wide array of other topics [16,18,36–38].
55 The aedeagus (intromittent organ) is covered in sclerotized spines that perforate the female
56 reproductive tract during copulation, inflicting significant scarring [9,39]. The degree of
57 reproductive tract damage negatively impacts both female longevity and female reproductive
58 success [6] but promotes male fertilisation success [16]. Reproductive tract damage facilitates
59 the transport of accessory seminal compounds into the female bloodstream, which improve
60 male competitive fertilisation success [40–42]. Therefore, the harm to females in this species
61 seems to be a side-effect of selection for competitive male fertilization success [18,42]. The
62 thickness of the female reproductive tract wall appears to have evolved under sexually
63 antagonistic coevolution to resist the harmful effects of male genital spines. Thus, across
64 populations [22] and species [43], female reproductive tract volume has been found to be
65 positively correlated with male penile spine length, providing one of the few empirical
66 examples of sexually antagonistic coevolution [22]. Moreover, male *C. maculatus* appear to
67 adjust their copulatory behaviour [44,45] and amount of harm imposed on females in
68 response to their social environment [37,46, but see 47].

69 Here, we investigate whether female *C. maculatus* exposed to greater risk of sexual
70 conflict can respond by adjusting the thickness of the reproductive tract to minimise
71 anticipated male harm. We used a manipulation of the social environment to vary the

immediate risk of sexual conflict: we had two treatments within which we manipulated both larval density, and the adult sex-ratio to simulate either a high or low sexual conflict risk. Additionally, we employed an experimental evolution design to test whether the thickness of the female reproductive tract diverged between populations of beetles evolving under a male- or female-biased sex-ratio. Assessment of reproductive tract morphology in this species has previously focused on either the thickness of the tract estimated from a small number of histological sections [43], or the total volume of tissue across the entire tract via 3-dimensional analysis of Micro-CT images [22]. Although tissue volume may capture large-scale changes in female investment, it does not capture fine-scale changes in tract morphology. Here, we developed a novel technique to measure variation in the thickness of the female reproductive tract at different locations along its length using Micro-CT data. We predicted that: 1) females in populations evolving under a male-biased sex-ratio will have thicker reproductive tract walls than those evolving under a female-biased sex ratio as a result of sexually antagonistic coevolution; and 2) females exposed to high-density larval environments and a male-biased social environment during development will develop thicker reproductive tract walls compared to females from low density larval environments exposed to a female-biased social environment, in anticipation of an increased risk of sexual conflict.

2. Methods

(a) Study population

The stock population of *C. maculatus* used for this study was derived originally from a population held by the CSIRO in 2005, which was itself founded by individuals found as agricultural pests. Both stock and experimental populations were maintained at 30°C under a

12:12 hour day/night regime for the duration of the experiment [48]. For further information regarding the stock population see [45].

(b) Experimental evolution

Experimental evolution lines were produced and maintained as described in McNamara *et al.* [18]. In brief, individuals from the stock population were used to create six experimental evolution lines. These lines were randomly assigned to one of two treatments, with either a male- or female-biased sex ratio. Each generation consisted of 120 individuals, with an 80:40 male to female ratio for male-biased lines, and vice versa for female-biased lines. To control for potential differences in larval competition between treatments, female-biased populations received 200g of mung beans (*Vigna radiata*) for oviposition, while male-biased populations received 100g. Each new generation was created by isolating 300 beans per population within ventilated 1.5mL Eppendorf tubes. After the required adults had emerged, sex-biased populations were again formed. This procedure was continued for 47 generations, after which populations were placed under common garden conditions for two generations with sex-ratio parity to remove the potential for any non-genetic parental effects. Following the second generation of common-garden breeding, beans were placed within ventilated Eppendorf tubes.

(c) Social manipulation

Isolated beans were assigned to one of two social manipulations designed to alter an individual's perception of future sexual conflict, either high-risk or low-risk. Evidence suggests that seed beetle larvae are able to determine population density before emergence from their beans via vibrations [49,50]. For this experiment we elected to use mung beans

(*Vigna radiata*) as the larval host species to increase surface area compared to their larger, traditional host species (*Vigna unguiculata)*, and thereby improve the transmission of vibrational cues to focal individuals. Therefore, in the high-risk treatment, five infested beans, each containing two larvae (infestation density can be assessed as eggs remain visible on the surface of the bean), were placed within an Eppendorf tube (resulting in ten larvae maximally per tube). Eppendorf tubes were checked daily for beetle emergence. Females that emerged synchronously with a male were discarded from the experiment to ensure focal females had standardised pre-mating social exposure and remained unmated. Females that emerged alone were placed within the lid of a 1.5mL Eppendorf tube, separated from four stock males and two stock females within the body of the tube via cotton mesh. The mesh allowed focal females to detect the presence of individuals but prevented them from copulating with the males. Previous studies have shown that this methodology elicits phenotypic responses to the social environment in both sexes of *C. maculatus* [44,51]. Thus, high-risk females experienced high larval density and a male-biased sex ratio. For the low-risk treatment, a single infested bean, containing a single larva, was placed within an Eppendorf tube containing four un-infested beans. Following emergence, females were placed within an Eppendorf tube separated from two stock males and four stock females. Thus, low-risk females experienced a low larval density and a female-biased sex ratio prior to mating. In the low-risk social treatment, we aimed to provide cues that were representative of naturalistic conditions. Given the high densities experienced by *C. maculatus* when infesting food stores, we therefore exposed females to a small number of males rather than no males. For both treatments, females were removed after 24 hours and then allowed to mate once with a stock male. Females were isolated for a further 24 hours post-copulation, and then euthanized by freezing. Although we might expect adjustments in the reproductive tract to most likely occur during larval and pupal development, we also included the adult

manipulation, because post-emergence sexual maturation is common in many insects, and this species has been found to respond to manipulations of sexual conflict at the adult stage [44,51].

(d) Micro CT-scanning and tomographic reconstruction

A total of five females per population-by-social-treatment combination were selected for Micro-CT scanning. Therefore, a total of 60 individuals were scanned for the purposes of this study. Sample tissue staining and scanning procedures followed those outlined by Dougherty et al. [22], with the exception that we used formalin for tissue fixation rather than paraformaldehyde (see detailed pre-scan methodology in the online supplementary material).

Samples were scanned using a ZEISS Xradia Versa 520 X-ray microscope housed at the University of Western Australia Centre for Microscopy, Characterisation and Analysis. Samples were suspended in 100% ethanol and mounted in heat-sealed pipette tips with wax-covered tops in groups of 3-5 for scanning. Abdomens were arranged vertically and scanned sequentially from the highest abdomen to the lowest. Source voltage and power of initial scans (n = 11) was set at 40kV and 3W. However, source stability was compromised for an extended period of time, so the remaining scans were conducted at 60kV and 5W (n = 43) to ensure that scan quality remained stable (details of the scanning procedures can be found in the online supporting material). A total of six samples could not be analysed due to low scan quality, leaving a sample size of 54 for image analysis.

(e) Image analysis

Images were analysed blind with regard to both the population of origin and social treatment. The analyses were performed using a combination of three custom-written FIJI [52–54] scripts and Amira 6.2 (Thermo-Fisher Scientific, U.S.A.). We first selected a consistent region of interest within each reproductive tract, which was marked by the entrance of the spermathecal duct into the reproductive tract at one end (Figure 1a) and the first occurrence of bursal teeth on the other (Figure 1b) This region was chosen because it sustains the greatest damage during copulation [22]. Within this region of interest, we computed the 3D thickness of the dorsal and ventral reproductive tract walls based on the local thickness definition proposed by Dougherty and Kunzelmann [55] (see online Supplementary material for detailed methodology). According to this definition the thickness at any point within an object, in our case the tract walls, is the diameter of the largest sphere that fits inside the object and at the same time contains the point (Figure 1c). Further, we differentiated investment in the upper and lower reproductive tract by placing a horizontal plane running through the lumen's centroid, which allowed us to reliably define the upper and lower regions of tracts among all sampled individuals (Figure 1d).

(f) Statistical Analyses

All statistical analyses were conducted in R (v 3.5.3) [56]. Normality of data was confirmed with Shapiro-Wilk's tests. Further, Bartlett's test for homogeneity of variance was found to be non-significant in all cases.

Pearson's correlation tests using the 'ppcor' R package [57] found significant correlations between the mean, minimum and maximum thickness values from the upper and lower reproductive tract. Repeatability analyses were conducted for all measurements by extracting measures of 9 individuals on each of three separate occasions and analysing the data using

the R package 'rptR' [58]. We found significant repeatability ($R > 0.6$) in mean and maximum tract thickness but not in minimum tract thickness (see Table S2 in the online Supplementary Material). We therefore conducted principal components analysis using the 'FactoMineR' R package, excluding upper and lower minimum thickness [59]. One principal component (PC1) had an eigenvalue greater than 1 and was extracted for further analysis. PC1 was used as the dependent variable using a Gaussian linear mixed model that included female weight as a covariate. Following Arnqvist [60], replicate population was included as a random factor within which an interaction between female weight and social treatment was fitted as a random slope. The random slope allows for the interaction to vary across the differing populations [60]. The source voltage was also included in this model as a random factor to control for any variance in trait estimates that might have arisen from the use of different voltage settings during scanning. The significance of our treatments, random factors and interaction effects were tested using Kenward-Roger F-tests using the R package 'pbkrtest'[61]. Non-significant interactions that did not improve model fit were subsequently removed from final models. Effect sizes were estimated as the standardised Pearson's correlation coefficients (Table 3) using the 'effectsize' package in R. Estimated marginal means for each level of the two treatments were produced via the package 'emmeans'. The data were explored for outliers using robust kernel-based outlier factor algorithms within the 'OutlierDetection' R package (k=3, bootstraps= 50,000). No outliers were identified.

3. Results

The first principal component explained 72% of the variance in reproductive tract thickness (Table 1) and was loaded equally by the mean and maximum thickness from both the upper and lower reproductive tract. There was no significant impact of evolutionary history or

social treatment on female tract (Table 2). All interaction combinations between evolutionary history, social treatment and weight were found to be non-significant and were dropped from the final model.

4. Discussion

We developed a novel Micro-CT imaging method to measure the thickness of the reproductive tract walls of female *C. maculatus* seed beetles, from populations that had evolved under a male- or female-biased population sex ratio for 47 generations, and which were subsequently exposed to a social environment which conveyed either a high or low-risk of sexual conflict. Previous studies of this species have found that males can adjust their harmfulness to females in response to their social environment [43–45, but see: 56]. There was no effect of evolutionary history on the overall thickness of the female reproductive tract, nor was there any effect of the social environment. Our data therefore suggests that female reproductive tracts may not respond to variation in the risk of sexual conflict.

Previous studies using *C. maculatus* have similarly failed to find evolutionary responses in females to experimental manipulations of sexual conflict. Gay et al. [57] showed that after 90 generations of enforced monogamy, the re-introduction of polyandry over 30 generations resulted in the evolution of harmful males but not resistant females. Similarly, after 8 generations of enforced monogamy it was found that the elaboration of male penile spines decreased, as might be predicted for a costly trait that functions in the context of competitive reproductive success. However, the morphology of the teeth found within the female genital tract failed to exhibit a correlated response to enforced monogamy [64]. Our results are also consistent with recent findings of McNamara et al. [18] who utilised the same

experimental evolution lines used in the current study. McNamara et al. [18] found that although males evolving under a male-biased sex ratio evolved to be more harmful, females from populations evolving under a male-biased sex ratio experienced comparable reproductive tract scarring to females from populations evolving under a female-biased sex ratio. We found no evidence that sexual conflict intensity results in an evolutionary divergence in female reproductive tract thickness. Collectively, these findings suggest that either females fail to coevolve as readily as males, or that female reproductive tract coevolution is more difficult to detect [18,63,64].

The findings of these experimental evolution studies are in contrast to comparative studies that have found evidence for sexually antagonistic coevolution between male harmfulness and female resistance to harm among *C. maculatus* populations and among *Callosobruchus* species more widely [22,43]. Further, the coevolution of resistance traits and male persistence has been identified in comparative studies of other arthropod taxa such as water striders (Heteroptera: Gerridae) [65] and diving beetles (Coleoptera: Dytiscidae) [66]. The apparent inability of female seed beetle reproductive tracts to respond evolutionarily to relatively short-term manipulations in sexual conflict might be attributable to the difficulty in detecting sexually antagonistic coevolution at a particular point in time [2,24,67]. This suggestion could explain why females seemingly did not respond to our selection treatments, as it is clear that divergence in male harmfulness was present in these populations 15 generations earlier [18]. We are unable to say whether females from populations experiencing differing levels of sexual conflict might have developed alternative methods of resistance, such as higher immunocompetence [68]. Previous studies utilising the same experimental evolution lines have shown that individuals derived from the male-biased lines exhibit reduced immune function [51]. These results are indicative of a resource trade-off between immune function and reproductive investment fuelled by the costs of high intensity sexual

conflict. Although the method by which Gay et al. [57] and McNamara et al. [18] altered sexual conflict intensity differed, both studies found that females from populations that experienced higher conflict were better able to counter-act the negative impact of mating on their fitness. However, it is clear that the method by which female *C. maculatus* accomplish this is not via genital morphology, female kicking behaviour, or through improved immunity [18,51]. Finally, it may be that inbreeding depression was responsible for impeding divergence among our lines. This seems unlikely however, because a hallmark of inbreeding is reduced fitness, which would have been observed as reduced fecundity for females from the female-biased lines. A previous study using the same experimental evolution lines [18] showed no evidence that females from female-biased lines experienced reduce fitness compared to those from male-biased lines.

We found that the social environment had no impact on female reproductive tract thickness. This is the only study to have investigated whether females are able to plastically respond to sexual conflict risk by altering reproductive tissue dimensions. There are several reasons why females might have failed to respond to our manipulation of their social environment. First, it is possible that females were unable to adjust the reproductive tract in response to our environmental cues. Females were given a proxy for sexual conflict risk via manipulations of larval density during development, but this cue may have been insufficient to provoke a plastic response. Second, the larval and pupal stages are critical to the development of insect reproductive organs [69]. This is demonstrated by the long-lasting impacts of larval food availability on adult reproductive output in both female and male insects [70,71]. Females were exposed to direct signals of increased sexual conflict risk (via sex-ratio) in their adult stage, post-pupation, but they may be unable to make plastic adjustments after adult emergence. Third, it is also possible that our measures of tract thickness do not capture important qualitative variation. For example, it may be that females

284 can also plastically adjust the elasticity of the reproductive tract through the incorporation of
285 resilin, a compound shown to improve tolerance to cuticle perforation in bed bugs (*Cimex*
286 *lectularius)*[72]. Finally, it may be that females plastically responded to our social
287 manipulation, but due to the logistical limitations placed on us with respect to the numbers of
288 females we could scan, we were unable to capture any differences among them. However,
289 given that our repeatability analysis revealed significant variation among females in
290 reproductive tract thickness, we can be certain that we are able to detect variation in tract
291 thickness using this novel technique. If there were an effect of the social environment on the
292 reproductive tract thickness, the effect size estimated in our analysis suggest that it is small
293 and would require a large sample size in order to detect significance.

294

295 Although our measure of female resistance traits is limited to a quantitative measure
296 of reproductive tract thickness, it nonetheless offers a significant advance over previous
297 studies. Previous studies utilising 3-dimensional analysis of Micro-CT scans have measured
298 total reproductive tract volume, which is effective at controlling for tract shape and size
299 effects, but cannot identify fine-scale changes in morphology within the reproductive tract
300 [22]. Our current method overcomes this challenge by allowing us to identify variation in
301 thickness in the upper and lower regions of the reproductive tract in 3-dimensional space.
302 Overall, our novel method for measuring reproductive tract thickness shows promise in its
303 ability to detect fine-scale differences in internal structures of the female reproductive tract,
304 and promises to be a valuable tool in the long-awaited study of female genital morphology
305 across numerous species.

306 In conclusion, we provide no evidence for plastic adjustments of reproductive tract
307 thickness, a trait known to have coevolved with male-imposed genital damage among

populations and species of *Callosobruchus*. Moreover, we found that populations that had evolved under intense sexual conflict failed to diverge in female genital morphology. The coevolution of male and female genital traits among populations suggests ample genetic variation in tract morphology may exist, however among-population genetic variance does not necessarily reflect within-population variation [73]. Therefore, the lack of divergence in tract morphology across our evolution lines may reflect a lack of within-population genetic variation for this trait. Studies of sexual selection acting on female genitalia typically lag behind those focussed on male traits [67,74]. Investigating female traits and their responses to male harmfulness can broaden our understanding of sexual selection and sexual conflict. Further research needs to focus on the female perspective if we are to quantify the pervasiveness and intensity of female responses to sexual conflict. The barriers to such research are slowly dissipating with the advent of innovative new technologies, such as Micro-CT scanning, that allow more effective measurement of female traits. As technologies become more accessible and cheaper to employ, increased sample sizes will be possible so that future studies have the power to detect variation in these minute structures. We believe that the present study provides a viable methodological approach for further investigations into plastic adjustments in female reproductive tracts, which is flexible enough to identify small-scale variation.

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529

Table 1 Fit and loadings for the first two principal components explaining variation in female reproductive tract thickness.

	PC1	PC2
Eigenvalue	**2.88**	0.78
% Variance	71.91	19.91
Upper mean	0.930	0.25
Upper maximum	0.777	0.60
Lower mean	0.877	-0.36
Lower maximum	0.811	-0.47

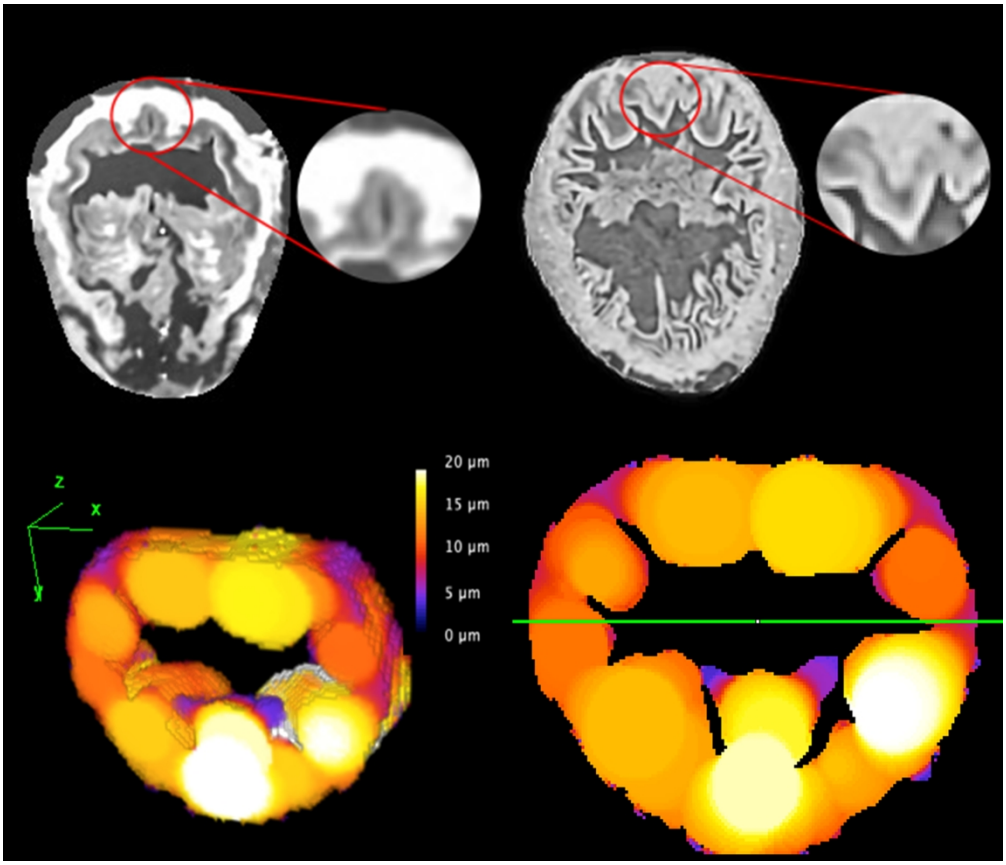
Table 2 Analysis of variance for fixed and random effects on a multivariate measure of female reproductive tract thickness (PC1) (N=54).

	F	df	p	Variance
Evolutionary History	0.51	1, 3.79	0.52	
Social Environment (SE)	1.18	1, 4.38	0.34	
Body Weight	3.95	1, 3.61	0.12	
Population replicate				4.31
Source voltage				<0.01

545 **Table 3** Estimated marginal means and effect sizes (Pearson's r) with 95% confidence
546 intervals of all fixed effects on a multivariate measure of female reproductive tract thickness
547 (PC1) (N=54).

	Mean	**95% CI**	**Effect size (r)**	**95% CI**
Evolutionary History (M-F)			-0.28	-0.76, 0.21
Female-bias	0.638	0.013, 1.26		
Male-bias	0.580	-0.17, 1.33		
Social Environment (L-H)			-0.43	-0.92, 0.05
High-risk	0.643	0.047, 1.24		
Low-risk	0.575	-0.15, 1.30		

548



Segmentation of the reproductive tract: (a) First appearance of the entrance of the spermathecal duct. (b) First appearance of the bursal teeth. Tract wall thickness: (c) 3D thickness heat map (d) Upper and lower regions of interest within the reproductive tract.

444x380mm (130 x 130 DPI)