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Highlights

- Sunshine virus is a paramyxovirus that infects snakes
- The virus was detected by PCR in a dam and a sire, both carpet pythons
- The dam then laid a clutch of 21 apparently healthy eggs, 14 eggs hatched
- Virus was found in the allantois, amnion and embryo from multiple eggs in the clutch
- No virus was detected in oral-cloacal swabs from the hatchlings of this clutch

Evidence for the vertical transmission of Sunshine virus

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1. Abstract

Sunshine virus is a paramyxovirus of pythons associated with neurorespiratory disease and mortalities. This report provides evidence for its vertical transmission. In a collection of over 200 Australian pythons, a dam and a sire, both carpet pythons (*Morelia spilota*), were PCR-positive for Sunshine virus at a time when the dam was likely to have been gravid. A clutch of 21 eggs was laid and three non-viable eggs were tested for the presence of Sunshine virus by PCR. One egg had been incubating for 34 days while the other two had been incubating for 49 days. The surface of all three eggs was negative for Sunshine virus but swabs of the allantois and amnion were positive in all three eggs. Embryo tissue samples were tested from the two 49 day old eggs. From one embryo, a sample of brain and a pooled sample of lung, liver, kidney and intestine were positive, while for the other embryo, a pooled sample of lung, liver, kidney, intestine and brain was positive. Fourteen of the 21 eggs hatched and all hatchlings were tested by PCR at least once between

the ages of 53 and 229 days old. All hatchlings were PCR-negative for Sunshine virus.

Keywords

Reptile; snake; python; *Morelia spilota*; carpet python; paramyxovirus; allantois; amnion; embryo

2. Introduction

Sunshine virus is a paramyxovirus that infects pythons and has been associated with outbreaks of neurological disease, respiratory disease and/or non-specific clinical signs such as lethargy and regurgitation (Hyndman et al., 2012b). It is distantly related to the other currently-known group of reptilian paramyxoviruses, the ferlaviruses (sometimes previously referred to as ophidian paramyxoviruses, or OPMV). The genus *Ferlavirus* clusters within the *Paramyxivirinae* subfamily of *Paramyxoviridae* (ICTV, 2014) but Sunshine virus does not cluster within either of the two currently-accepted paramyxoviral subfamilies: *Paramyxovirinae* and *Pneumovirinae* (Hyndman et al., 2012a).

Carpet pythons (*Morelia bredli* and a variety of subspecies of *Morelia spilota*) are commonly kept in captivity throughout the world, including Australia where they are native. They reliably breed when kept under suitable conditions and produce moderately large clutches of eggs (Elliott, 2014). A successful mating will start with copulation and insemination by a fertile male into a female with

appropriate follicular development. Ovulation follows and a few weeks later, the female will shed its skin (ecdysis). Approximately three to four weeks after this shed, known as a pre-lay shed, oviposition will usually occur. After approximately 55-60 days of incubation, hatchlings will typically start emerging from viable eggs. There are minor variations in this breeding cycle depending on the (sub) species of carpet python. At the time of this writing, Sunshine virus has been detected in Australian carpet pythons more than any other species of snake (Hyndman et al., 2014).

Sunshine virus has been detected by PCR from oral and cloacal swabs, so it is assumed that horizontal transmission can occur from oral and cloacal secretions (Hyndman et al., 2012b). Prior to this report, there has been no evidence that Sunshine virus can transmit itself vertically.

Vertical transmission has been defined by many sources. In *Fenner's Veterinary Virology* (2011), vertical transmission is an "infection that is transferred from dam to embryo, or fetus, or newborn before, during, or shortly after parturition". In *Field's Virology* (2007), the vertical spread of viruses is where "viruses infect either the immature fetus or the new born during the birth process". And as a final example, the *Saunders Comprehensive Veterinary Dictionary* (2012) defines vertical transmission as being "from one generation to the next, perhaps transovarially or by intrauterine infection of the fetus". In the case of reptilian paramyxoviruses, three published

literature reviews were unable to find evidence that supported or refuted the natural occurrence of the vertical spread of ferlaviruses from dam to embryo (Hyndman et al., 2013; Pasmans et al., 2008; Ritchie, 2006).

Vertical transmission of paramyxoviruses in non-reptilian hosts has been demonstrated with Nipah virus in domestic cats (Mungall et al., 2007) and Hendra virus in flying foxes (Halpin et al., 2000; Williamson et al., 2000) and guinea pigs (Williamson et al., 2000), however, the body of work with the greatest relevance to reptiles are the studies on Newcastle disease virus (NDV) in poultry. In a review of the vertical transmission of NDV, it is stated that hens infected with virulent strains usually stop laying eggs (Alexander and Senne, 2008b) making vertical transmission of limited significance. However there are still examples in the literature that provide insights into the transfer of NDV from one generation to the next. In one study, low doses of virulent NDV were experimentally-inoculated into the allantoic cavities of 155 specific pathogen free (SPF) embryonated chicken eggs (Chen and Wang, 2002). In this experiment, NDV was isolated from three of 71 hatchlings. The embryos in the other 84 eggs died. This report does not form evidence of vertical transmission as the eggs (and not the dams) were experimentally-inoculated. In another study, NDV was detected in chicken embryo liver and cell lines that had both originated from the same set of eggs (Capua et al., 1993). Subsequent testing of the hens that laid these eggs, and their

progeny, revealed NDV in the cloacal swabs of both generations. While it is possible the hens horizontally transferred NDV to their *hatchlings*, it seems likely that the hens transmitted NDV into the *embryos* during embryo and egg development (satisfying vertical transmission). There is no evidence of vertical transmission in two other significant paramyxoviral pathogens of poultry: avian metapneumovirus (Gough and Jones, 2008) and avian paramyxoviruses 2-9 (Alexander and Senne, 2008a). In summary, there is evidence that at least some paramyxoviruses utilise vertical transmission to propagate.

In this report, we provide evidence of the *in ovo* presence of Sunshine virus in three eggs that had been laid by a dam that had been diagnosed with Sunshine virus infection at a time the snake was likely to have been gravid.

3. Materials and Methods

3.1 History

In 2013, Sunshine virus was detected by PCR (and sequencing) in a private Australian collection of over 200 pythons consisting of carpet pythons (*Morelia bredli* and various subspecies of *M.spilota*), green tree pythons (*M.viridis*), Children's pythons (*Antaresia childreni*) and

148 black-headed pythons (*Aspidites melanocephalus*). PCR testing and
149 sequencing was performed at Murdoch University, Australia using
150 methods previously described (Hyndman et al., 2012b). The first
151 animal from this collection to be diagnosed with Sunshine virus was
152 a six year old female Darwin carpet python (*Morelia spilota*
153 *variegata*) that had been apparently-healthy since joining the
154 collection five years earlier. In 2013, this snake presented with a
155 rapid onset (days) of generalised weakness, occasional stertorous
156 respiration, skin blisters containing clear fluid, and intracytoplasmic
157 inclusion bodies in heterophils and monocytes (azurophils). Prior to
158 this diagnosis of Sunshine virus, there had been no concerning signs
159 of ill-health in this collection. The keeper of this collection reliably
160 placed new animals into quarantine for 12-months. The source of
161 this infection was unknown.
162
163 In the eight months following the first diagnosis of infection,
164 another 15 similarly-affected and/or in-contact snakes from this
165 collection were tested for Sunshine virus by PCR, and seven of these
166 were positive. One of these was an apparently-healthy 3.5 year old
167 female carpet python (*M.s.variegata*/*M.s.mcdowelli* hybrid) that
168 was PCR-positive for Sunshine virus on a combined oral-cloacal swab
169 and PCR-negative on blood 55 days before laying her first ever
170 clutch of eggs; a clutch of 21 eggs. This dam had been in contact
171 with the apparently-healthy sire (*M.s.variegata*) of this clutch for
172 approximately one and a half months during a three month period
173 that ended approximately two months before oviposition (Figure 1).

The sire had tested positive for Sunshine virus on blood and a combined oral-cloacal swab (tested separately) 57 days prior to oviposition. All 21 eggs were placed into an incubator and based on external examination, all appeared to be viable. This clutch of eggs shared an incubator with other clutches of eggs but there was never any direct contact between this clutch and any other clutch. No egg or hatchling ever made contact with either the dam or the sire after this time.

3.2 Sample Collection

Thirty four days into incubation, an egg that was suspected to be non-viable was dissected to retrieve samples for Sunshine virus testing. This sampling was performed by the attending veterinarian for diagnostic purposes. Three swabs were collected from this egg: one from the surface of the egg, the second from the allantois, and the third from the amnion. Cotton-tipped applicators were used to swab these areas (see Figure 2) and then swab tips were broken off into 3 mL plain blood tubes. The tubes were then partially filled with 1.5 mL of isotonic saline solution. Fifteen days later (49 days into incubation), two more eggs of normal size but mottled appearance, were transferred into a -20 ^{o}C freezer for future sampling. Sixty four days later, swabs were collected as before from the surface, the allantois and the amnion of each of these two eggs. In addition to this, tissue samples were collected from the embryos in these two

eggs. To prevent tissue samples being contaminated with the extra-embryonic membranes, the surface of the embryo was sprayed with a benzalkonium-biguanide disinfectant combination (F10®SC Veterinary Disinfectant, Health and Hygiene, South Africa) which was then wiped off with cotton swabs. Following embryo surface decontamination, a separate set of sterile instruments was used to collect samples of brain, kidney, lung, liver, heart and intestine. Tissue samples were placed into plain blood tubes. All samples were sent to Murdoch University for PCR testing for Sunshine virus.

In addition to the samples that were retrieved from eggs, combined oral-cloacal swabs were opportunistically-collected from all 14 of the hatchlings from this clutch of 21 eggs. Three of the remaining seven unhatched eggs were non-viable and were tested for Sunshine virus (see above), while the other four were not sampled and did not hatch. One hatchling was tested at the ages of 53 and 229 days old; another hatchling was tested at the ages of 53, 74 and 229 days old; two hatchlings were tested at the age of 74 days old; and nine hatchlings were tested at the ages of 74 and 229 days old. The dam and the sire that had previously been tested on days 55 and 57 *prior* to oviposition, respectively, were each tested for a second time 133 days *after* oviposition (equal to the hatchlings' age of 74 days). Swabs were collected as previously described (Hyndman et al., 2012b). Briefly, a cotton-tipped applicator was pre-moistened in isotonic saline and then the inside of the mouth was swabbed. This same swab was then used to swab the cloaca. The swab tip was

then broken off into a 3 mL plain blood tube and was submerged in 1.5 mL of isotonic saline. A number of surfaces of the incubator were also swabbed but this was not done until 133 days after oviposition.

3.3 Polymerase Chain Reaction (PCR) and Sequencing

Containers that contained swab tips immersed in isotonic saline were vigorously vortexed for at least 15 seconds and then a 200 µL aliquot of the saline was used for nucleic acid extraction using the Purelink™ Viral RNA/DNA Mini Kit (Cat. No. 12280-050, Invitrogen, Victoria) according to the manufacturer's instructions. Fresh tissues were processed using the MELT™ Total Nucleic Acid Isolation System (Cat. No. AM1983, Ambion, Texas) according to the manufacturer's instructions. Total nucleic acid from both extraction procedures was eluted into 30 µL of elution buffer. For one-step reverse transcription (RT)-PCR, 1 µL of extracted nucleic acid was added to 0.8 µL of SuperScript® III RT/Platinum® *Taq* Mix (Cat. No. 12574-026, Invitrogen, Victoria), 10 µL of 2x Reaction Mix, 1 µM (final concentration) of each of SunshineS2 (5'-TTCAAGGAGATAACCAGG) and SunshineAS2 (5'-CGGGATTCCCATAGAC) (Hyndman et al., 2012b), and made up to 20 µL using PCR-grade water. Cycling conditions were 45 ^{o}C x 45 m, 94 ^{o}C x 2 m, 40 x (94 ^{o}C x 20 s, 51 ^{o}C x 30 s, 72 ^{o}C x 20 s). PCR products

were visualised using agarose gel electrophoresis and sequencing of appropriately-sized PCR products (230 nucleotides) was accomplished using an AB3730xl DNA Analyser (Applied Biosystems, California).

4. Results

4.1 Fate of Clutch

A timeline of parent pairings, oviposition and results of PCR testing for Sunshine virus are presented in Figure 1. In total, 21 eggs were laid and of these, 14 hatched. One hatchling died at 53 days old without any premonitory signs of disease and necropsy was unremarkable. This animal was PCR-negative for the presence of Sunshine virus (see below). A second hatchling was killed by the household cat at 74 days old and a third was found missing from its cage between 74 and 229 days old without ever being found again (presumed eaten by the household cat as well). At the time of writing, the hatchlings were approximately 11 months old and have been feeding, growing and behaving as expected for this species.

4.2 Polymerase Chain Reaction and Sequencing

The results of PCR testing of each animal are summarised in Figure 1. Both parents of this clutch were positive for Sunshine virus by PCR at least once. Swabs of the allantois and the amnion from all three of the eggs that were tested were PCR-positive for Sunshine virus. Additionally, the embryo itself was tested from two of these eggs and in both cases, embryonic tissues were PCR-positive for Sunshine virus. In contrast to these findings, blood, a combined oral-cloacal swab, brain, and a pooled sample of kidney, liver, lung and intestine, from the hatchling that died at 53 days of age (see above), were all PCR-negative for Sunshine virus. Tissues from this hatchling were not examined histologically. Furthermore, combined oral-cloacal swabs from all hatchlings, at all times tested, were PCR-negative.

All PCR-positive results were sequenced and there was no sequence variation between any of the positive results in this study. After excluding the primers from the sequence data, the remaining 196 nucleotides were 99% identical (195/196) to Sunshine virus (GenBank accession number JN192445.1). The single nucleotide difference was a silent mutation.

5. Discussion

There is almost no published information on the vertical transmission of any virus in any group of reptile. There are reports of inclusion body disease (IBD)-positive boa dams giving birth to IBD-

positive offspring but these reports are either unpublished (Rachel Marschang, personal communication) or provide little detail (Chang and Jacobson, 2010). Most published reports merely acknowledge the absence of information. In this report, evidence for the natural occurrence of the vertical transmission of Sunshine virus is described. In a large private collection of Australian pythons, the parents of a clutch of carpet python eggs were shown to be PCR-positive for Sunshine virus. This virus was then also detected *in ovo* from three of the eggs in this clutch. Interestingly, the virus was not detected in any of the hatchlings, including a hatchling that died suddenly at 53 days of age.

Although it is our opinion that Sunshine virus was vertically transmitted to the eggs, an alternative explanation could be that the eggs were uninfected when they were laid, and environmental contamination of Sunshine virus resulted in the horizontal transmission of the virus into the eggs (trans-shell infection). Although we feel this was unlikely, we do not have sufficient data to categorically disprove this. Translocation of a paramyxovirus across a snake egg shell has not been demonstrated but studies exist that have shown that other microbes are capable of trans-shell infection in reptiles. In one study, the trans-shell infection of *Salmonella* sp. occurred in 25 out of 46 *Pseudemys elegans* (red-eared slider turtle) eggs after 24 hours of exposure under laboratory conditions (Feeley and Treger, 1969). In another study, bacterial and fungal trans-shell infections were commonly identified in non-viable ("slug") *Caretta*

caretta (loggerhead turtle) eggs (Wyneken et al., 1988). In poultry, motile bacteria such as *Pseudomonas* sp., *Alcaligenes* sp. and *Salmonella enteritidis* have been shown to effectively infect eggs (De Reu et al., 2006) but the same could not be said for the paramyxovirus *Newcastle disease virus* (NDV). Under laboratory conditions, Williams and Dillard (1968) showed that NDV was only able to penetrate the cuticle (the mucus layer external to the shell) and the shell of <4% of uncracked chicken eggs after up to 48 hours of NDV exposure. Penetration through the cuticle, the shell and the outer shell membrane occurred in 10% of cracked eggs but for both cracked and uncracked eggs, NDV did not penetrate through the inner shell membrane.

Based on the fastidious cleaning routines of the private breeder, the inability to detect Sunshine virus on the surface of the eggs and in the incubator (although they were sampled later), the presence of Sunshine virus in both parents, we feel that the simplest explanation for the *in ovo* presence of Sunshine virus is that the virus was vertically transmitted from parent to egg.

A limitation of this study is that non-viable eggs were stored at -20 ^{o}C until sampling could be performed. Because of this, there was no opportunity to examine infected embryo tissues histologically to look for evidence of embryo toxicity that may have been seen under light microscopy.

In the author's laboratory, Sunshine virus has been detected by PCR in ovary tissue in an Australian carpet python before (unpublished data) and it may seem logical to assume that the eggs were infected transovarially, however, the role that the sire played in the *in ovo* presence of Sunshine virus warrants consideration. In the aforementioned definition of vertical transmission in *Fenner's Veterinary Virology* (2011), the dam is specifically identified as the source of infection for the progeny but the results of our study cannot rule out the possibility that the sire may have been the source of infection. It is possible that the ovulated eggs were uninfected (despite it being likely that the dam was infected at this time) and only became infected at the time of fertilisation by the sire's sperm. Semen as a source of infection for two mammalian paramyxoviruses (both rubulaviruses) has been investigated. Following experimental inoculation, porcine rubulavirus was found in semen collected from boars (Solis et al., 2007) and in people, mumps virus has been detected in semen (Jalal et al., 2004). For both rubulaviruses, epididymo-orchitis was a potential complication of infection. The presence of Sunshine virus in testicular tissue or semen has not been demonstrated but from a comparative virology perspective, this is an intriguing area worthy of investigation.

Sunshine virus could not be detected in any of the hatchlings from this clutch and it can only be cogitated why this may have been. One possible explanation is that Sunshine virus has a high embryo mortality rate and not all the eggs were infected. That is, the eggs

376 that died may have died from Sunshine virus infection, and the
377 surviving hatchlings were uninfected during incubation. Another
378 explanation is that the viral load delivered to each egg was unequal.
379 Conceivably, the eggs with higher viral titres died, while those with
380 lower titres hatched and cleared the infection. In a study on the
381 vertical transmission of NDV in chickens, Pospisil et al. (1991)
382 detected the virus in chicken embryos and young (hatchling to 25-
383 day old) but not older chickens. The viral load in the hatchlings
384 decreased as the chicks aged. Levels of NDV could not be detected
385 in chicks after they had reached 25-days old. In our study, the
386 hatchlings were at least 53 days old at the time of the first sampling.
387 Yet another explanation is that the hatchlings were infected at the
388 time of sampling but were not shedding sufficient quantities of
389 Sunshine virus to be detected by the PCR used in this study. The
390 PCR-results of the hatchlings have important biosecurity
391 ramifications on a collection. The results presented here were not
392 able to demonstrate these animals as reservoirs of virus. For
393 Sunshine virus, multiple PCR-negative test results are needed to
394 increase the confidence that an animal is uninfected (Hyndman et
395 al., 2012b) and although most of these hatchlings were tested at
396 least twice over a six month period, it is possible that infected
397 animals may still have escaped detection.
398
399 The results of this study provide evidence that Sunshine virus may
400 be able to transmit vertically from parent to egg. The significance of
401 this finding is not yet clear. The possibility that this virus can

propagate itself using this mode of transmission should be carefully considered by herpetologists and reptile veterinarians alike if the virus is to be eradicated from a snake collection.

6. Conclusion

Sunshine virus is a paramyxovirus of pythons associated with neurorespiratory and non-specific clinical signs of ill health. Prior to this report, the only information available on its transmission was derived from PCR testing of oral and cloacal swabs. Based on this, it was assumed that Sunshine virus could be transmitted by oral and cloacal secretions. Data are presented here that supports the vertical transmission of Sunshine virus from parent to offspring. A dam and a sire, both carpet pythons, were both PCR-positive for the presence of Sunshine virus at a time near to when the dam would be expected to have been gravid. A clutch of 21 eggs was laid and three non-viable eggs were tested for the presence of this virus by PCR. Sunshine virus was detected in extra-embryonic membranes (allantois and amnion) and embryonic tissues but not on the surface of each egg. Hatchlings from this clutch were PCR-negative when tested at the ages of 53, 74 and/or 227 days old. It is unknown whether the hatchlings were infected prior to PCR testing or whether they hatched uninfected.

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8. Figure Captions

Figure 1. Timeline of parent pairings, oviposition and PCR testing for Sunshine virus of eggs, hatchlings and parents. All numbers are the days before (-) or following oviposition except h.a. = hatchling age. The dam (in box, left) and sire (in box, right) were in breeding contact for approximately one and a half months during the three months from days -147 to -57. Neither snake was PCR tested during this time (unfilled images). On day -55, the dam (above) was PCR-positive (image coloured red) and on day -57, the sire (below) was PCR-positive (red). A clutch of 21 eggs was laid on day zero. On days 34 and 49 of incubation, three eggs were PCR-positive (red). Fourteen of the 21 eggs hatched. On day 112 (hatchling age = 53 days), three of the 14 hatchlings were PCR-negative (image coloured green). On day 133 (hatchling age = 74 days), the dam (above) was PCR-negative (green), the sire (below) was PCR-positive (red) and 13 of the 14 hatchlings were PCR-negative (green). The fourteenth hatchling was not tested. On day 288 (hatchling age = 229 days), 11 of the 11 remaining hatchlings were PCR-negative (green). Between days 133 and 288 (hatchling ages 74 to 229), three hatchlings were either lost or were killed by the keeper's domestic cat. Unfilled images represent animals/eggs that were not tested for the presence of Sunshine virus. Images filled in red or green represent animals that were tested and were PCR-positive or PCR-negative, respectively.

543 Figure 2. Swabbing the surface (A) and allantois (B) of a non-viable
544 egg from a carpet python, which 55 days prior to oviposition, was
545 PCR-positive for Sunshine virus. Following the removal of the
546 embryo (C), the amnion (D) was swabbed.

Figure 1

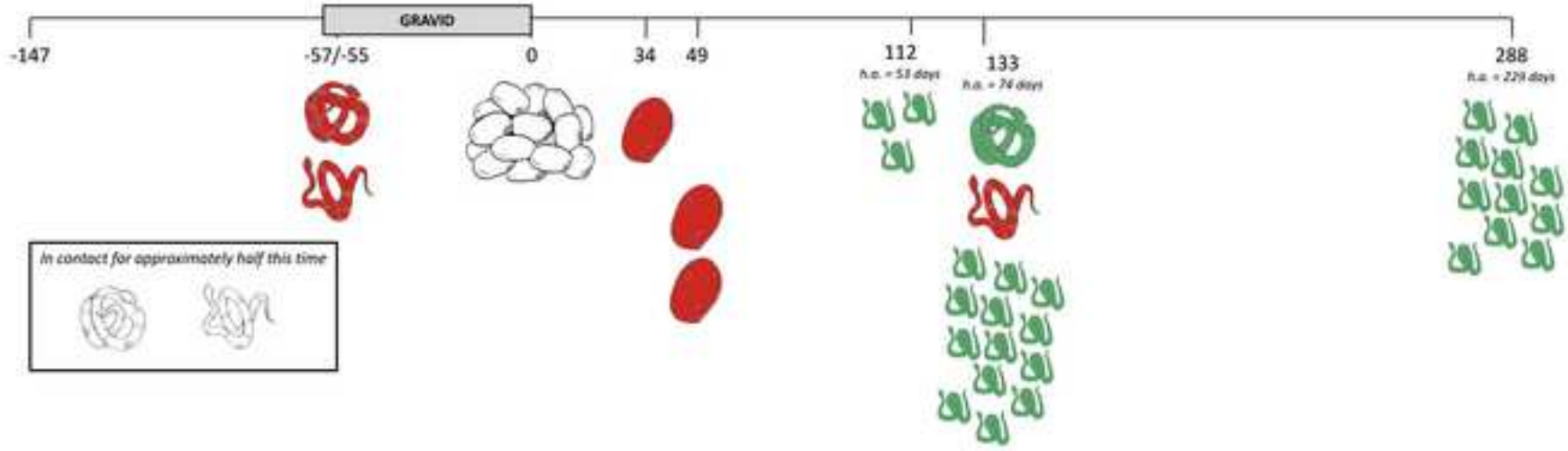

Figure 2a

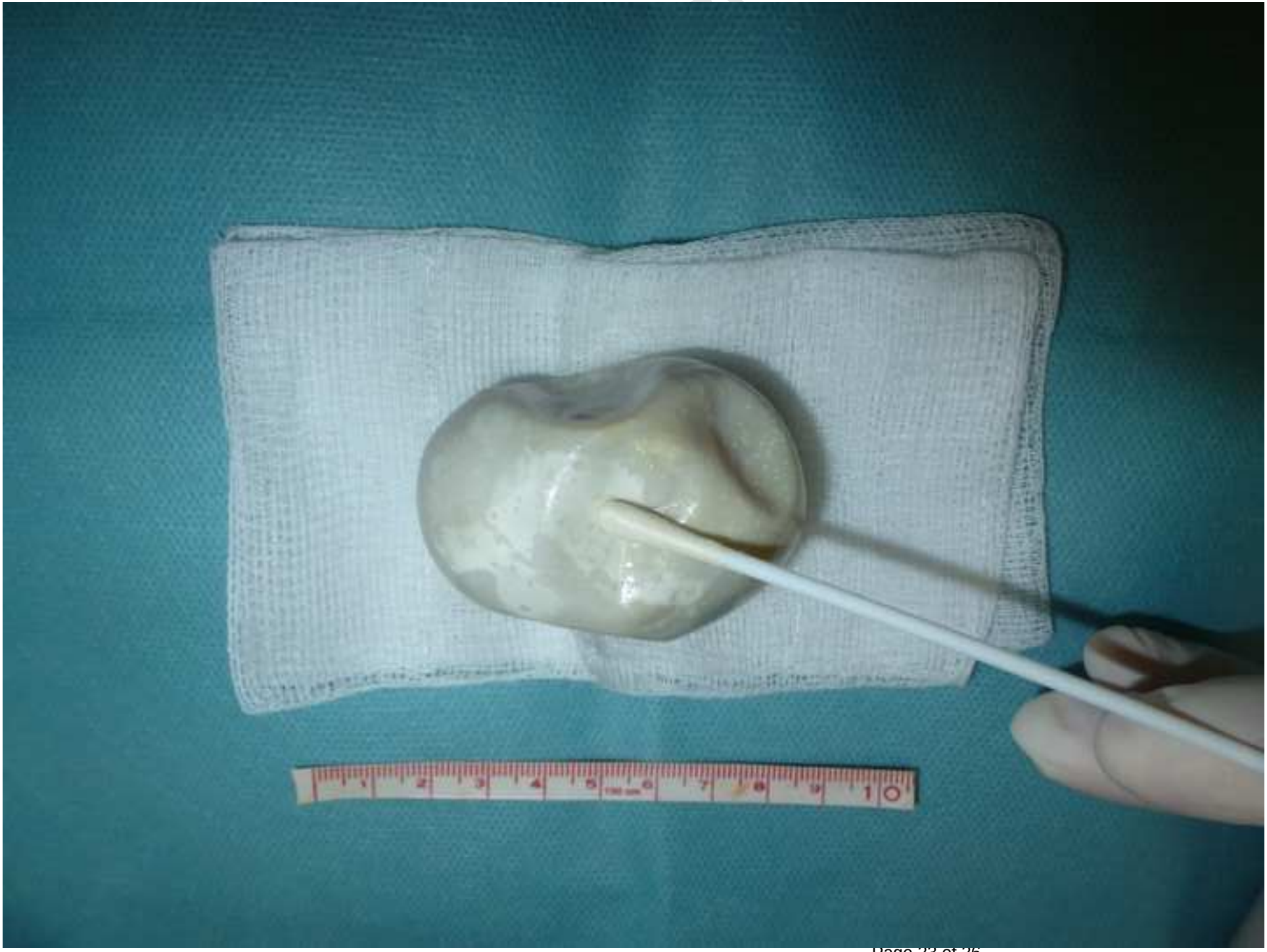

Figure 2b

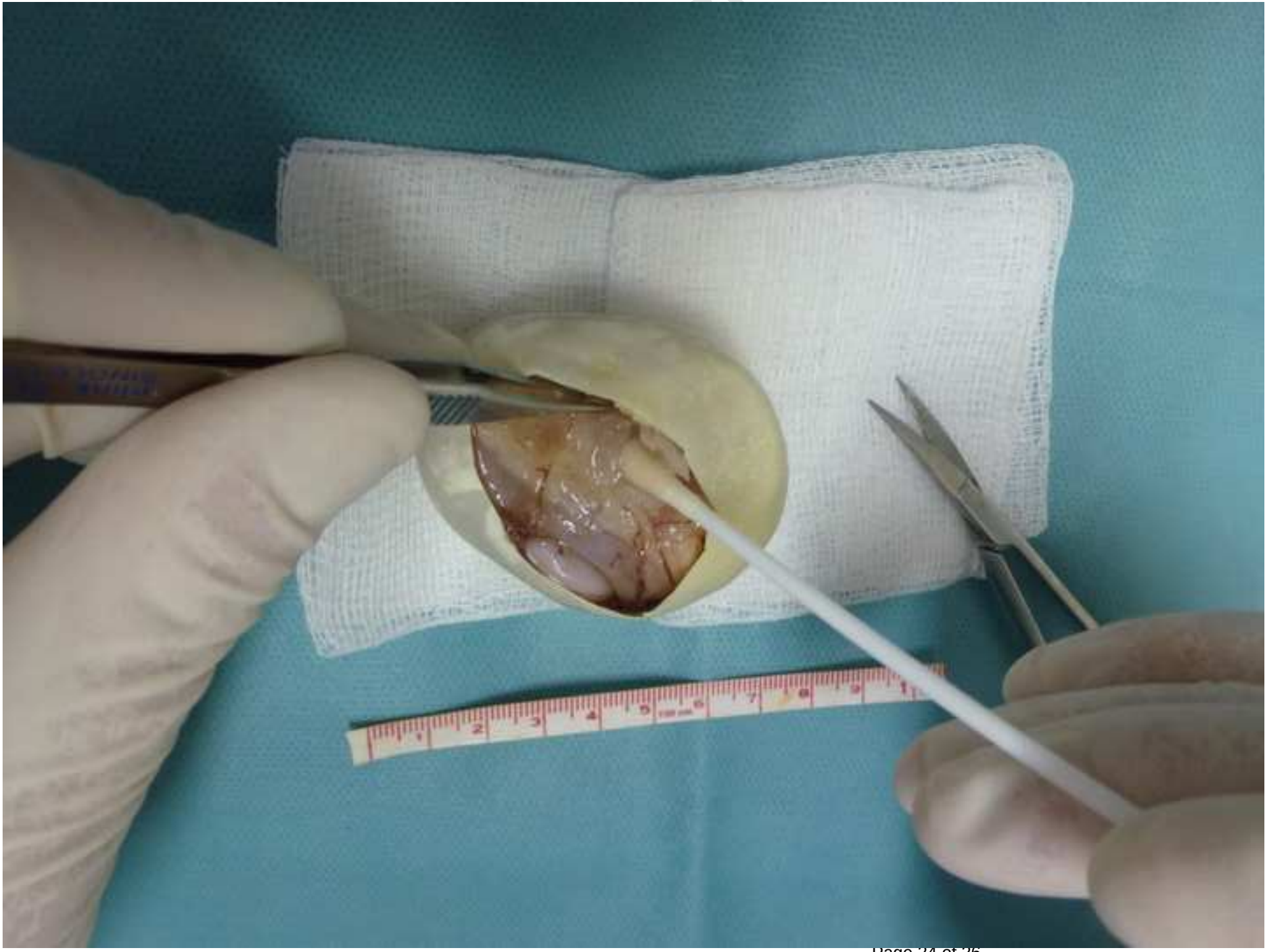

Figure 2c

Figure 2d