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Vaginal Calculi in the Eastern North Atlantic Common Dolphins Delphinus Delphis, Induction Mechanisms and Possible Effects on Fecundity

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The proper management of cetacean populations requires life history and demographic parameters to be estimated at population level. In this study we focus on a reproductive pathology that has the potential to alter reproductive rate: the vaginal calculi or stones. The present work documents vaginal calculi prevalence and structure in the eastern North Atlantic common dolphin *Delphinus delphis* in order to infer their likely mechanisms of induction and possible effects on fecundity. The work is based on routine examinations and necropsies of stranded marine mammals reported by the French stranding scheme from 1972 to 2012. Vaginal calculi were described and measured, and their composition was analyzed by Fourrier-Transformed Infra-Red (FTIR) spectroscopy. Necropsies and reproductive tract examinations were performed on 435 female common dolphins since 1972 along the French coasts, of which 14 showed vaginal calculi, representing 3.2% of the examined females. All females with *calculi* were older than 7, and there was no relationship of *calculus* size with age. Histopathology revealed lesions due to an inflammatory response to the presence of the *calculi*: chronicle vaginitis, variable endometritis and cystitis. Calculus size varied from 1 to 21 cm in maximum dimension and 4–1,460 g in mass. Their internal structure was homogeneous, particularly due to the absence of core material, hence corresponding to the definition of primary *calculi*. All *calculus* spectra showed almost identical compositions, with struvite (magnesium ammonium phosphate hexahydrate) representing on average 87% of *calculus* mass. Dysfunction of the uro-genital tract, such as vesico-vaginal fistulae, would be the likely initial pathological condition that led to the formation of these stones. Both the initial chemical condition in the vagina and the resulting formation of a *calculi* are obstacles to successful reproduction.

Keywords: pathology, fecundity, stranding, common dolphin, demographic, spectroscopy

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INTRODUCTION

The proper management of cetacean populations requires life history and demographic parameters to be estimated at population level. In addition to reproductive parameters *sensu stricto*, pathologies of the genital tract can be relevant as well, in that they can be associated with sterility (Boyd et al., 1999). In this study we focus on a reproductive pathology that has the potential to alter reproductive rate: the vaginal calculi or vaginal stones.

Vaginal stones have been well documented in mammals like Guinea pigs, moles, bats and primates, including man (Harrison, 1969; Raghavaiah and Devi, 1980; Oguzkurt et al., 2009). In human medicine, 82% of vaginal calculi are due to fistulas resulting from gynecologic surgery, 8% to obstetric procedures, 6% to pelvic radiotherapy, and 4% to trauma (Lee et al., 1988; Singhal et al., 2007). The general conditions described as contributing to *calculi* induction are cystic lesions due to an obstructed genital tract or to the presence of embryological remnants. Reflux of urine into the vagina during voiding may mimic a cystic lesion. Solid lesions are uncommon and are usually fibroids or neurofibromas. Malignant lesions usually occur in elderly patients and account for only 1–2% of gynecological malignancies. Most malignancies are usually squamous cell carcinomas, although adenocarcinomas and melanomas do occur. Younger patients may get rhabdomyosarcoma (Allan et al., 2011).

These stones may be primary or secondary. Primary stones are formed in the vagina due to the deposition of urinary salts as a result of continuous urinary leakage into the vagina resulting from vesicovaginal fistulae and concomitant bacterial infections (Hildebrand, 1987). The internal structure of primary stones is homogeneous without foreign bodies in the nucleus, and its section appears smooth. The structure is mainly made of struvite (magnesium-ammonium-phosphate). A secondary vaginal stone is formed around a foreign body in the vagina and results from the reaction of the organism involving hormonal and calcium mobilization (Warner et al., 1979). Secondary stones are extremely polymorphic and show internal organization with a nucleus composed of foreign bodies secondarily embedded in crystals that are similar in composition to the apatite of dental *calculus*. Both primary and secondary vaginal *calculi* can occur as single or multiple stones.

Vaginal *calculi* are reported to alter reproductive parameters. Firstly, *calculi* over 20 ml would induce mating failure in dolphins by reproductive tract occlusion (Essapian, 1961). Secondly, the chemical environment required for crystal formation would also lead to sterility (McFee and Osborne, 2004). However, this harmful chemical environment is only permanent in the case of physiological dysfunction associated with primary stones, whereas these conditions stop in the case of secondary stones when the foreign bodies are expulsed or isolated.

Many cases of vaginal stones were reported in marine mammals. Several delphinid species have been reported to display vaginal stones: the dusky *Lagenorhynchus obscursus* (Van Bressem et al., 2000), Pacific white-sided *L. obliquidens* (Harrison, 1969), pan-tropical spotted *Stenella attenuata* (Sawyer and Walker, 1977), bottlenose *Tursiops truncatus* (McFee and Osborne, 2004), and common *Delphinus delphis* (Sawyer and Walker, 1977; Bernirschke et al., 1984; Woodhouse and Rennie, 1991) dolphins. However, the prevalence of this condition and its potential effect on reproductive outputs at population level are unknown.

The goal of the present work was to address the issue of vaginal calculi prevalence and structure and infer their likely mechanisms of induction and possible effects on fecundity. To do this, we described the prevalence of this pathological condition in the eastern North Atlantic common dolphin and its relation with age and reproductive status. Besides, we analyzed composition of *calculi* by using Fourrier Transformed Infra-Red (FTIR) spectroscopy. In small cetaceans, the examination of stranding events represents a major source of samples from which health condition and reproductive parameters can be estimated. The present work is based on routine examinations and necropsies of stranded marine mammals reported by the French stranding scheme from 1972 to 2012. In a second step, all *calculi* found during necropsies were described in terms of size, internal structure and chemical composition.

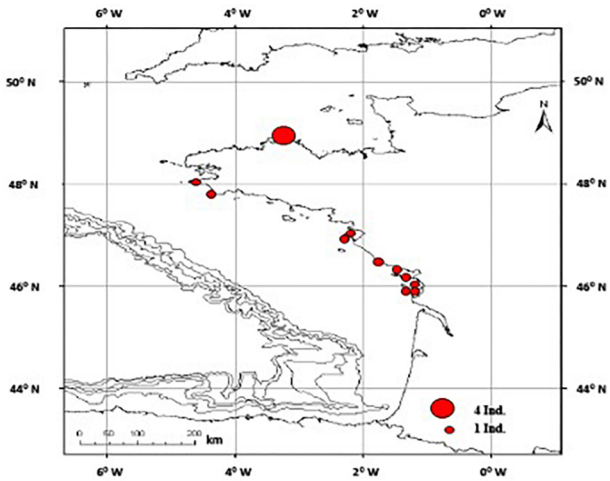

FIGURE 1 | Location map of sampled female common dolphin with vaginal calculi (n = 14).

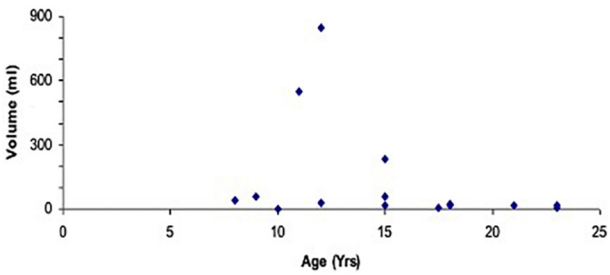

FIGURE 2 | Vaginal *calculus* size as a function of dolphin age (n = 14).

FIGURE 3 | Largest vaginal calculi observed (collection number 10202178).

TABLE 1 | Veterinary and histopathology appearance (*n* = 14).

Calculus #	Stranding Date	Age	Nature	Diameters			Mass (g)	Volume (ml)
				D1 (cm)	D2 (cm)	D3 (cm)		
1675	13/02/84	10	Single	2.6	2.1	1.2	4	2
1760	14/04/85	17,5	Single	4.3	3.1	1.9	12	8
10202157	19/02/02	21	Single	5.9	4.2	1.5	21	16
1265	07/08/81	24	Multiple*	4.8	1.9	1.6	7	4
				5.2	3.2	1.9	24	16
1630	25/01/84	15	Single	6.7	4	1.3	28	18
1797	24/01/86	18	Multiple**	–	–	–	31	19
1709	26/11/84	15	Single	6.2	4.2	2.1	37	25
2131	06/01/89	15	Single	7	5.3	3.4	44	28
10202165	19/02/02	8	Single	6.4	4	2.6	41	42
1600	11/09/85	12	Single	8.9	4.8	3.9	88	61
1807	03/03/86	9	Single	8.9	4.8	3.9	50	61
9604023	16/04/96	24	Single	11.8	7.0	6.1	348	236
10202169	19/02/02	11	Single	16.8	8.7	7.5	875	551
10202178	19/02/02	12	Single	21.1	9.4	8.1	1,460	845

2 calculus—multiple calculus (n = 22), very polymorphic of less than 1cm3 each.*

MATERIALS AND METHODS

Origin of the Samples

Biological material was made available by the French stranding scheme which monitors and examines marine mammal strandings since the early 1970's. The network is composed of field correspondents trained to use standard examination protocols (Kuiken and Hartmann, 1991; Jauniaux et al., 2005), under supervision by *Observatoire PELAGIS*, at the University of La Rochelle. Yearly reports of stranding events are made available online at http://observatoire-pelagis.cnrs.fr/publications/rapports/.

Age Determination

Tooth preparation was adapted from the protocol described by Lockyer (1995). Teeth were immersed in a decalcifying agent (DC3© Labonord, Z.I. de Templemars, f-59175 Templemars, France) before sectioning and staining in toluidin blue (Martoja and Martoja, 1967) and then washed overnight in running tap water. Sections were mounted in a synthetic medium (Isomount Labonord®) and examined under a stereomicroscope with transmitted polarized light (Olympus® BH2, Olympus Optical Co., Ltd.). Growth layer groups (glg) were counted, assuming that one glg equals one year (Gurevich et al., 1980; Perrin and Myrick, 1980; Klevezal, 1996). Three independent readings were done for each tooth section (Hohn and Fernandez, 1999). All readings were recorded to the nearest whole year and age was expressed as mean for each individual.

Individual Reproductive Status and Ovarian Characteristics

Individual reproductive status was determined by the examination of the entire reproductive tracts on the basis of the criteria described and illustrated by Collet and Harrison (1981). All *Corpus Albicans* (CAs) and *Corpus Luteum* (CLs) were counted and measured in the entire ovaries (Perrin and Donovan, 1984). Females were categorized as "immature" when they had no CA, as "resting mature" when they had at least one CA and showed no sign of gestation or lactation (no CL, no fetus, no milk in the mammary gland, no expanded uterine horn), as "pregnant" when a CL was present and a fetus was found, as "lactating" when milk was found in mammary gland. Vaginal tissue was fixed in buffered formaline and processed for histopathological assessment and *calculi* were collected and dry stored (Perrin and Donovan, 1984).

Calculus Morphometrics

Firstly, *calculi* were measured (Mitutoyo CD-15DC; 0.01 mm), weighed (Precisa 300C; 0.01 g) and their volume determined by water displacement in a graduated cylinder. Secondly, shape,

TABLE 2 | Morphometric measurements (mm) and microscopic observations (*n* = 14).

Calculus collection number	Left ovary scars	Right ovary scars	Uterin horn	Endometrial	Vagina	Bladder	Cyst location
1265	14	4	Edematous stroma	–	Chronic vaginitis	–	Uterin horne
1600	0	1	Chronicle endometritis	Edematous propria	Chronic vaginitis		–
1630	15	4	–	Chronicle endometritis	Chronic vaginitis	Cyst	–
1675	–	–	–	–	–	–	–
1709	3	0	Endometritis	Superficial chronicle endometritis	Acute vaginitis	Cyst	Left ovary surface
1760	7–9	13	Endometritis	Chronicle endometritis	Chronic vaginitis	–	–
1797	13	8	–	–	Chronic vaginitis	–	–
1807	15	10	Moderate endometritis	–	Chronic vaginitis	–	–
2131	–	–	–	–	–	–	–
9604023	–	–	Mild endometritis	Mild endometritis	Chronic vaginitis	–	–
10202157	14	4	Mild endometritis	Mild endometritis	Chronic vaginitis	–	Left ovary
10202165	13	7	Mild endometritis	Mild endometritis	Chronic vaginitis	–	Left ovary
10202169	-	3	Mild endometritis	Mild endometritis	Chronic vaginitis	–	Left ovary
10202178	22	4	Endometritis	Endometritis	Chronic vaginitis	–	Left ovary

surface aspect and sections were described both macroscopically and microscopically. Polarized microscope was used in order to describe crystal structures (Olympus BH2, Olympus® Optical Co., Ltd.).

Chemical Composition

The composition of each *calculus* was analyzed by Fourier Transformed InfraRed spectroscopy (FTIR). This technique is based on the absorption of infrared radiation by *calculi* powder added with potassium bromure matrix in excess. Every individual spectrum was compared with reference spectra to identify the main components (Maurice-Estepa et al., 2000). The quantitative composition of each individual female spectrum was calculated from surface area of peaks (Kuzmanovski et al., 2003).

RESULTS

General

Necropsies and reproductive tract examinations were performed on 435 female common dolphins since 1972 along the French coasts, of which 14 showed vaginal *calculi,* representing 3.2% of the examined females. They were found along the coasts of the Bay of Biscay (n = 10) and the western Channel coasts (n = 4) (**Figure 1**). As many as 12 of these individuals were reported during the winter to early spring (from January to April).

Only one *calculus* was present at a time in most cases except in three females where multiple stones were found. All females with *calculi* were older than 7, and there was no relationship between *calculus* volume and age (**Figure 2**). Smaller *calculi* were rather polymorphic in shape, but larger ones all tended to develop an ellipsoid shape (**Figure 3**).

Reproductive Tract Examination and Histopathology

The reproductive tract examinations revealed that all females with vaginal *calculi* were resting mature individuals as they all showed ripe follicles or CA on the ovaries (**Table 1**). None of these females were pregnant, but ten of the eleven females for which ovaries were examined showed active CL suggesting that ovulation had occurred recently. CA counts were about twice higher on the left ovary (10.6 ± 5.3) than on the right one (5.7 ± 4.0). Histopathology revealed lesions due to an inflammatory response to the presence of the *calculi*: chronic vaginitis, variable endometritis and cystitis (**Table 1**). Finally, none of the females were lactating.

Description of *Calculi*

The morphometric analysis shows a great variability in *calculus* size (1–21 cm maximum dimension) and mass (4–1,460 g; **Table 2**). Microscopically, all *calculi* had a smooth surface and showed no specific internal organization particularly due to the absence of core material.

Identification of Main Components by FTIR Spectroscopy

The 14 vaginal *calculi* were analyzed by FTIR spectroscopy. All *calculus* spectra (e.g., **Figure 4**) showed almost identical compositions. All *calculi* were composed of struvite (magnesium ammonium phosphate hexahydrate), calcium carbonate, whewellite, ammonium, and phosphate (**Table 3**). Struvite represented on average 87% of *calculus* mass (**Table 3**).

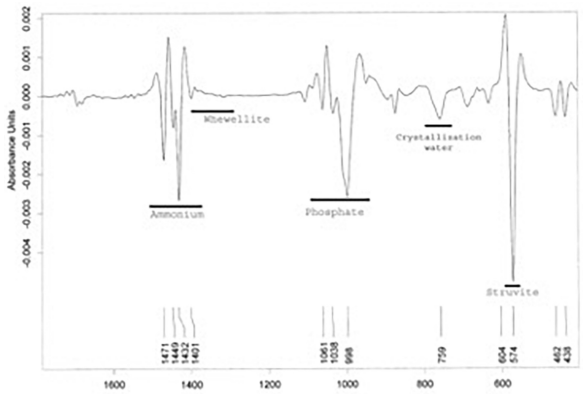

FIGURE 4 | Exemplified spectrum analysis by Fourrier Transformed Infra-Red (FTIR) spectroscopy with main component identified.

TABLE 3 | Component proportion of Vaginal calculi as revealed by FTIR spectroscopy (n = 14).

Components	Occurrence (n = 14)	Average crystal%	Range
Struvite	14	87.7	84.5–90.9
Dittmarite	8	6.3	4.6–8
Proteins	14	3.6	3.1–4.1
Carbapatite	14	3.3	2.3–4.3
Whewellite	11	1.4	0.9–1.9
Weddellite	3	1	–
Urate acid ammonium	3	1	–

DISCUSSION

Vaginal *calculi* were only found in resting mature females with otherwise apparently functional reproductive tracts. This cannot be attributed to insufficient scrutiny during examination, because from January to April, the period when most of the sample set came from, pregnant females normally show large fetuses that cannot readily be overlooked at necropsy.

Furthermore ten out of eleven of the females examined for reproductive status had a CL on one ovary, showing that an ovulation took place recently. This proportion of ovulating females is much higher than the average figure previously reported for the Bay of Biscay population of common dolphins (39% of mature females have a CL on one ovary, Dabin et al., 2008) and would suggest that females with vaginal *calculi* had higher ovulation rates than mature females with no vaginal stone, presumably because they can neither become pregnant nor lactate and therefore would stay in hormonal conditions favorable to repeated ovulation.

Vaginal *calculi* were extremely variable in size inrespective of the individual's age. Their internal structure was homogeneous with no core material. All *calculi* had a similar composition

mainly made of struvite. They all can be considered as primary vaginal stones as defined by Hildebrand (1987).

The chemical conditions associated with *calculus* crystallization can be inferred from the compositions revealed by FTIR spectroscopy. High ammoniac concentration, alkaline urine, supersaturated urine with important concentration of magnesium ammonium phosphate are the likely conditions for the formation of these primary *calculus* (Warner et al., 1979). Continuous urinary leakage into the vagina caused by vesico-vaginal cysts and fistulae, associated with a bacteriological infection with ureasic bacteria resulting in increased ammonium concentration in the vagina are the initial pathological conditions required for struvite *calculi* to form (Hildebrand, 1987).

In a previous case in captivity, calculi were detected using echography and obstructed successful copulation (Essapian, 1961). These calculi were smaller than the calculi found in the common dolphin described here. Therefore, comparing to Essapian's findings, 12 individuals of the present study would be physically unable to successfully copulate as a result of genital tract obstruction. In addition to this mechanical effect on reproduction, fistulae and chemical conditions required for *calculi* crystallization would also be sufficient to cause sterility even before any *calculus* would eventually develop (McFee and Osborne, 2004).

In the present common dolphin sample set, 3.2% of all examined females had vaginal stones. Calculi were only found in mature females. Considering that the chemical environment leading to the formation of vaginal stones would prevent fertilization even before stone formation, this figure is likely an underestimation of the proportion of females made infertile because of this initial dysfunctional condition of the urogenital tract. A higher ovulation rate in females with *calculi* suggested by the higher proportion of females with a CL would result from their inability to become pregnant and thereafter lactating.

A closer systematic examination of uro-genital tract should be conducted on all stranded common dolphin females, notably to look at possible vesico-vaginal fistulae even in the absence of fully developed vaginal stones, in order to better estimate the proportion of infertile females among the adult population.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because this work comes from the exploitation of dead stranded animals.

AUTHOR CONTRIBUTIONS

WD: French stranding network manager who discovered most of the vaginal stones in stranded common dolphin necropsy. BR: technician student, who participated in calculi analysis during an internship. MD: doctor of the Necker Hospital, specialty of vaginal stones in women. VR: observatory director and article supervisor. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.