



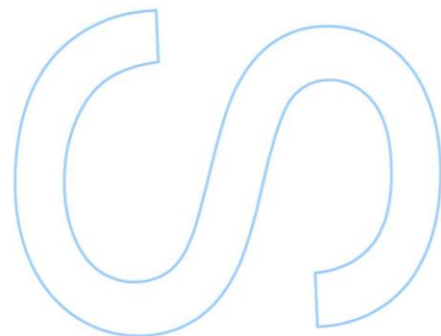
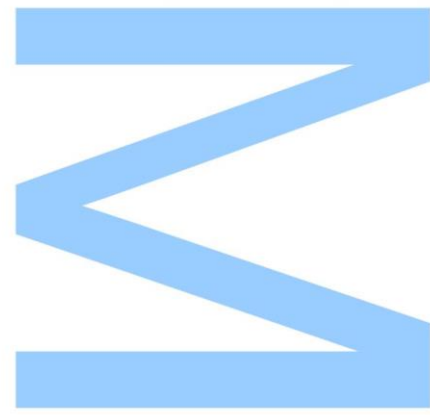
Worm pipefish endocranial volume along a natural gradient of sexual selection intensity

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Orientador

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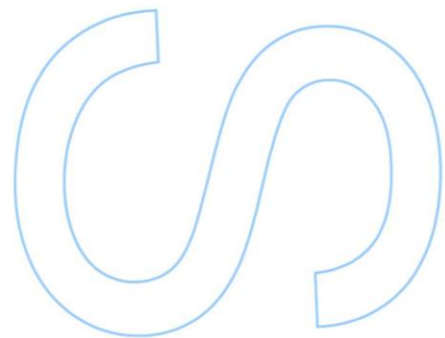
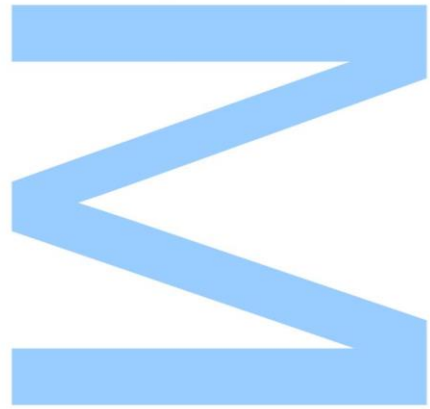




Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, ____ / ____ / ____



To my grandfather, Manuel.

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Resumo

Dentro da distribuição geográfica de uma espécie, as populações experimentam diferentes forças seletivas, uma vez que estão expostas a diferentes ambientes. No entanto, não existem muitas informações sobre os efeitos da seleção sexual. Em *Nerophis lumbriciformis*, uma distribuição parabólica da intensidade da seleção sexual foi recentemente descrita na sua ampla distribuição biogeográfica e, em regimes de temperatura que se aproximam dos limites fisiológicos da espécie, há sinais de aumento da seleção sexual. Ainda, um estudo recente dentro da família *Syngnathidae* apoia a ideia de que a seleção sexual pode desempenhar um papel importante na evolução do tamanho do cérebro.

Tendo em consideração estes dados, quisemos testar, ao nível intraespecífico, se padrões semelhantes ocorrem com a espécie *N. lumbriciformis*. Neste estudo, a tecnologia microCt é utilizada, e o nosso principal objetivo é detetar se a seleção sexual é capaz de promover dimorfismo sexual no tamanho do cérebro (através da medição do volume endocraniano como um *proxy* alternativo para o volume do cérebro), especialmente em populações onde se sabe que a seleção sexual é mais intensa. Além disso, quisemos detetar nessas populações se os machos ou as fêmeas apresentam cérebros maiores. Medimos também os otólitos lapillus uma vez que o seu crescimento é influenciado pelas condições ambientais.

Em relação ao nosso objetivo principal, não fomos capazes de detetar diferenças no tamanho do cérebro, utilizando o tamanho corporal como co-variável, entre as três populações amostradas e / ou entre os sexos. Tentamos utilizar outras co-variáveis como tamanho da cabeça, focinho e abdómen, mas os resultados mostraram sempre semelhança entre as populações amostradas e entre os dois sexos. Também não encontramos diferenças significativas quando se analisou o tamanho dos otólitos, após o ajuste com o tamanho corporal. Esta ausência de diferenças pode, em parte, ser devida ao número de indivíduos analisados, mas também pode derivar de determinadas características da história vital de *N. lumbriciformis*, nomeadamente o período de dispersão larvar (larvas pelágias) que impede a estruturação populacional característica de muitas espécies de *Syngnathidae*.

De modo geral, aqui tentámos mostrar como, com auxílio de microCt, é possível explorar mesmo as mais crípticas das características sexualmente selecionadas de uma espécie, como o cérebro, e tentar medir a influência que o processo de seleção sexual tem sobre elas.

Palavras-chave: *Syngnathidae*; *Nerophis lumbriciformis*; seleção sexual; dimorfismo sexual; tamanho do cérebro; microCT, otólitos.

Abstract

Within a species' geographic distribution, populations experience different selective forces since they are exposed to different environments however, it doesn't exist much information regarding the effects of sexual selection. In the pipefish *Nerophis lumbriciformis*, a parabolic distribution of sexual selection intensity was recently described across its wide biogeographical distribution and, in temperature regimes that approach the physiological limits of the species, there are signs of increased sexual selection. Moreover, a recent study within the *Syngnathidae* family supports the idea that sexual selection might play an important role in brain size evolution.

Taking into account these data, we want to test, at the intraspecific level, if similar patterns occur with *N. lumbriciformis*. In this study microCt technology is used, and our primary goal is to detect if sexual selection is able to promote brain size sexual dimorphism (by measuring endocranial volume as an alternative proxy for brain volume), especially in populations where sexual selection is known to be intensified. Furthermore, we want to detect in those populations, if either males or females display the larger brains. As a secondary goal, we will measure lapillus otoliths as their growth is influenced by environmental conditions.

Regarding our main objective, we were unable to detect differences in brain size, using body size as a covariate, between the three sampled populations and/or between sexes. We tried to use other covariables such as head, snout and abdomen size, but the results always showed a similarity between the sampled populations and between the two sexes. We also found no significant differences when analysing the size of the otoliths, after adjusting for body size. This absence of differences may, in part, be due to the number of individuals analysed, but it can also derive from certain characteristics of the vital history of *N. lumbriciformis*, namely the period of larval dispersion (pelagic larvae) that prevents the population structure characteristic of many species of *Syngnathidae*.

Overall, here we tried to show how, with the aid of microCt, it is possible to explore even the most cryptic of the sexually selected characteristics of a species, such as the brain, and try to measure the influence that the process of sexual selection has on them.

Keywords: *Syngnathidae*; *Nerophis lumbriciformis*; sexual selection; sexual dimorphism; brain size; microCT, otolith.

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List of Abbreviations

μ CT, microCT – Micro-computed tomography

VOI – Volume Of Interest

ROI – Region Of Interest

ANCOVA - Analysis Of Covariance

LT – Total Length

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1. Introduction

The climate is changing, our planet is rapidly heating, and organisms are being constantly challenged to adapt to these shifting conditions (Vitousek, 1994; Santos *et al.*, 2018). As a result, phenology and population dynamics are being affected in all studied marine, freshwater and terrestrial groups (Hughes, 2000; Root *et al.*, 2003; Rajon & Plotkin, 2013;). In response to ongoing changes, in order to avoid local extinctions, animals can disperse or adapt, either through phenotypic plasticity or genetic changes (Hoffmann & Sgrò, 2011; Williams *et al.* 2008). In fact, according to a study on marine fish conducted by Perry *et al.* (2005), climate change is having visible impacts on species distributions. Also, many evidences confirm that a wide diversity of taxa already experienced heritable changes as a result of climate shifts (Barrett *et al.*, 2011).

Within a species' geographic distribution, populations experience different selective forces since they are exposed to different environments (Cunha *et al.*, 2019). Surprisingly, most of the available information regarding species adaptation and survival under changing conditions commonly revolves around natural selection, often neglecting to address the potential effects of sexual selection (Candolin & Heuschele, 2008). Sexual selection, a process by which individuals compete for access to mates and fertilization opportunities (Andersson, 1994), can be a relevant determinant of population viability, impacting its ability to cope with environmental change. For instance, a process of non-random mating, where specific phenotypes consistently display higher fitness, can potentially lead to genome alterations, possibly contributing to purge deleterious mutations (Cally *et al.*, 2019), thus leading to an increased protection against extinction. However, the role of sexual selection in adaptation remains disputed as the few available studies show conflicting evidence (Chenoweth *et al.*, 2015). These contradictions reveal the youth of this field of study.

There is at least one characteristic about sexual selection that seems undisputed: it is a dynamic process able to vary temporally or geographically (Boughman, 2001; Mobley & Jones, 2009; Monteiro *et al.*, 2017b). As an example, Monteiro *et al.* (2017b) recently showed in the pipefish *Nerophis lumbriciformis*, that sexual selection intensity follows a parabolic variation along a latitudinal and temperature cline. Populations inhabiting locations towards both edges of distribution (north and south), where temperature regimes approach the physiological limits (cold and hot) for the species, showed parallel signs of increased sexual selection. Namely, the competing sex displayed heightened levels of expression of secondary sexual characters (higher facial coloration) and increased investment in reproduction (more and larger eggs). Moreover, near the southern limit of distribution, a unique mating system (an inverted lek) was

revealed in the worm pipefish (Monteiro *et al.*, 2017a), an observation that perfectly matches the high sexual selection intensity hypothesized for a population located near the edge of distribution. Lek mating systems (Höglund & Alatalo, 1995), where competing individuals gather in particular spots in order to be chosen by the opposite sex, are known to cause strong directional selection imposed by mate choice.



Figure 1 – *Nerophis lumbriciformis* female (A) and female (B) specimens where it is possible to observe coloration patterns, mostly in the head region.

In the worm pipefish (**Figure 1**), females with higher coloration display heavier gonads, and can produce fuller broods of larger eggs (Monteiro *et al.*, 2017a). Thus, in order to increase their fitness, males should be able to navigate to the mating areas located in the intertidal area (Monteiro *et al.*, 2006), find female aggregations and then appropriately choose the more coloured females (Monteiro *et al.*, 2017a). Simultaneously, females should also be able to find the mating arenas, compete with their rivals and secure the most rewarding position that could increase their chances of being selected by a male (Monteiro *et al.*, 2017a). One might wonder then if, especially

in areas where sexual selection is intensified, besides an escalation in the expression of secondary sexual characters (in the competing sex – females, in the case of the worm pipefish), we could also find an increase in the cognitive abilities of either the choosier sex (males), the competing sex (females), or both.

Recently, Tsuboi *et al.* (2017) uncovered a female-biased sexual dimorphism pattern in brain size within the *Syngnathidae* family (seahorses, pipefish and seadragons). Females of polyandrous pipefish, subject to higher sexual selection pressures, displayed larger than expected brains when compared to those of polygynandrous and monogamous syngnathids. Males seemed to follow the opposite trend. These fascinating results, obtained at the interspecific level, were interpreted as supporting the idea that sexual selection might indeed play an important role in brain size evolution (Tsuboi *et al.*, 2017).

Given the described latitudinal/temperature parabolic variation in sexual selection intensity described in the worm pipefish (Monteiro, Cunha, *et al.*, 2017), we believe that we could test, at the intraspecific level, if similar patterns are present when contrasting populations scattered along the species distribution. We hypothesize that, if sexual selection does indeed support alterations in brain size, we should be able to detect them by contrasting extant worm pipefish populations experiencing distinct sexual selection pressures. Specifically, following the results of Tsuboi *et al.* (2017), we expect to find increased female-biased brain size dimorphism in populations inhabiting areas near the edges of distribution. Alternatively, if male mate choice requires increased cognitive abilities than those supporting female-female competition processes, we could expect the inverse scenario, with a male-biased brain size dimorphism. In either case, if the results mimicked the parabolic distribution uncovered by Monteiro *et al.*, 2017b, then the most probable cause supporting brain size dimorphism would be sexual selection.

To directly measure brain size variation along the worm pipefish distribution, one would need access to fresh specimens, either for immediate brain dissection or for appropriate fixation (e.g. paraformaldehyde) that could allow subsequent processing. Dissection of very small organs, such as pipefish brains, is often a complex task that requires special care not to alter the brain morphological structure. One alternative, used by Tsuboi *et al.* (2017), would be to measure brain volume indirectly, using brain weight as a reliable proxy. Another alternative (among many others) would be to measure endocranial volume as an alternative proxy for brain volume. The use of endocranial volume as proxy for brain size is not a novel approach and has been previously tested in vertebrates such as birds (Iwaniuk & Nelson, 2002) or primates (Isler *et al.*, 2008). This approach would allow for the use of previously sampled specimens, without much caution regarding the type of fixation process (the cranium, a bony structure, remains

unaffected when specimens are conserved with either ethanol or paraformaldehyde, the two most commonly used fixing agents). Then, to avoid, as much as possible, any bias potentially introduced during the estimation of the endocranial volume, we would need to be able to accurately trace the intracranial surface in order to obtain an accurate calculation.

Three-dimensional representations of internal anatomical structures are often required to perform phenotypical analysis (comparative, functional, or ontogenetic), since it allows for the visualization of samples in their native state, with minimal or no pre-processing steps (Metscher, 2009). Micro-computed tomography (microCT), or X-ray microtomography (Metscher, 2009), is a powerful technique for the visualization and meticulous analysis of biological materials as it is a non-destructive technology which allows the creation of virtual slice images detailing the inside of the specimen, without damaging the biological material (du Plessis & Broeckhoven, 2019). MicroCT is mostly used for imaging but this technique can allow for more precise analysis of the 3D structure (du Plessis & Broeckhoven, 2019). Due to the differences in photon attenuation levels in each tissue type, this technique allows to distinguish soft from hard tissues (Ritman, 2004; Mizutani & Suzuki, 2012). Hard tissue, such as bone, that is constituted with calcium phosphate minerals, attenuates X-rays more intensely than other softer tissues, such as cartilage, nerves, blood vessels and muscles (Descamps *et al.*, 2014). Because soft tissues are mainly composed by elements such as carbon, hydrogen and oxygen (with low atomic numbers) and similar water content, their differentiation is difficult (Mizutani & Suzuki, 2012).

In this work, by using microCT, we primarily aim to detect if sexual selection is able to promote brain size sexual dimorphism in the worm pipefish, especially in populations where sexual selection is known to be intensified. Furthermore, we want to detect in those populations, if either males or females display the larger brains. As a secondary goal, given the ability to perform three-dimensional representations of specimens sampled from populations inhabiting distinct temperature regimes, we will measure otoliths (calcareous aggregations found in the inner ear of teleost fishes) as their growth is influenced by environmental conditions.

2. Material and Methods

2.1 Species biology

2.1.1 *Syngnathidae* family

The *Syngnathidae* family, which includes pipefish, seadragons, pipehorses and seahorses (Monteiro *et al.*, 2005b), is composed by 320 described species (Fricke *et al.*, 2020). All syngnathids share a remarkable common trait: male pregnancy (Wilson *et al.*, 2001; Stölting & Wilson, 2007). However, some species differ in the section of the body where males incubate the eggs, which can be located either in the abdomen (*Gastrophori*) or tail (*Urophori*) (Herald, 1959), as well as in the architecture of the brooding area. The species whose males possess a brood pouch (marsupium) provide oxygen and nutrients to the offspring through a placenta-like structure (Haresign & Schumway, 1981; Berglund *et al.*, 1986, Berglund, 2000, Monteiro *et al.*, 2005b). According to Dawson (1985), within the *Syngnathidae* family, the brooding structures vary in complexity in five steps, from: (1) a simple unprotected ventral area where eggs are attached, (2) individual membranous egg compartments, (3) protection of eggs in a pouch with pouch plates, (4) bilateral pouch folds that grow together into a closed pouch, to (5) the most complex and completely enclosed brooding pouch of seahorses (Wilson *et al.*, 2003). The process of fertilization and development of the embryos occurs in the interior of the pouch after the eggs are deposit by one or more than one female, depending on the species (Kornienko, 2001). Due to this particular mode of reproduction, syngnathids have become model organisms for the investigation of the sexual selection process (Clutton-Brock & Vincent, 1991; Kvarnemo & Ahnesjö, 2002; Berglund & Rosenqvist, 2003; Wilson *et al.*, 2003; Jones *et al.*, 2005).

In natural environments, several genetic mating systems have been described in syngnathids, such as monogamy in seahorses (Kvarnemo *et al.*, 2007), polyandry in more than one species of pipefish (McCoy *et al.*, 2001; Monteiro *et al.*, 2017a) and polygynandry in several species of pipefish (Jones & Avise, 2001; Monteiro *et al.*, 2015). Sexual selection intensity seems to be tightly linked with the genetic mating system since secondary sexual characteristics develop more frequently in females of the more polyandrous species (Jones & Avise, 2001).

Syngnathids are more often found in shallow coastal waters with some submerged vegetation, but also coral reefs (Cervigón, 1991). Their bodies are generally small (Ruiz *et al.*, 2000) and totally covered by bone plates that are joined, forming belts (rings) (Kornienko, 2001). Both seahorses and pipefishes are slow swimmers and cryptic predators (Berglund, 2000). Syngnathid snouts can have big differences in size and

shape and the adult bodies can also vary between 27,3 to 600 mm (Dawson, 1982; Short *et al.*, 2020). These fishes are carnivores (Cervigón, 1991) and efficient predators that ambush small preys (Manning *et al.*, 2019). They are successful due to their feeding technique which is often faster than the reaction time of their fastest preys (Gemmell *et al.*, 2013). This technique is called pivot feeding (Van Wassenbergh *et al.*, 2011) and preys, normally small crustaceans (Ryer & Orth, 1987; Tipton & Bell, 1988; Teixeira & Musick, 1995), are ambushed with a fast vertical 'flick' of the head that positions the mouth near to the prey and, simultaneously, the buccal cavity expands and creates an inhalant current (Bergert & Wainwright, 1997). Since syngnathids have no teeth, preys are swallowed whole (Kendrick & Hyndes, 2005).

2.1.2 *Nerophis lumbriciformis*

The species *Nerophis lumbriciformis* (Jennys, 1835) is a small and slim pipefish that can be found in the rocky intertidal to about 30 m, normally among seaweeds at high tide or underneath loose stones during the ebb tide (Monteiro *et al.*, 2001). The worm pipefish is distributed from the Atlantic coast of Norway to the Kattegat, from Belgium southwards to Morocco (Dawson, 1986) and may also occur in estuaries (Almeida, 1994). It is one of the smallest endemic western European pipefish (Monteiro *et al.*, 2005a) and it feeds on small crustaceans (copepods, amphipods and isopods), sucking and swallowing the preys through its distinguishing upwards pointing snout (Monteiro *et al.*, 2005a). *Nerophis lumbriciformis* presents no caudal, pectoral and pelvic fins, and there is no relief between dermic rings (Monteiro *et al.*, 2005a).

The worm pipefish is a sex-role reversed species (Monteiro *et al.*, 2003), where males are the choosier sex and females compete for male access. There is clear sexual dimorphism, with females attaining larger sizes, presenting more extensive coloration in the head and trunk, and being more active than males during courtship (Monteiro *et al.*, 2005b; Monteiro *et al.*, 2006). Besides, while females grow, a keel-like structure develops in the trunk, where a characteristic pattern of stripes amplifies during inter- or intra-sexual interactions (Monteiro & Lyons, 2012). Males present a more uniform coloration and their brooding area is in the abdomen, where a band of tightly compacted bright orange sticky eggs are deposited by females (Monteiro & Lyons, 2012).

In this species, females can mate with more than one male, however, a male receives eggs exclusively from one female (Monteiro & Lyons, 2012). According to a study conducted by Monteiro *et al.* (2017a), using the criteria established by Bradbury (1981), the worm pipefish mating system resembles to a lek and in this case, unusually, males choose females, being the first case registered in the marine environment. In lek mating

systems, organisms gather in specific locations with the single intention of reproducing (Monteiro *et al.*, 2017a).

The courtship process always occurs in close contact with the substratum (Monteiro *et al.*, 2002). The sequence of courtship and spawning in this species is divided in three main stages (initial courtship, spawning and embrace; Monteiro *et al.*, 2002). The initial courtship usually lasts for 30 minutes, and it's characterized by repeated occurrences of quivering by the female (Monteiro *et al.*, 2002). In the second phase, the female lays the eggs in long strings, with a mucilaginous layer, in the part that is in contact with the male's incubating ventral surface (Monteiro *et al.*, 2002). The last phase lasts between 10 to 15 minutes and it's characterized by the male embracing and wrapping his body around the female, with both sexes always staying in contact with the substratum (Monteiro *et al.*, 2002). This is a unique characteristic of this species since, in other syngnathids, both sexes tend to swim in the water column during courtship and egg transference.

Once the eggs are transferred, parental care is entirely paternal (Monteiro *et al.* 2002), with males carrying their offspring attached to their flattened ventral surface (Monteiro *et al.*, 2001). The eggs are directly in contact with the seawater, and when juveniles are born, after approximately 30 days (Monteiro & Lyons, 2012), no more care is provided (larvae are planktonic; Monteiro *et al.*, 2006). *N. lumbriciformis* larvae present a primordial marginal fin, a translucent coloration and fluctuate to find food near the surface (Monteiro *et al.*, 2003).

The genus *Nerophis* displays one of the simplest brooding structures within the family, probably similar to that of an ancestral syngnathid (Wilson *et al.*, 2001). This genus can help to comprehend the evolution of sex-role reversal and the relationship between the mating system and degree of sexual dimorphism (Monteiro *et al.*, 2006), since it belongs to one of the most basal lineages (Herald, 1959; Wilson *et al.*, 2001).

2.2 Sampling

Considering the parabolic distribution of the species that was studied by Monteiro *et al.* (2017b), we chose to sample three populations that represented the full extent of the species geographical distribution (**Figure 2**). Therefore, we selected a population located near the northern edge of distribution (Peterhead, Scotland), a population from the center of distribution (Roscoff, France) and a population from the south (Viana do Castelo, Portugal).

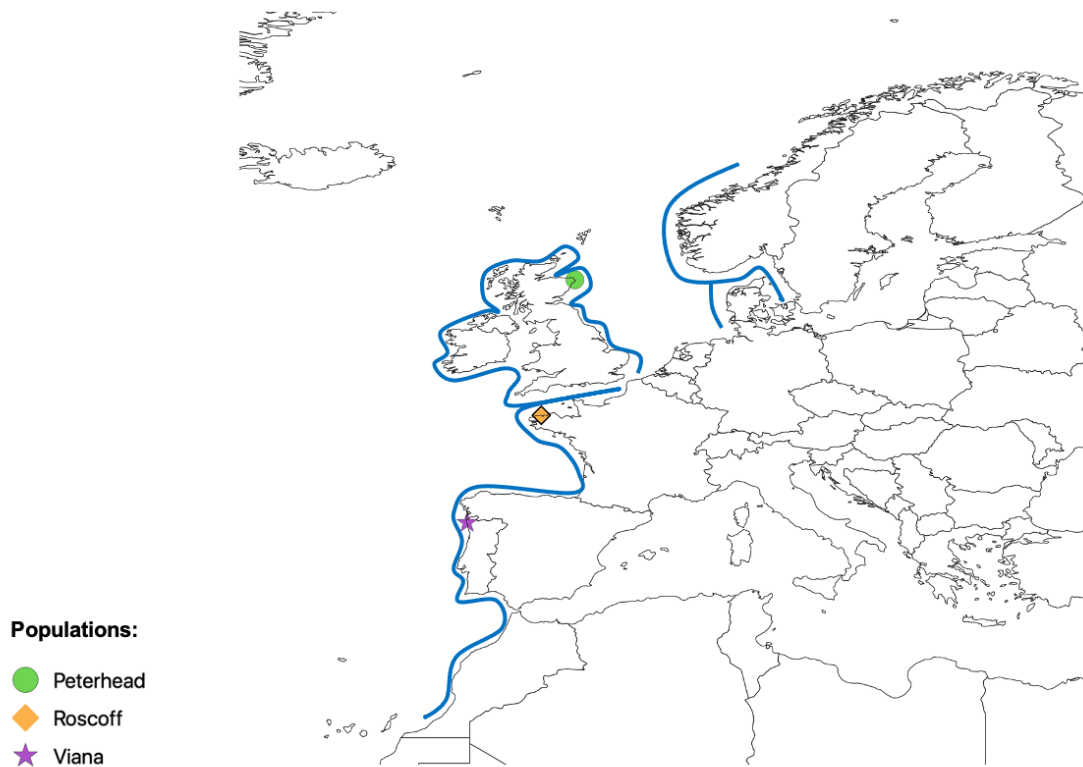


Figure 2 – Geographical distribution of *Nerophis lumbriciformis* (according to Dawson, 1986) and sampling locations (made with QGIS v3.10).

Individuals were collected by hand in the intertidal zone and preserved in ethanol, as part of a previous research project. A total of 30 pipefish were selected: 10 individuals per population, 5 of each sex. From the preserved pipefish of each population, we tried to select individuals of different sizes.



Figure 3 – *Nerophis lumbriciformis* specimens, photographed alongside an external ruler. (A) Sample Roscoff female 04; (B) Sample Peterhead female 03;

We photographed the full body of all pipefish, alongside an external ruler (**Figure 3**). These photographs were then used to measure pipefish body length and abdomen length (from the operculum to the dorsal fin), using ImageJ (Schneider *et al.*, 2012). Then, in the laboratory, pipefish heads were separated from the bodies with the help of a scalpel and stored in Eppendorf tubes with ethanol to preserve the samples.

2.3 MicroCT data processing and image analysis

CT images were acquired from samples fixed and stored in 70% ethanol in polypropylene tubes using a SkyScan 1276 microCT (Bruker, Kontich, Belgium) at the Bioimaging Scientific Platform from i3S. Projection images were obtained using the SkyScan software version 1.0.11 (Bruker, Kontich, Belgium), with a resolution of 8 x 8 x 8 μm . X-ray tube potential was 55 KV and tube current 200 μA , with exposure time of 435 ms. A total of 901 projection images were acquired over 360 degrees with a rotation angle of 0.4 degrees and average of 4 projections.

Tomographic images (3D datasets) were obtained by reconstructing the projection images using the NRecon software (version 1.7.4.2, Bruker, Kontich, Belgium). This software uses a filtered back-projection algorithm developed by Feldkamp and co-workers (Feldkamp *et al.*, 1984). For the reconstruction, the image alignment with respect to the actual center of rotation was specifically chosen for each sample. For every sample, the ring artifact reduction chosen was 7 and the beam hardening compensation of 20%.

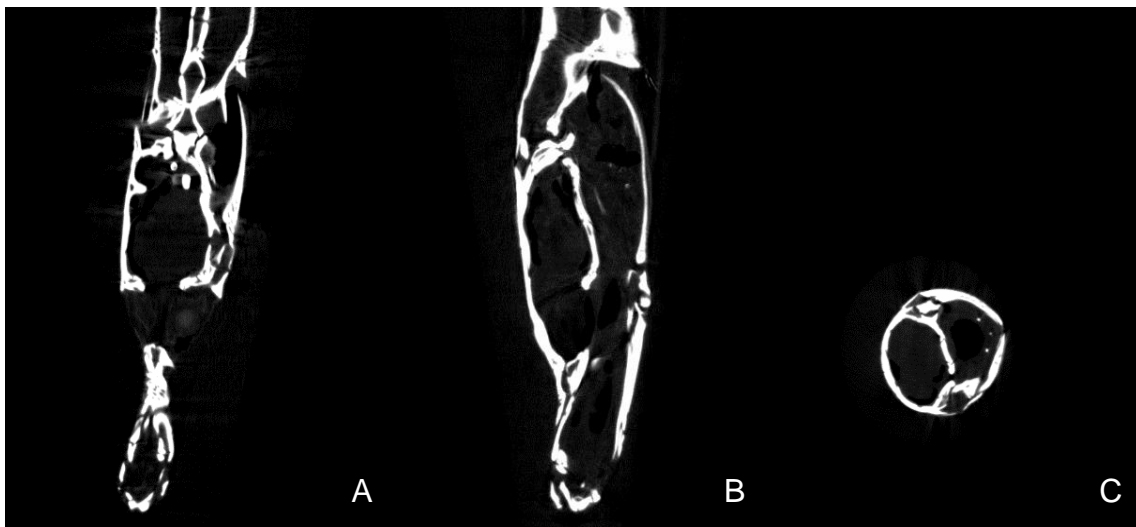


Figure 4 – μCT scan of a pipefish head (*Nerophis lumbriciformis*), whose images were obtained with DataViewer software (version 1.5.6.2, Bruker, Kontich, Belgium) using a threshold from 16 to 96 grey scale values. (A) Sagittal plan (Z-Y) image; (B) Coronal plan (X-Z) image; (C) Transaxial plan (X-Y) image.

Further visualization and inspection of the reconstructed 3D datasets was performed using the software DataViewer (version 1.5.6.2, Bruker, Kontich, Belgium) and CTVox (version 3.3.0r1383, Bruker, Kontich, Belgium). The DataViewer software was used to extract the images for constructing the figures for this thesis (**Figure 4**) as well as to select the volumes for further image analysis with the CTAn software. The CTVox software was used for the preparation of the videos of the 3D dataset (**Figure 5**).

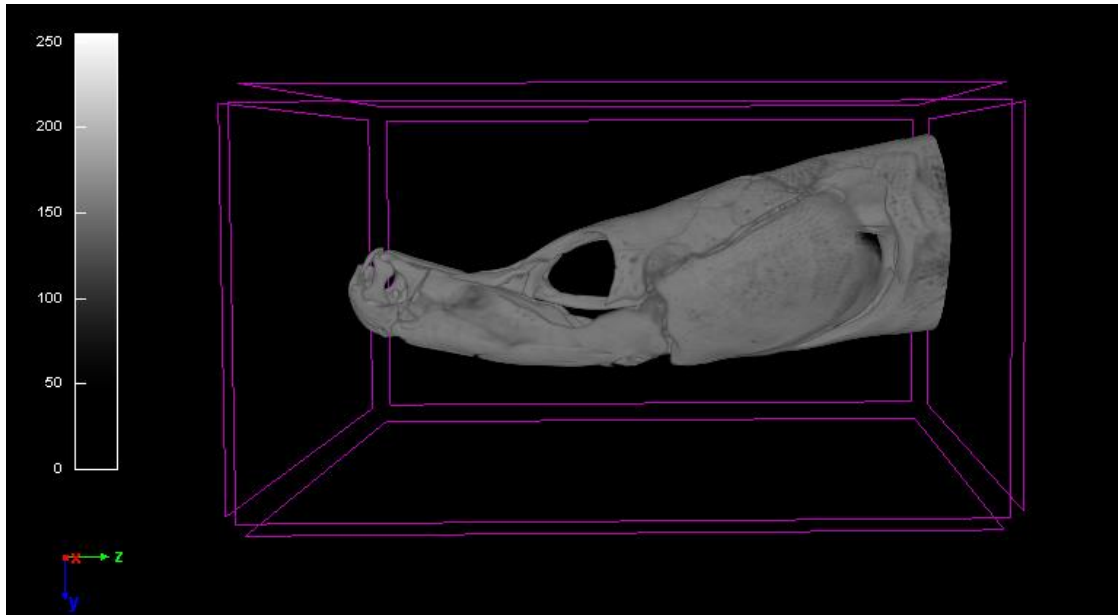


Figure 5 – 3D reconstruction image of a μ CT scan of a pipefish (*Nerophis lumbriciformis*) head, obtained with CTVox software (version 3.3.0r1383, Bruker, Kontich, Belgium), where it is possible to observe a clipping box with six surfaces surrounding the volume of interest that allows to cut the volume in all directions.

The CTAn software (version 1.20.3.0, Bruker, Kontich, Belgium) was used for image segmentation and analysis (**Figure 5**). For every sample the volume of interest (VOI) of the internal cranial space was selected by manually drawing the region of interest (ROI) of the cranial space every ten slice images. The respective volume was calculated from the VOI with a previous segmentation of the 8-bit greyscale bitmap (BMP) images using a threshold from 0 to 127 grey scale values.

Since microCT is a high-resolution technique that allows the visualization of the 3D structure of the specimen, we searched for the otoliths (**Figure 8**) in order to measure them. After analyzing all samples, we chose the lapillus for our measurements since they were easier to identify in all specimens, due to their shape and size. The average of left and right otolith major diameter was obtained using CTAn software (version 1.20.3.0, Bruker, Kontich, Belgium) following the same methodology used to calculate endocranial

volume, using the 8-bit greyscale bitmap (BMP) image stack and a threshold of 0 to 87 grey scale values.

Snout and head sizes were obtained by using the measure tool that is available on CTAn software (version 1.20.3.0, Bruker, Kontich, Belgium).

Finally, mean values and standard deviation were calculated for each measurement, sex and population and are displayed in **Table 1**.

Table 1 - Mean values and standard deviation calculated for each measurement: Endocranial volume (cubic millimetres) and body size (LT), head, snout and abdomen (centimetres).

Population	Sex	LT (cm)	Endocranial volume (mm ³)	Head (cm)	Snout (cm)	Abdomen (cm)
Peterhead	Males	12.738 ± 1.372	4.700 ± 1.210	0.940 ± 0.094	0.300 ± 0.038	3.481 ± 0.567
	Females	12.766 ± 1.372	4.514 ± 0.924	0.915 ± 0.064	0.360 ± 0.043	3.182 ± 0.354
Roscoff	Males	10.281 ± 0.800	3.968 ± 0.547	0.908 ± 0.075	0.340 ± 0.073	2.991 ± 0.165
	Females	10.792 ± 1.487	3.728 ± 0.658	0.827 ± 0.088	0.318 ± 0.071	2.648 ± 0.437
Viana	Males	11.811 ± 0.404	4.213 ± 0.367	0.905 ± 0.024	0.318 ± 0.059	3.145 ± 0.265
	Females	12.712 ± 0.678	4.766 ± 0.837	0.981 ± 0.088	0.377 ± 0.055	3.323 ± 0.218

2.4 Data analysis

In order to detect hypothetical differences in endocranial volume between the sexes and populations, we used an ANCOVA (analysis of covariance), using body length as the covariable (as we will show later, we also tried using distinct covariables, such as head, snout and abdomen size). ANCOVA assumptions (linearity of regressions, normality of residuals and homogeneity of variances) were examined beforehand. A similar process was used for the analysis of otolith diameter. All analyses were performed in R software (version 4.0.3).

3 Results

3.1 Endocranial volume

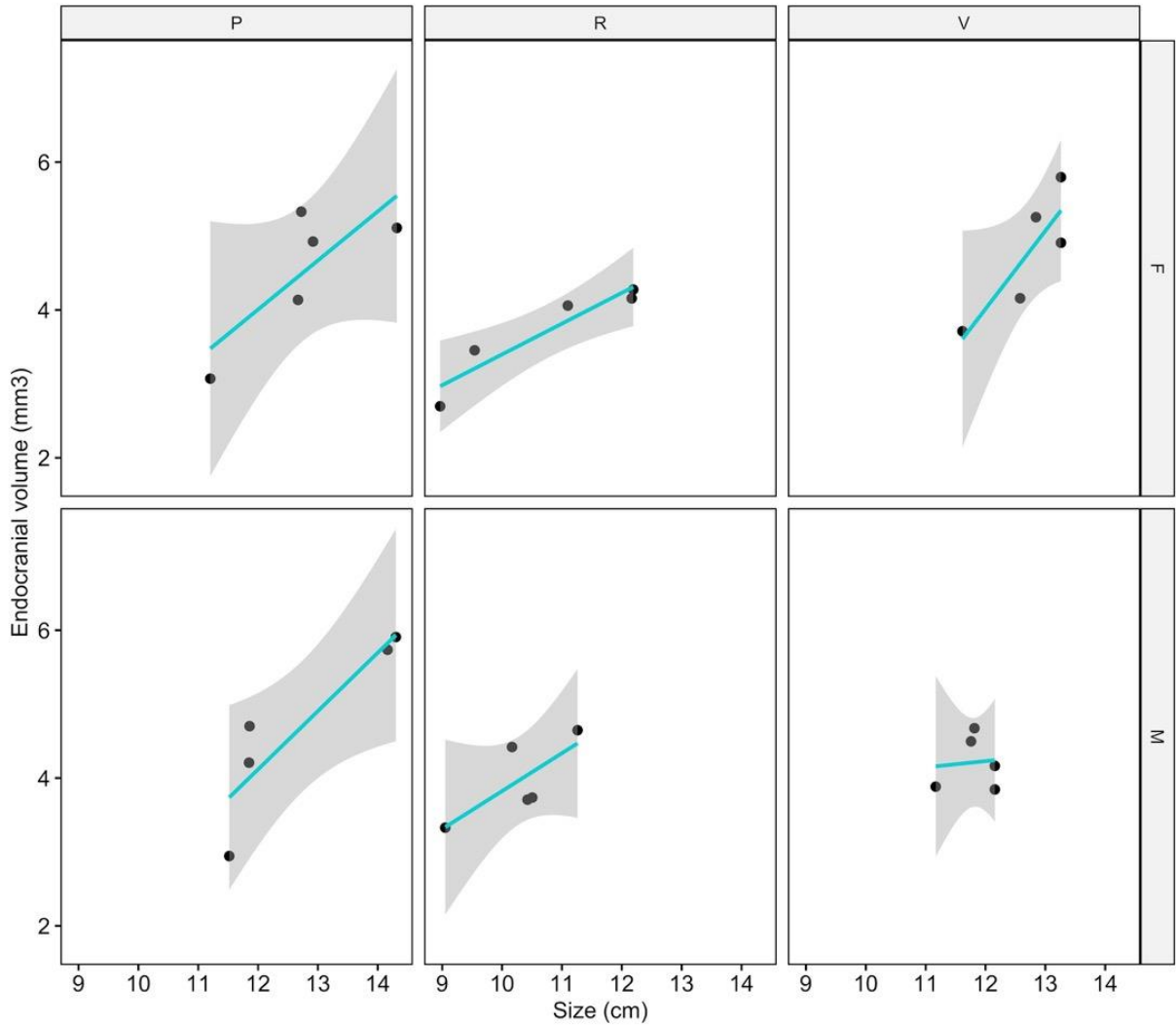


Figure 6 – Analysis of linearity between the covariate (body length, in centimetres) and the outcome variable (endocranial volume, in cubic millimetres) at each level of the grouping variable (Sex and Population), obtained with R software (version 4.0.3).

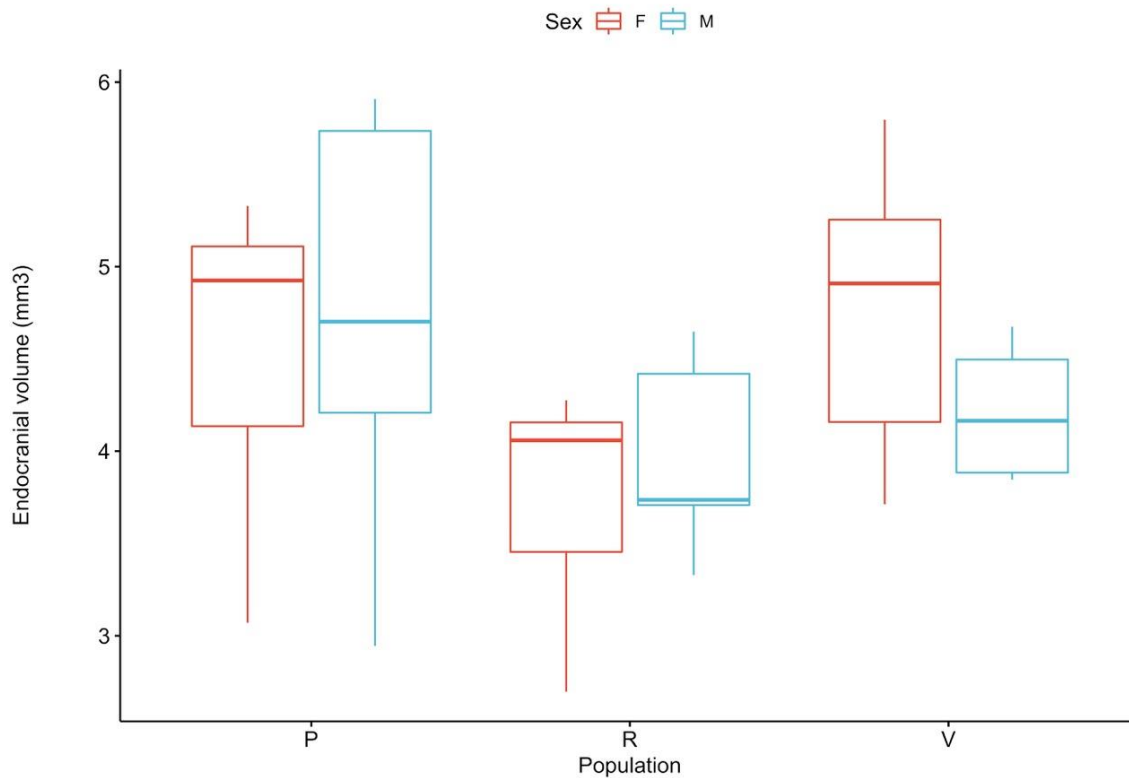


Figure 7 – Variation of endocranial volume (cubic millimetres) calculated for males and females, in each sampled population (P - Peterhead, R - Roscoff and V- Viana), obtained with R software (version 4.0.3).

ANCOVA assumptions were met. A visual inspection of linearity between the covariate (body length) and the outcome variable (endocranial volume) at each level of the grouping variable (Sex and Population) showed that there seems to be no ostensive deviations from linearity (**Figure 6**). There was homogeneity of the regression slopes as the interaction term was not statistically significant ($F(2,18)=1.058$, $P=0.092$). The Shapiro Wilk test (conducted on residuals) was not significant ($W=0.970$, $P=0.533$), so we can assume normality of residuals. The Levene's test was not significant ($F(5,24)=0.132$, $P=0.983$), so we can assume homogeneity of the residual variances for all groups. Finally, there were no obvious outliers.

After adjustment for body size, there was no significant interaction between our factors ($F(2,23)=0.803$, $P=0.460$) and no significant differences between the levels of our factors (Population: $F(2,23)=1.971$, $P=0.168$; Sex: $F(1,23)=2.219$, $P=0.154$) (**Figure 7**).

Results remained unchanged even when we tried to use other covariables such as head, snout size or abdomen size (data not shown). We tested these variables as populations could potentially differ in their growth patterns (i.e., if the growth of certain body parts does not follow an isometric pattern). As an example, populations in the north, due to a shorter breeding season (Monteiro *et al.*, 2017b), could hypothetically favour

the evolution of longer abdomens (where female ovaries are located), in order to maximize reproductive success.

We decided to present some additional data related with pipefish morphology. Although our data is extremely preliminary, since we were able to perform a 3D reconstruction of the worm pipefish body (or sections of the body), we imagined that it would be relevant to show some interesting details of this pipefish morphology. So, uncharacteristically, we opted to present a very brief context to the results on body shape and otoliths in this section, far from the introduction (to avoid removing attention from our main goal – the analysis of endocranial volume throughout a gradient of sexual selection intensity).

3.2 Seahorses versus pipefishes

Syngnathid fishes show a very specialized morphology (Stölting & Wilson, 2007; Wilson & Orr, 2011). Their feeding technique is known by 'pivot feeding' due to the animals' movement when eating their preys (Leysen *et al.*, 2011). Some similarities are already known between pipefish and seahorses: fused jaws that have no teeth and a slim and long snout (Leysen *et al.*, 2011). However, some characteristics distinguish the two groups.

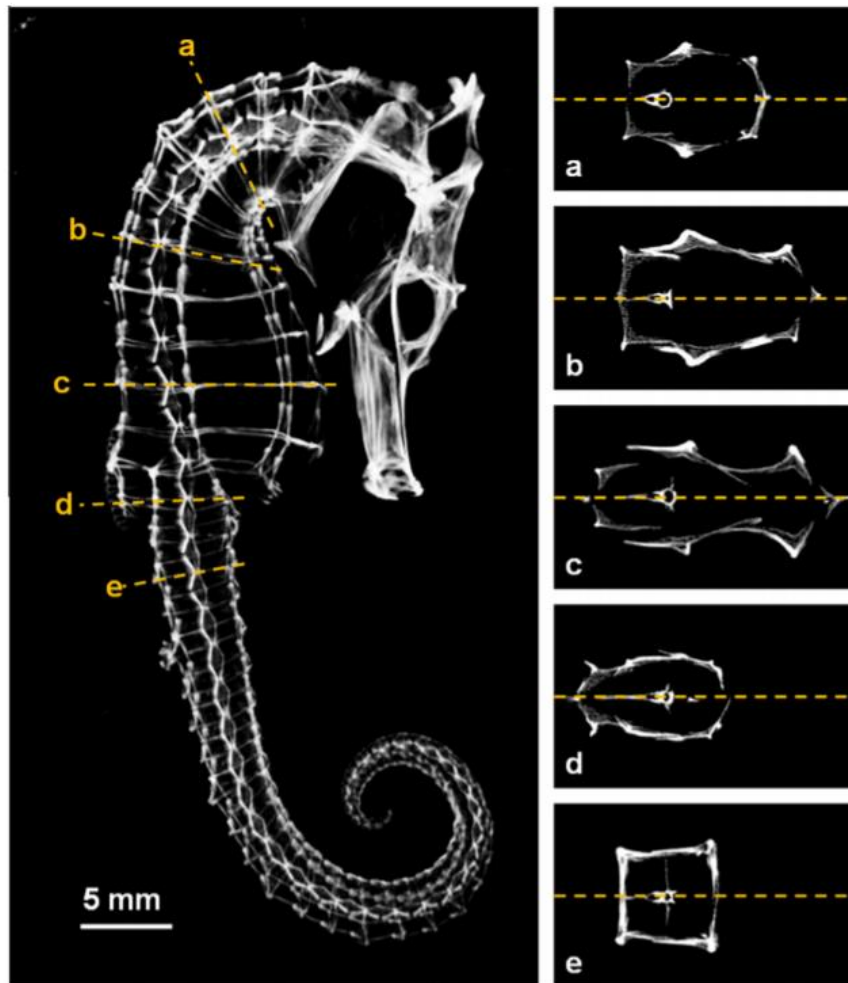


Figure 8 – μ CT scan of a juvenile seahorse skeleton (*Hippocampus kuda*) illustrating the cross-sections of several different segments along the length of the fish: (a–c) heptagonal segments at different locations of the torso; (d) hexagonal segment at the dorsal fin (torso–tail intersection); (e) square-like segment of the prehensile tail. Adapted from Porter *et al.* (2013).

Some exceptional characteristics can be found in seahorses (**Figure 8**) such as the horsy profile (due to the inclined posture of the head relative to the trunk), the coronet (bony prominence on the top of the head with variable shape, depending on the species), upright swimming posture, arched trunk, an elongated tubular snout, eyes that move independently, a fully closed brood pouch, and a flexible prehensile tail (Lourie *et al.*, 1999; Foster & Vincent, 2004; Wassenbergh *et al.*, 2011; Porter *et al.*, 2013;). In order to swim vertically, seahorses use their dorsal fin for impulsion and two pectoral fins for maneuverability, since they don't possess a caudal fin (Consi *et al.*, 2001; Ashley-ross, 2002). Also, they developed a segmented array of bony plates that cover the body and function simultaneously as an armor to protect from predators while also allowing for muscular force transmission and body mobility (Hale, 1996; Praet *et al.*, 2012; Porter *et al.*, 2013).

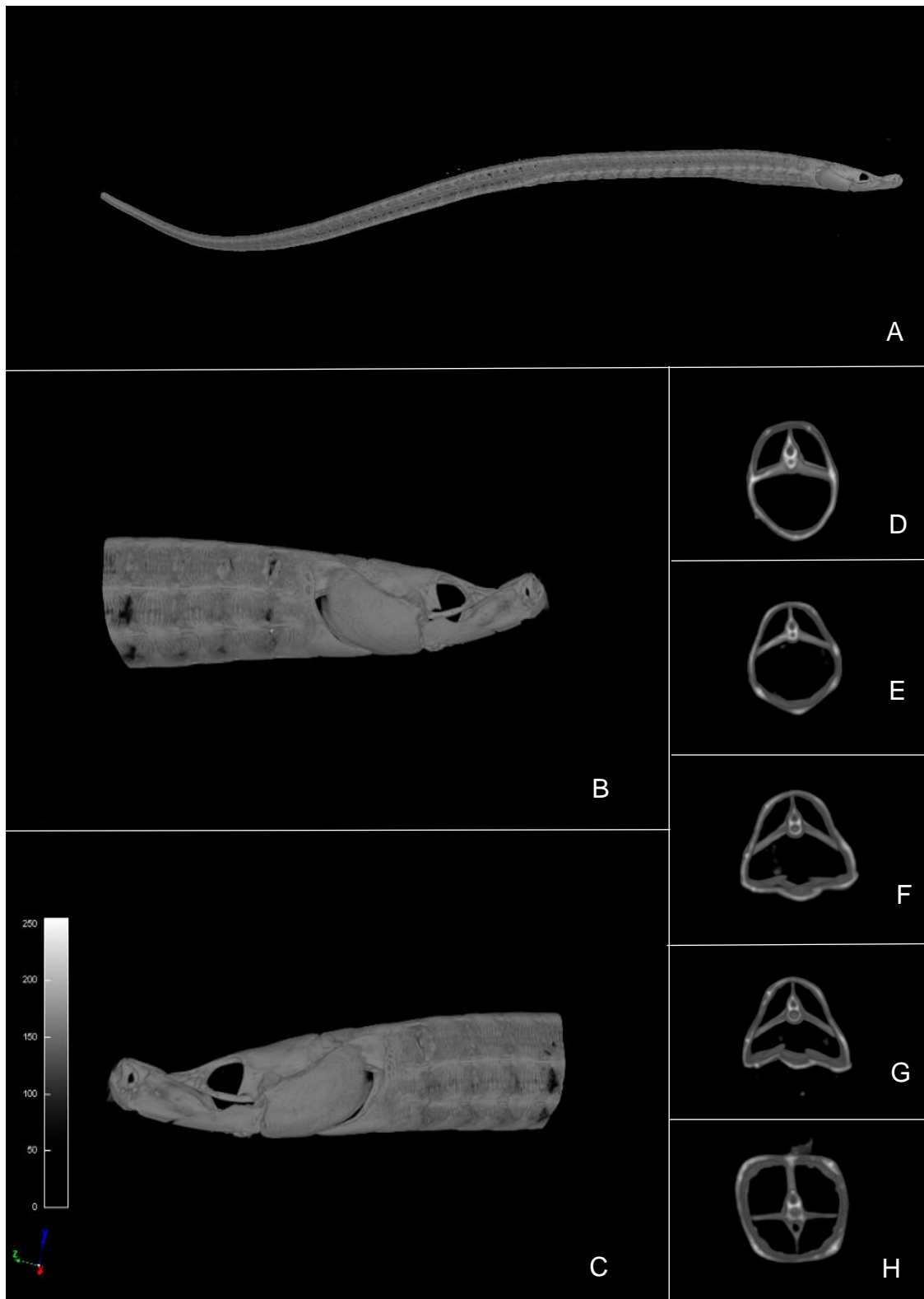


Figure 9 – μ CT scan of a pipefish (*Nerophis lumbriciformis*) whose images were obtained with CTVox software (version 3.3.0r1383, Bruker, Kontich, Belgium). (A) longitudinal image of full body 3D representation; (B-C) right and left head side images where it is possible to visualize an array of bony plates. (D-H) cross-sections of several segments along the length of the pipefish.

Regarding pipefishes (**Figure 9**) (subfamily Syngnathinae), they also have some unique features that allow distinction from seahorses such as a tubular and long body (**Figure 9A**) and a small head without spikes (Leysen *et al.*, 2011). Besides this, pipefish also present a long snout (**Figure 9B and 9C**) composed of modified neurocranial and suspensory elements, with jaws positioned at the distal end of the snout and turned slightly upwards (Flammang *et al.* 2009; Small *et al.*, 2016). They are heavily armoured and the opercular part can expand to the sides (Bergert & Wainwright, 1997). When pipefish feeds, they swallow preys through an oral opening with reduced dimensions, much like a pipette (Flammang *et al.* 2009). As it is possible to observe in **Figure 9**, some pipefish species, such as *Nerophis lumbriciformis*, have an array of bony plates that, similarly to seahorses, can protect them from predators. With microCT technique, that was used in this study, it was possible to observe the bony structure of the plates that are larger in the opercular region. Moreover, it seems clear that, despite the morphology of the rest of the body, the head is very similar between seahorses and pipefish, probably as a result of similar feeding habits.

3.3 Otoliths

Otoliths, also known by ear stones, are calcified structures that exist in pairs (Campana, 1999) and are confined in three compartments associated with the ear in teleost fishes (Popper *et al.*, 1988). Otoliths are made, in their majority, by calcium, oxygen and carbon (Campana, 1999). There are three pairs of otoliths: in most fishes, the bigger otolith is the saccular (the sagitta). The second largest in most fishes, and largest in most ostariophysian fishes, is the lagenar otolith (the asteriscus), and the utricular otolith is the smaller one (the lapillus) (Paxton, 2000). Regarding their function, otoliths are supposed to be related with audition and vestibular system, that conveys gravity information (Popper & Fay, 1993; Paxton, 2000).

Otoliths are often used as a timekeeper since they register fish age (Campana, 1999) through the formation of annual (Casselman, 1987) and daily growth rings (Pannella, 1971; Campana & Neilson, 1985). Otoliths were already used, in syngnathids, to estimate their age (Pereira, 2009; Parkinson *et al.*, 2012). Nevertheless, otoliths can provide more information than age. For instance, Bignami *et al.* (2013) showed that ocean acidification alters fish otoliths (increased otolith size and density), possibly altering alter the perception of auditory information and impacting the dispersal, survival, and recruitment of pelagic larvae. As the worm pipefish has a pelagic stage before recruiting to the intertidal, we imagined that a comparison of otolith size between our

studied populations could potentially signal environmental differences other than those directly related with temperature. In this species of fish in particular, according to a study conducted by Pereira (2009), the biggest pair of otoliths is sagitta, followed in size by lapillus (**Figure 10**) and asteriscus.

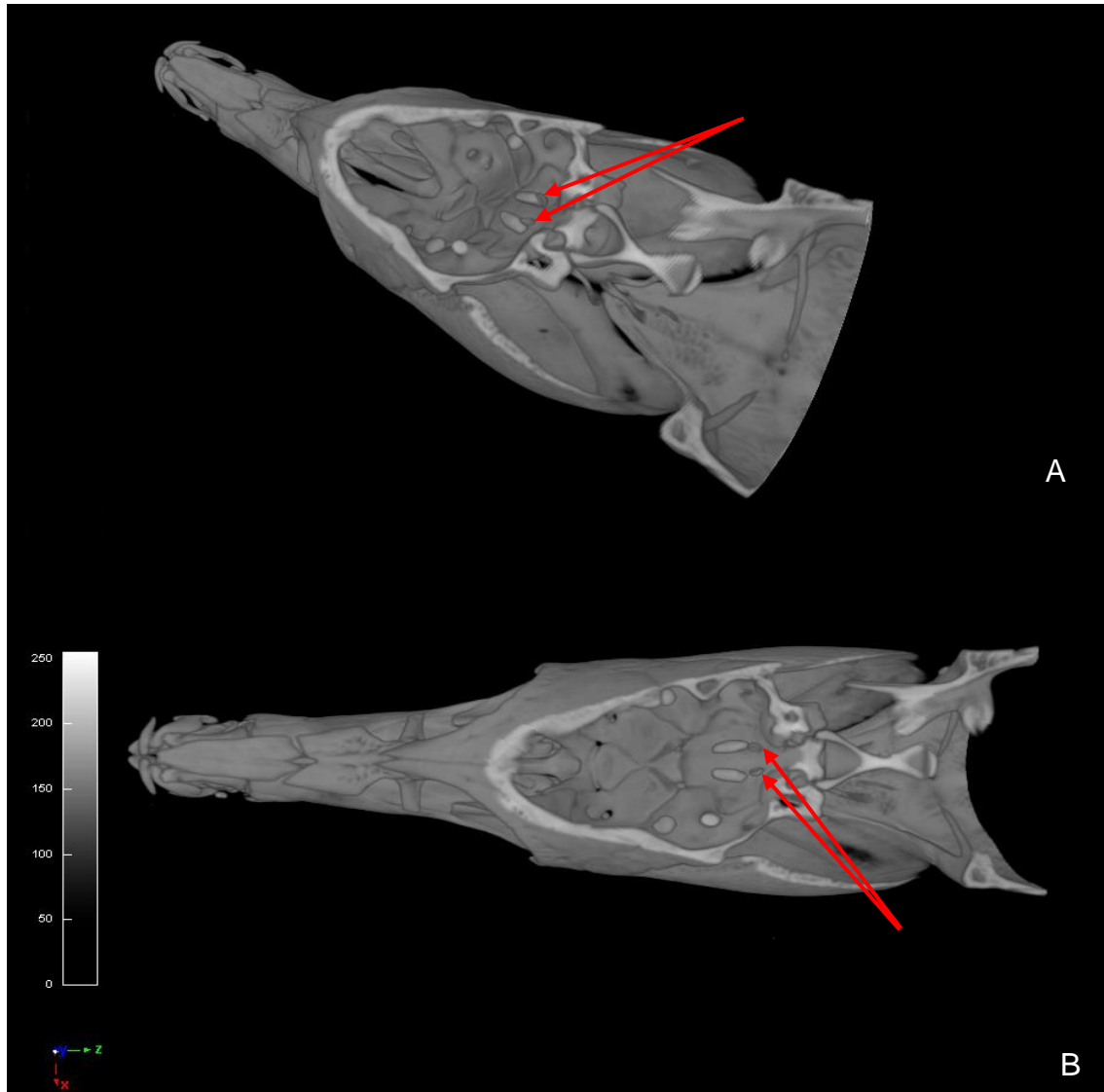


Figure 10 – μ CT scan of a pipefish head (*Nerophis lumbriciformis*), whose images were obtained with CTVox software (version 3.3.0r1383, Bruker, Kontich, Belgium). (A, B) images of inside of the head structure where it is possible to observe (indicated with red arrows) the pair of otoliths that was measured (lapillus).

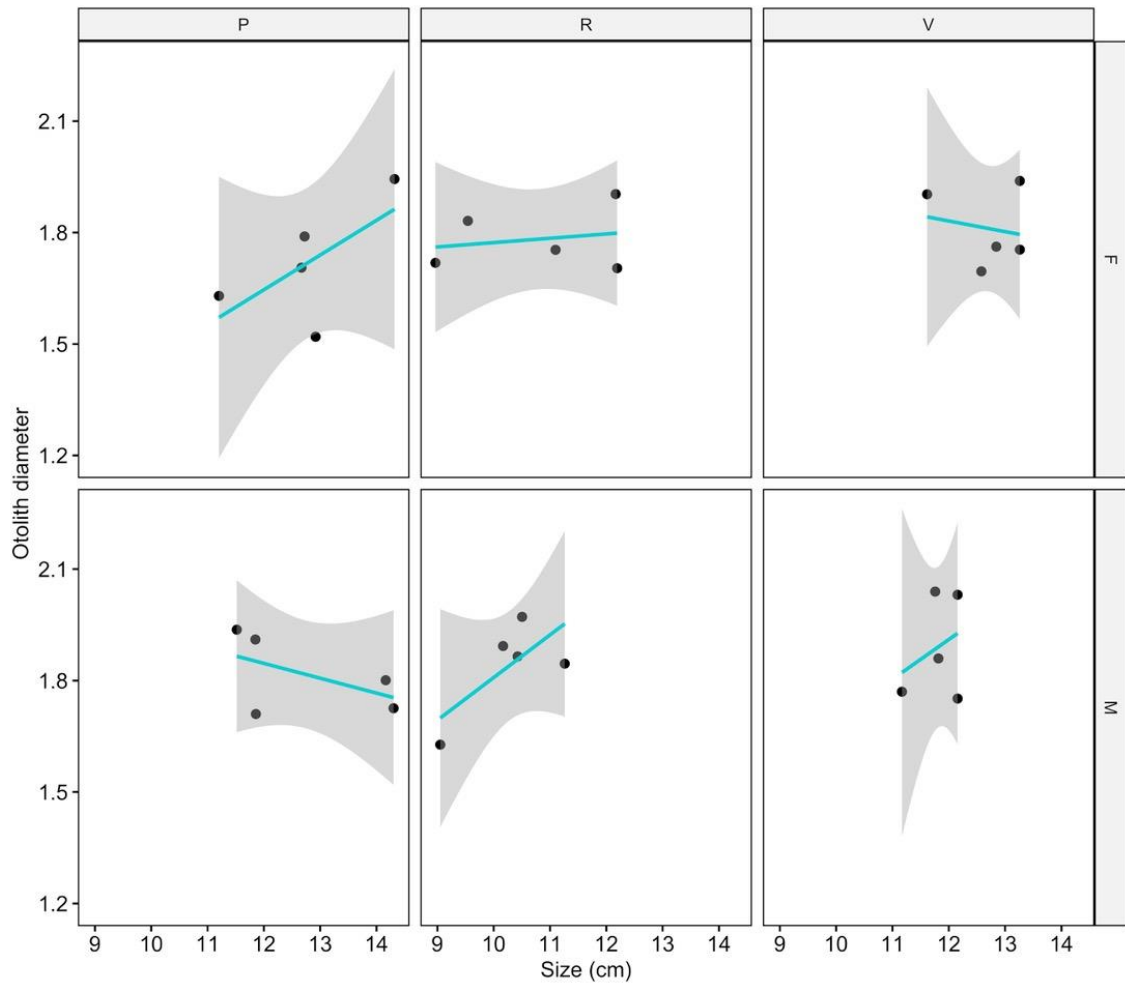


Figure 11 – Analysis of linearity between the covariate (body length, in centimetres) and the outcome variable (otolith diameter, in millimetres) at each level of the grouping variable (Sex and Population), obtained with R software (version 4.0.3).

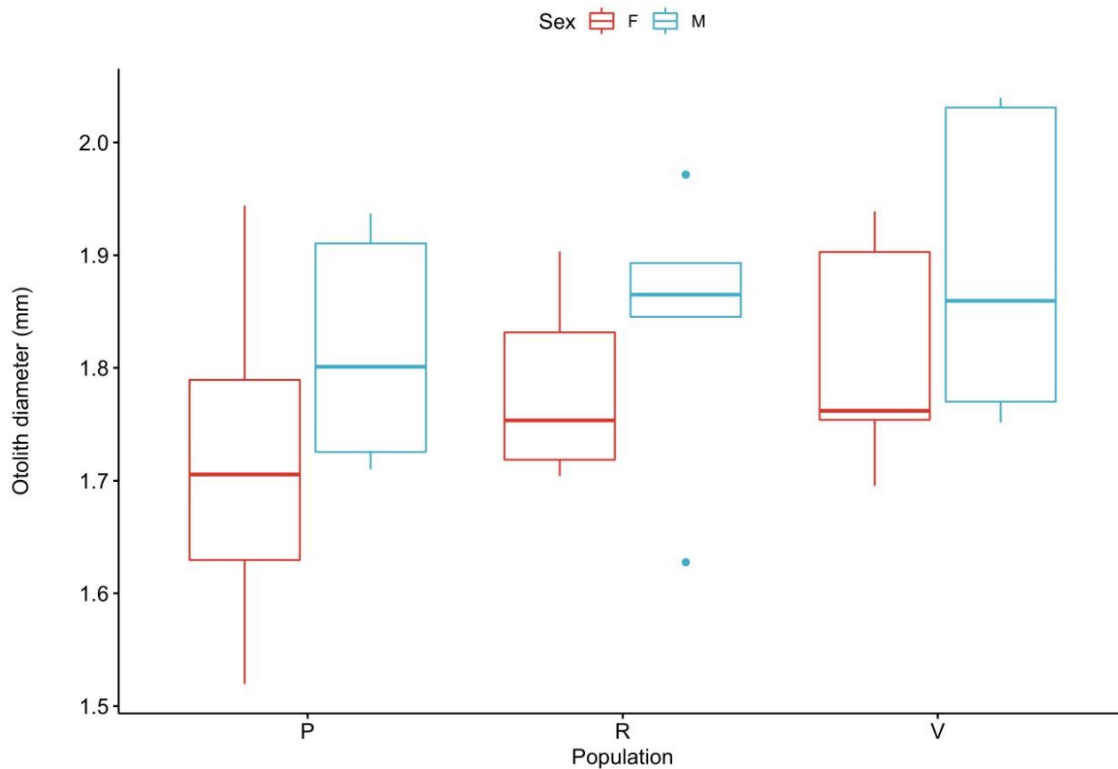


Figure 12 – Variation of otolith diameter (millimetres) calculated for males and females, in each sampled population (P - Peterhead, R - Roscoff and V- Viana), obtained with R software (version 4.0.3).

ANCOVA assumptions were met. A visual inspection of linearity between the covariate (body length) and the outcome variable (otolith diameter) at each level of the grouping variable (Sex and Population) showed that there seems to be no ostensive deviations from linearity (**Figure 11**). There was homogeneity of the regression slopes as the interaction term was not statistically significant ($F(2,18)=0.199$, $P=0.092$). The Shapiro Wilk test (conducted on residuals) was not significant ($W=0.958$, $P=0.267$), so we can assume normality of residuals. The Levene's test was not significant ($F(5,24)=0.248$, $P=0.937$), so we can assume homogeneity of the residual variances for all groups. Finally, there were no obvious outliers.

After adjustment for body size, there was no significant interaction between our factors ($F(2,23)=0.050$, $P=0.952$) and no significant differences between the levels of our factors (Population: $F(2,23)=1.493$, $P=0.246$; Sex: $F(1,23)=3.721$, $P=0.066$) (**Figure 12**).

4 Discussion

In this work, using micro-computed tomography, we aimed to detect if sexual selection can promote brain size sexual dimorphism in the worm pipefish (especially in populations where sexual selection is known to be more intense) and, we also wanted to detect, in those populations, if either males or females displayed the larger brains.

Although Peterhead presented the highest median values for endocranial volume, and Roscoff presented the lower median values (see **Figure 7**), these differences in endocranial volume values between each population were not significant. This lack of significance derives primarily from the fact that the sampled pipefish in Roscoff were generally smaller (see **Table 1**). Once body size was used as a covariate in our analysis, these apparent differences disappeared. Likewise, although it may seem that the median values in all female populations are higher when compared with those from males, body size corrections removed any difference between the sexes. Overall, we were unable to detect differences in brain size (using endocranial volume as a proxy) between populations and/or between the two sexes.

The lack of differences in brain size might be due to at least three non-exclusive reasons: 1) our sampling was too low to allow the detection of subtle brain size differences between the sexes and populations and 2) sexual selection, in the worm pipefish, does not promote brain size dimorphism and does not influence brain growth in populations experiencing higher selective intensities, and 3) worm pipefish larvae dispersion hinders the development of a clear population structure along its distribution. According to Tsuboi *et al.* (2017), who compared several syngnathid species, sexual selection seems to promote the evolution of larger brains in females of more polyandric species. Since a polyandric mating system naturally enforces a higher sexual selection pressure in females, we hypothesized that worm pipefish populations (polyandric mating system, see Monteiro *et al.*, 2017a) experiencing higher sexual selection could show intersexual differences in brain size. In order to be more confident on our results (a lack of differences between sexes and populations), we will need to expand our sampling scheme, by simultaneously analyzing more pipefish in each population and expand the number of sampled populations. Initially, we planned to have a wider number of populations and individuals but our expectations were cut short by the limitations imposed by the current pandemic.

Another possible explanation for the lack of differences (in brain size) in our sampled populations might be due to the particular life history of the worm pipefish. Contrarily to most syngnathids, which display high levels of population differentiation

(e.g., Mobley *et al.*, 2010; Sogabe and Takagi, 2013; Fedrizzi *et al.* 2015), even at small geographical scales, the worm pipefish displays an almost genetically homogeneous population, with indications of extensive gene flow (Monteiro, personal communication). This fact is probably due to the dispersal potential of the species' pelagic larvae. If larvae are able to travel long distances, as evidence suggests, then brain size increase supported by sexual selection might just be impossible at small scales.

A similar line of reasoning (sampling effort and panmictic population) might also help interpret the results emanating from our otolith analysis. Nevertheless, we believe that, since worm pipefish recruit to the intertidal zone a few months after they are born (and remain there for the rest of their lives), the lack of differences between sexes and populations could be due to an insufficient power of our analysis due to the small number of used individuals. **Figure 12** hints at a possible otolith growth towards the southern limit of distribution, with males and females following distinct slopes. Again, these conjectures will force us to increase our sampling, once the pandemic ends.

Micro-computed tomography is a very powerful tool able to highlight inter- and intra-population differences resulting from evolutionary processes such as sexual selection. Here, we tried to show how one can look for the results of a sexual selection process by looking into often cryptic sexually selected characters traits, like the brain.

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