



## Looking for the “Woman in Red” effect in a pipefish without a closed brooding pouch

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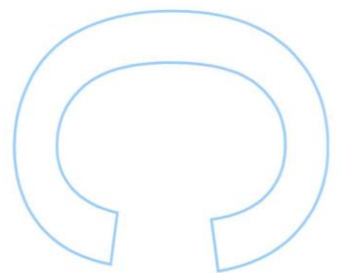
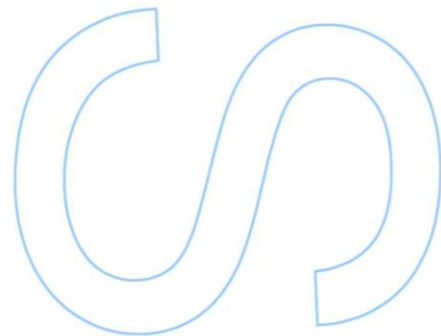
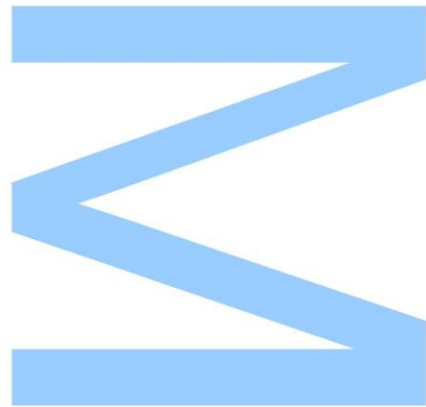
Departamento de Biologia,

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### **Orientador**

Professor Doutor Nuno Miguel Pinto de Sousa Monteiro

CIBIO-INBIO, Researcher, FCUP



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

O Presidente do Júri,

Porto, \_\_\_\_ / \_\_\_\_ / \_\_\_\_

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## Abstract

The “Woman in Red” effect, a post-copulatory sexual selection mechanism where the presence of a better female encourages the male to reduce its investment in the current pregnancy, was recently uncovered by Cunha et al. (2018). This mechanism highlights the fact that the marsupium is potentially much more than a brooding area, also serving as an arena of competition between the sexes.

Here, we tested the importance of the marsupium in the expression of the “Woman in Red” effect. To this end, we used pregnant male worm pipefish (*Nerophis lumbriciformis*) which, unlike the black-striped pipefish (*Syngnathus abaster*) with which the initial experiment was conducted, do not have a closed marsupium (male worm pipefish display a simple brooding area where the attached eggs remain in contact with seawater). Closely replicating the conditions used in Cunha et al. (2018), we allowed pregnant males to see females that were larger and more attractive than their original, already high-quality, mates. Despite the similarities between experimental conditions, pregnant worm pipefish males exposed to these extremely attractive females produced offspring that showed no significant change in length and no noticeable increase in abortion rates, showing that males of this species seem unable (or unwilling) to reduce the investment in a brood, despite the potential for access to a better mating partner.

Our apparent inability to show alterations in male reproductive investment in a species without a brood pouch can imply i) that the marsupium (a closed space whose conditions are tightly controlled by the pregnant male) may have a critical role in the expression of the “Woman in Red” effect, and ii) that this post-copulatory sexual selection mechanism is not widespread within the *Syngnathidae* family.

Given that the “Woman in Red” effect is a very recent discovery, whose mechanistic determinants remain mostly unknown, further research is required, namely by incorporating data from other syngnathid species with distinct brooding structures, in order to gain a broader understanding of this remarkable post-copulatory sexual selection mechanism.

**Keywords:** Post-copulatory sexual selection; *Nerophis lumbriciformis*; “Woman in Red” effect; Male pregnancy; Marsupium; Parental care.

## Resumo

O efeito da “Mulher de Vermelho”, um mecanismo de seleção sexual pós-cópula onde a presença de uma fêmea de melhor qualidade estimula o macho a reduzir seu investimento na sua gravidez atual, foi descoberto recentemente por Cunha et al. (2018). Esse mecanismo realça o facto do marsúpio poder potencialmente ser mais do que uma área de incubação, servindo também como uma arena de competição entre os sexos.

Neste trabalho, testámos a importância do marsúpio na expressão desse efeito. Para este fim, usamos machos grávidos de marinhas da espécie *Nerophis lumbriciformis* que, ao contrário da marinha *Syngnathus abaster*, usado na experiência inicial, não tem um marsúpio fechado (os machos de *Nerophis lumbriciformis* possuem uma área de incubação simples onde os ovos se fixam, permanecendo em contato constante com a água do mar). Replicando as condições usadas em Cunha et al. (2018), permitimos que os machos, já grávidos, vissem fêmeas maiores e mais atraentes do que suas parceiras originais. Apesar das semelhanças entre as condições experimentais das duas experiências, os machos grávidos expostos a estas fêmeas extremamente atraentes não mostraram nenhuma variação significativa no tamanho ou taxas de aborto dos seus descendentes, o que mostra que os machos da espécie *Nerophis lumbriciformis* parecem relutantes ou incapazes de reduzir o seu investimento na descendência, apesar das perspectivas de acesso a um melhor parceiro reprodutivo.

A nossa aparente incapacidade em mostrar alterações no investimento reprodutivo paterno, numa espécie sem marsúpio, parece sugerir i) que o marsúpio (um espaço fechado cujas condições são intimamente controladas pelo macho grávido) pode ter um papel crítico na expressão do efeito da “Mulher de Vermelho”, e ii) que este mecanismo de seleção sexual pós-cópula não está uniformemente disseminado por toda a família *Syngnathidae*. Dado que o efeito da “Mulher de Vermelho” é uma descoberta muito recente, cuja mecanística necessária à sua expressão permanece em grande parte desconhecida, é necessária mais investigação, nomeadamente através da incorporação de dados de outras espécies de singnatídeos com estruturas de incubação distintas, a fim de se obter uma compreensão mais ampla deste notável mecanismo de seleção sexual pós-cópula.

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**Palavras chave:** Seleção sexual pós-cópula; *Nerophis lumbriciformis*; Efeito “Mulher de Vermelho”; Gravidez masculina; Marsupio; Cuidados parentais.

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# 1. Introduction

Understanding sexual reproduction entails recognizing the often-significant role of a potent evolutionary agent: sexual selection. This process helps decide which individuals will be more prone to reproduce, therefore increasing the frequency of genes that will ultimately contribute to the expression of reproductive advantages (Birkhead & Pizzari, 2002). This mode of non-random reproduction, in turn, leads to the inevitable increase of favourable characteristics in a population, as mating individuals, better favoured by mates, will out-reproduce competitors (Starr *et al.*, 2012).

Darwin first described sexual selection (Darwin, 1871) as a facet of natural selection (Darwin, 1859), thus justifying the persistence and ubiquity of adaptations not immediately perceivable as enablers of increased survival. Ostentatious and energetically onerous body structures, such as imposing antlers, or vivid plumages that seem to reduce crypsis, could now be understood as having meaningful evolutionary value. These characters, often differing between the sexes, ultimately dictate not only which individuals are able to survive but also who will reproduce, despite the inherent costs (Andersson, 1994; Andersson & Simmons, 2006).

Under conventional sex roles, not all males are able to reproduce and those that successfully find a mate often breed with the most desirable females. Thus, it seems simple to anticipate that sexual selection is often grounds for conflict. This is where the expression of secondary sexual characteristics comes into play: the males use these unique characters as effective tools in competition with rivals (intrasexual selection), fighting for the opportunity to mate with the best females, and/or use their sexual characters as a way of conveying information to females about their intrinsic quality (intersexual selection). Thus, under conventional sex roles, secondary sexual characters, such as the peacock train or the deer antlers, are especially pronounced, or solely present, in males, leading to obvious dimorphism between the sexes. In species with reversed sex roles, where competition is stronger among females, and mate choice is more evident in males, secondary sexual characters tend to be present in females (Berglund *et al.*, 2005; Silva *et al.*, 2010). It should be noted that sexual dimorphism, one of the possible results of sexual selection, is not restricted to the development of weapons or ornaments (Berglund *et al.*, 1996). For

instance, sexual size dimorphism can also result from the sexual selection process (Blanckenhorn, 2005), reflecting the adaptation of both sexes to their different reproductive roles.

Contrarily to what was initially supposed by Darwin (and those that followed, for nearly a century), sexual selection is a continuous process which may stretch well past the moment of copulation (after all, copulations are not always translated by successful fertilization). With the work of Parker (1970), highlighting how competition between males can endure within the female's body, a new, broad avenue for sexual selection research ensued, now known as post-copulatory sexual selection.

Post-copulatory selection mechanisms are usually much less conspicuous than pre-copulatory sexual selection, often occurring within the female's body, at a minute scale. This fact might help explain why only after technological limitations were overcome did researchers start to describe the vast array of selection mechanisms that extend beyond copulation. Probably the most well-known form of post-copulatory sexual selection is sperm competition, where two or more males continue to compete for the fertilization of a female's egg through their spermatozoa. Sperm competition might involve very different mechanisms such as altered sperm (Firman & Simmons, 2009) or penis morphology (Rönn et al., 2007; Schilthuizen, 2003; Shackelford & Goetz, 2007), the use of mating plugs (Quammen, 2012), variation in ejaculate volume (Schulte-Hostedde & Millar, 2004) or even female stimulation that leads to sperm ejection (Heather & Robertson, 2005). Post-copulatory selection can also occur through cryptic female choice when females are able to store and separate sperm from multiple males, and then select which male will sire their offspring (Moore et al., 2009).

Not all post-copulatory sexual selection mechanisms are cryptic. A striking instance of an easily observable mechanism is infanticide (Hrdy, 1974). Occurring much after copulation, infanticide is an obvious example of how sexual selection is able to operate long after successful fertilization. In certain species, of which the lion is probably the best-known example, males often kill the offspring of the previous overthrown competitor, thus stimulating females to become readily available to reproduce again (Packer, 2000).

In some species, where the risk of infanticide is high, females have been shown to be able to reduce their reproductive costs by aborting their developing embryos.

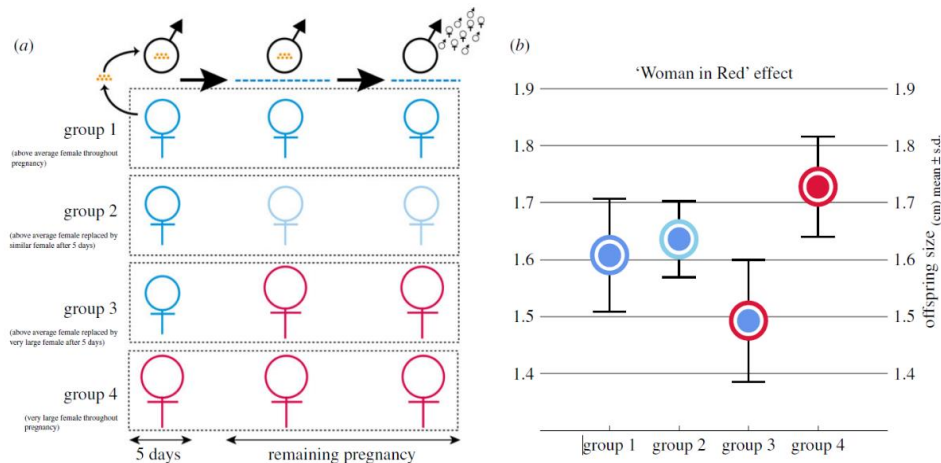
This post-copulatory sexual selection mechanism, first described by Hilda M. Bruce in female mice (*Mus musculus*) (Bruce, 1959), was later termed the “Bruce effect”: a mother’s pregnancy block induced by the exposure to the scent of a new, possibly dominant, male when the father is not present. Exposure to pheromones of new fertile adult males leads to the abortion of the developing foetus as a means for the mother to save energy and resources that she would inevitably lose due to the death of its offspring (Bruce, 1959; Hofmann et al., 1987). The Bruce effect was later found to extend not only to other rodent species (Clulow & Langford, 1971; Eleftheriou et al., 1962; Mallory & Brooks, 1980) but is suspected to occur in other mammals such as horses (Berger, 1983), lions (Packer & Pusey, 1983) and even primates, like geladas (Roberts *et al.*, 2012).

As the Bruce effect is directly linked to pregnancy, recently, Cunha et al. (2018) looked for a Bruce-like effect in a species where pregnancy occurs but does so in males rather than females. For that, they used a pipefish (*Syngnathus abaster*), a member of the *Syngnathidae* family, the only known taxa where male pregnancy occurs (Kuitert, 2000; Wilson et al., 2001). During copulation, the female will transfer her eggs into the males incubating surface or pouch, where the eggs are fertilized solely by that male, ensuring its paternity status (Woodall et al., 2011). Then, the male incubates the embryos, providing protection, aeration, osmoregulation, while also provisioning their development (Stölting & Wilson, 2007).

The occurrence of post copulatory sexual selection in syngnathids was first hinted by Paczolt and Jones (2010) when they showed that males had the ability to cause fitness trade-offs between consecutive broods. An already pregnant male was capable of increasing offspring abortion in pregnancies from less fit mothers, retaining resources for future pregnancies. In essence, Paczolt and Jones (2010) showed that the brood pouch (or marsupium), where some syngnathid species develop their embryos, has an additional role besides allowing for nutrient transference between the father and developing embryos: it acts as an arena of conflict between the sexes.

Cunha et al. (2018) went a step further and explicitly tested how males would react, once already pregnant, when exposed to a female of very high quality. Although unable to find a true Bruce effect (as there is no systemic infanticide risk in syngnathids and no complete abortion of the brood was ever recorded), Cunha et al. (2018) showed that *S. abaster* males were able to impact their pregnancy by

producing fewer and notably smaller offspring when exposed to more attractive females (Figure 1 – group 3) when compared to a normal pregnancy (Figure 1 – Group 1 and 2). They coined this post-copulatory sexual selection mechanism as the “Woman in Red” effect.



**Figure 1** - Cunha's (2018) experimental setup (a) and main results (b). The male mated freely a female and, 5 days after egg transference, a divider was introduced, separating the sexes (Group 1 and 4). Afterwards a new female was introduced: an above-average female similar to the partner (Light blue) – Group 2; or a very large female (Woman in Red) – Group 3. Groups 1 and 4 have the same female before and after the divider was inserted. In sub-figure b) the inner circle colour represents the mother, while the outer colour represents the stimulus female that remained with the male during pregnancy. Error bar represents standard deviations (Image from Cunha et al. (2018)).

In order to complement the results from Cunha et al. (2018), this dissertation will focus on understanding whether the “Woman in Red” effect is widespread in the *Syngnathidae* family (i.e., linked to male pregnancy) or if it is dependent on the presence of a brood pouch. To this extent, we used the pipefish *Nerophis lumbriciformis* as a model species because, as with every syngnathid, it displays male pregnancy but, unlike *S. abaster*, this species has no brood pouch. Worm pipefish males have a groove in their abdomen to which the eggs are glued (Dawson, 1985; Monteiro et al., 2002). A fully enclosed pouch in a male provides not only nutrients (Paczolt & Jones, 2010) but also immunity to developing embryos (Roth et al. 2012). Therefore, the absence of a fully enclosed pouch can limit the amount of control the male has over its brood, which might, in turn, lead to the absence of the “Woman in Red” effect. Contrarily, the lack of a pouch can also lead to an easier disposal of eggs in the case of deliberate abortion as pregnant *N. lumbriciformis* males are capable of scraping along surfaces (in the environment or an enclosure) when in stress, possibly in an attempt to discard the eggs or to

overcome the lack of control that comes from the lack of a marsupium. This difference can present an advantage to species that are adapted to lower temperatures and consequently possess longer gestations (Gillooly & Dodson, 2000) since the male can abort the brood of a less desirable female more readily.

## 2. Methods

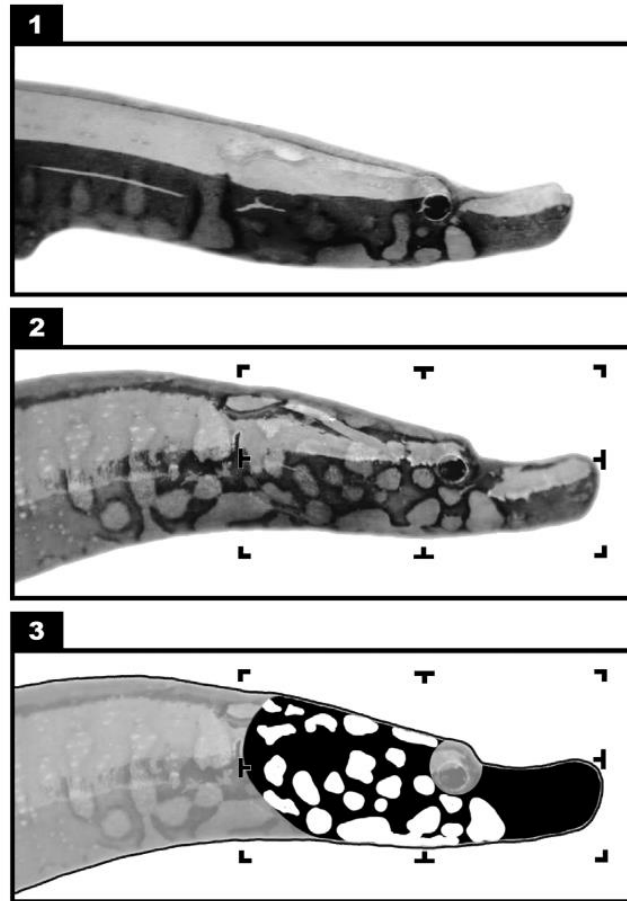
### 2.1 *Nerophis lumbriciformis*

The worm pipefish belongs to the family *Syngnathidae*, whose name derives from Greek, *σύν*, meaning “together”, and *γνάθος*, meaning “jaw”, thus describing the fused, tube-shaped elongated snout that is characteristic to the family.

*Nerophis lumbriciformis* (Jenyns, 1835), possesses an elongated worm-like body, with an almost cylindrical shape right to the end of the tail which flattens at the tip. The tail is much longer than the rest of the body. It also possesses a skeleton riveted with dermal armoured plating forming rings along the length of the body. Adults are sexually dimorphic with female adults being larger and possessing an abdomen with a more pronounced oval shape. The abdomen can become especially prominent during the breeding season, with the female's ovaries becoming turgid and bloated. Contrarily, males possess an abdomen that is either flat or slightly bending inwards forming a concave shape where the female eggs are laid, glued to and inseminated after successful mating (Monteiro et al., 2002).

The adult fish can be individually distinguished from one another by their facial markings (Monteiro et al., 2005a), consisting of a variable number of white spots of distinct sizes (Figure 2), a characteristic that we used during our experiments. These white spots become more prominent as the breeding season begins and are linked to the rank of the females within reproductive grounds, that are shaped as inverted leks (Monteiro et al., 2017a). More ornamented the females tend to have higher reproductive success and heavier gonads.

*N. lumbriciformis*' distribution spreads through the Atlantic coast, from Norway to Marroco (Dawson, 1986; Miller & Loates, 1997; Nieto, 1991) (Figure 3).



**Figure 2** - Facial pigmentation in adult *Nerophis lumbriciformis*: 1 - Male; 2 - Female; 3 - head region used for individual recognition and calculation of individual facial ornamentation (Image from Monteiro et al. (2006)). Females are usually far more ornamented than males (Coloured Male and Female see appendix picture S1).



**Figure 3** – *Nerophis lumbriciformis* geographical distribution, according to Dawson (1985)

## 2.2 Sampling and materials

All specimens were collected by hand during low tides (lifting or moving boulders and searching for any pipefish taking shelter underneath), at the Norte beach of Viana do Castelo (41°41'55.9"N 8°51'23.2"W) (Figure 4). In this location, the species is found throughout the year. The collection period of sexually mature pipefish necessary for the study was extended throughout several months (from October to January).

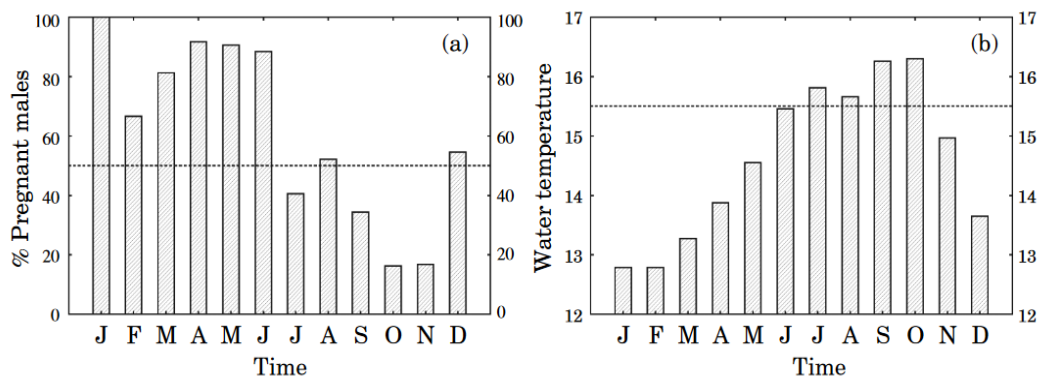
Water temperatures in Portugal are comparatively higher than those found in *N. lumbriciformis*' ideal habitats, farther north. Despite this, pregnancies throughout the year are not uncommon (Figure 5 (a)). The collection was undertaken during the species-specific reproductive period, commonly linked to periods of colder waters (Figure 5 (b)). An early collection period ensures that few females have already successfully mated, thus increasing our chances of using, in our experiments, females with high reproductive capacity. This happens because females have a limited number of eggs they can deposit per breeding season, and as such, we need to collect these females before they are given the chance to run out of eggs (Monteiro et al., 2017a). As higher-ranking females (rarer but highly sought after by both males in the wild and for this experiment) tend to be the first to empty their egg reserves relatively early in the breeding season, we tried to collect the sample as soon as possible.

Captured pipefish were transported to the lab in an isothermal container and, after an acclimation period, they were individually kept in 3 or 10 litre aquaria. We prepared artificial seawater, regularly kept at 33 ppm of salt, whose quality was ensured by continuous biological and physical filtration. The temperature was maintained constant at  $13 \pm 2$  °C. Pipefish were daily fed, *ad libitum*, with freshly hatched *Artemia* nauplii.





**Figure 4** - Sampling site for *Nerophis lumbriciformis* specimens.



**Figure 5** – (a) Percentage of pregnant males found in the wild according to the time of the year. The colder months of December and January represent the beginning of the *Nerophis lumbriciformis* breeding season. (b) Mean monthly surface water temperature in northern Portugal (Image from Monteiro et al. (2005b)).

### 2.3 Laboratory experiments

Taking into consideration the experiment devised by Cunha et al. (2018), briefly described in Figure 1, we programmed a similar experimental setup (conducted in the same aquarium system used in the “Woman in Red” experiment) aimed at trying to find evidence of the “Woman in Red” effect (or anything similar) in the worm pipefish. Our setup was simpler as, unlike Cunha et al. (2018), we were not interested at ascertaining if the expression of a Bruce-like effect could result from

the presence of an unfamiliar female – they have already shown that it did not (treatment 2 in Cunha et al. (2018)). Thus, we ended up with just three distinct treatments. In Treatment 1 (N=12), a male worm pipefish was paired with a female (larger, but not exceeding 1 cm of the male size) and they were kept together to the end of pregnancy. In Treatment 2 (N=8), a male was kept with a female similar to those of treatment 1 but, after egg transference was complete, the female was swapped by a very large female, the potential “Woman in Red” (at least 2 cm larger than the male). The pair were kept together up to the moment of parturition. In Treatment 3 (N=4), a male was paired with a very large female right from the beginning. Knowing that the number of replicates can have a direct impact on statistical power, we will attempt to justify the reasons that forced us to present a lower than initially intended number of replicates (especially in treatment 3) in the conclusion section.

Unlike in the experiment with *S. abaster* (see Cunha et al., 2018), as worm pipefish only accept eggs in a single event from one female (e.g., if a female deposits only a few eggs, the male is not able to mate again in order to fill up the remaining free brooding surface), we did not physically separate males from females during the extent of the pregnancy. Indeed, since eggs are exposed in the males, we were curious to see if a female acting as the “Woman in Red” could physically interact with the male so as to force the detachment of the eggs.

As we were able to collect, in the wild, males that were already pregnant, we also followed their pregnancies (N=8). This treatment of wild males would serve as a more natural control for the results of our lab-coerced matings. If, for instance, matings in the lab resulted in fewer or smaller eggs, we would be able to notice it when comparing with those of males that received eggs in the field.

During and at the end of each pregnancy, we measured several variables such as female facial ornamentation, egg number and size, offspring number and size, as well as offspring size variation within a brood.

### 2.3.1 Facial Ornamentation

Females display varying levels of investment in their eggs, which is a variable that can be partially ascertained through the measurement of the female’s facial ornamentation (Monteiro et al., 2017a). A female possessing high-quality eggs will tend to have more pronounced facial ornamentation and, therefore, be more attractive to the males (Figure 6 (a)). This ornamentation, a secondary sexual characteristic that conveys desirability, changes throughout the year, being most expressed as the individual prepares for its mating season during the colder months (Monteiro et al., 2017a).

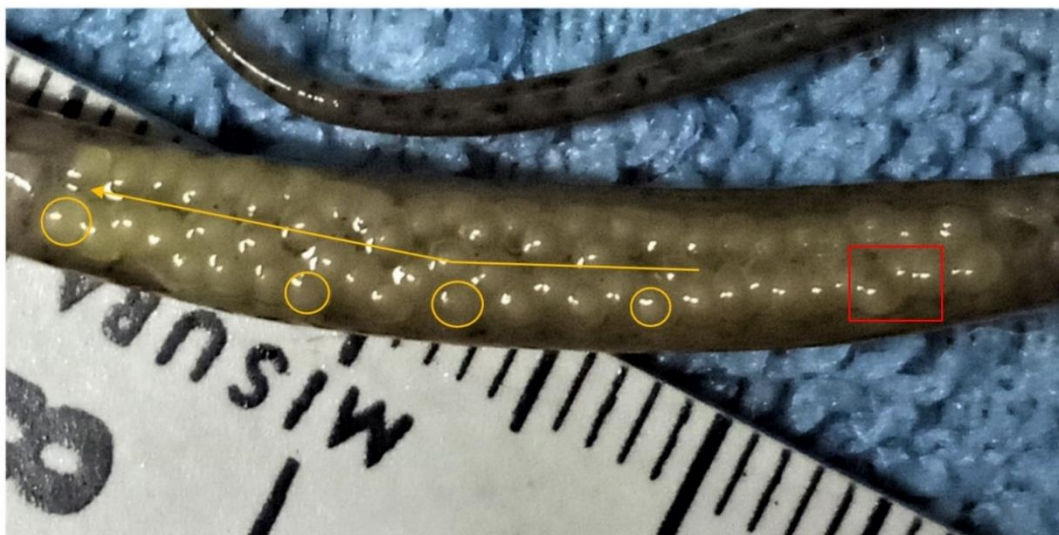
As a means of detecting differences in ornamentation in our experimental groups (which we will detail below) the mothers’ heads were photographed after each successful pregnancy. The percentage of facial colouration was determined as in Monteiro et al. (2005). Furthermore, the average facial ornamentation data of a total of 28 females from the wild, obtained from a previous collection period, were also added, for comparison with our laboratory-kept females.



**Figure 6** - (a) Difference between low (top) and high (bottom) ranked adult females. Higher ranked females within a lek will naturally possess a more pronounced facial ornamentation and therefore be more appealing to potential male partners, despite not necessarily being the largest females.

### 2.3.2 Eggs

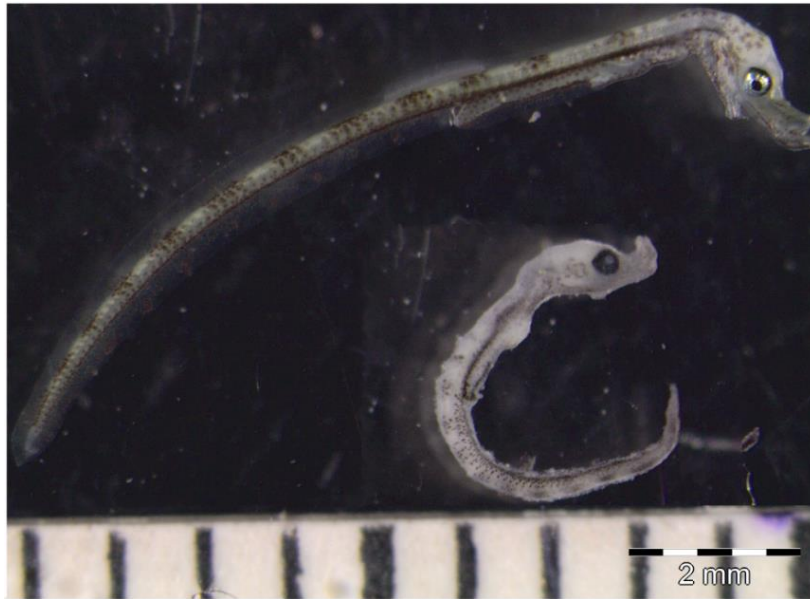
At the time of impregnation, each male was gently captured and had its egg surface photographed for posterior analysis in ImageJ. In each pregnant male, eggs were counted and measured (perpendicularly to the body line of the father), as an assessment of brood size and quality. Egg measurements can be tricky, as eggs can sometimes be compressed (especially in large broods). So, to avoid measurement errors, a total of ten non-compressed eggs were selected in every brood (Figure 7).



**Figure 7** – Egg measuring methodology. Example of four eggs (Yellow circle) chosen for their perfectly round shape, measured perpendicularly to the male's body line (Yellow arrow); Red Square – Example of a compressed group of three eggs, unsuitable for size analysis.

### 2.3.3 Newborns

Upon the birth of the entire brood, newborns were collected (using a small pipette), counted and individually photographed, alongside a ruler, for length measurements (ImageJ v. 1.8). As we observed newborns showing signs of incomplete development in all treatments, without any apparent pattern (as we will discuss in the results section), we decided to select only the offspring that seemed able to potentially survive (Figure 8).



**Figure 8** – *Nerophis lumbriciformis* newborns. Top newborn was born apparently healthy, displaying a well-formed snout. Bottom newborn is a still-born, displaying signs of an incomplete embryonic development.

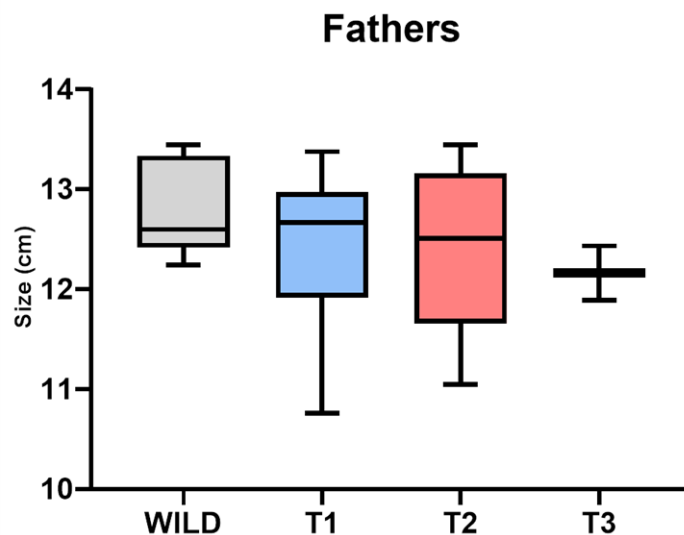
### 2.3.4 Statistical analysis

All statistical analysis was conducted either on Prism 8.0.1 or Statistica 10. Whenever using parametric tests (e.g., ANOVAs, analysis of variance), we first looked for normality and homogeneity of variances. We indicate, in the results section, whenever these assumptions were not met and why we decided to proceed with the analysis. We used one-way ANOVAs (with Tukey’s multiple comparison test, when appropriate) to assess potential differences between our treatments when looking at 1) the size of the males or the size of the used females, 2) female ornamentation (average percentage of spots covering the face), 3) egg size and egg number, 4) newborn number or size, and 5) size variation within the brood (coefficient of variation). When testing if groups differed in the percentage of abnormal embryos, we used a  $\chi^2$  proportion comparisons test. Furthermore, to specifically test if treatment 1 and 2 differed in newborn size, we used an unpaired t-test. Lastly, as egg number could be hypothesised as having an impact on offspring size, we used an ANCOVA (analysis of covariance) using egg size as a covariable.

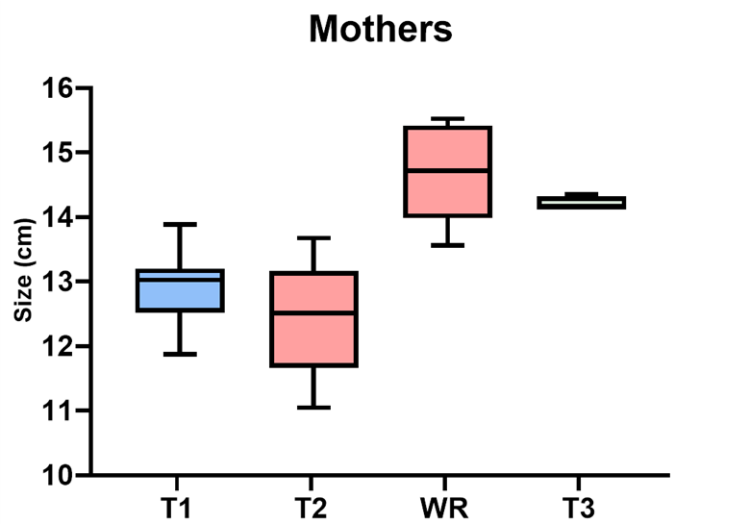
### 3. Results and Discussion

No significant differences were observed between the size of the males used in our treatments (one-way ANOVA;  $F(3, 24) = 0.746$ ,  $P = 0.535$ ) (Figure 9). As males used in the experiments showed similar body sizes, we are confident that any hypothetical difference found between treatments should not be attributed to male input (as we are especially keen on detecting female, rather than male, influence on offspring development).

Female size, however, differed between treatments (one-way ANOVA;  $F(3, 28) = 17.48$ ,  $P < 0.001$ ). As intended, the females that acted as mothers in treatments 1 and 2 were smaller than the females used to portray the role of the “Woman in Red” (treatments 2 and 3), as observed through a post-hoc Tukey’s multiple comparisons test (Figure 10).



**Figure 9** - Average size of the males used in our experimental treatments. WILD (Grey) represents the group of newborns born from males collected already pregnant and therefore with an unknown mother; T1 (Blue) represents treatment one where newborns were born of a father that was left with the same female throughout the duration of the pregnancy; T2 (Red) represents treatment 2 where newborns were born of a father whose coupling partner was switched to a female that was significantly larger (Woman in Red); T3 represents treatment 3 where newborns were born of a father who mated and remained with a “Woman in Red” female. Error bars depict maximum and minimum values, boxes show lower and upper quartiles, and middle line depicts the median.

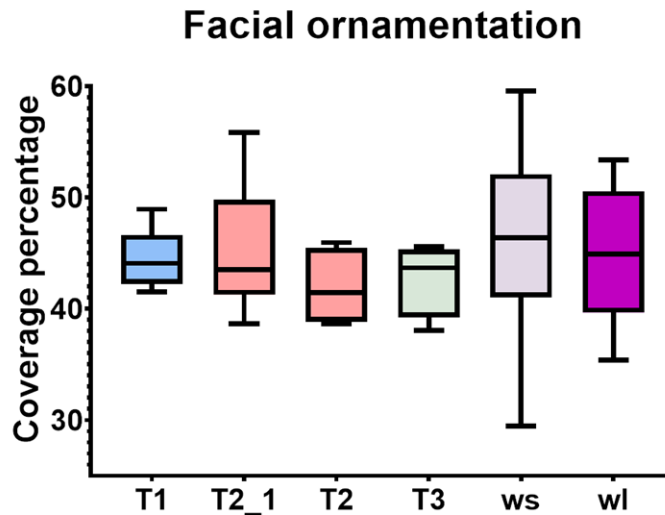


**Figure 10** - Average length of the females used in our experimental treatments. Mothers of treatment one and two are similar to each other, as are the mothers used in treatments three and the “Woman in Red” (WR) females. However, these two groups depict a significant size difference between them. Error bars depict maximum and minimum value, boxes portray lower and upper quartiles, and middle line show the median.

### 3.1.1 Facial Ornamentation

After every female’s average facial ornamentation coverage passed normality testing (Shapiro-Wilk test), they were submitted to a test of homogeneity of variance. The data showed no homoscedasticity among the treatments (Cochran C = 0.402,  $P < 0.005$ ). Nevertheless, according to Lindman (1970), the F statistic can be robust enough to accurately support variance analysis when there is no significant correlation between means and variances across the cells of the design, which was the case (Spearman correlation,  $r = 0.746$ ,  $P = 0.089$ ). So, we were able to confirm that there was no difference in facial ornamentation between females of different treatments (One-way ANOVA;  $F(5,50) = 0.430$ ,  $P = 0.825$ ) at the time of the males’ impregnation (Figure 11).





**Figure 11** - Facial ornamentation (as a percentage of facial coverage) used in our treatments. T2\_1 depicts the facial ornamentation of treatment 2 mothers and ws (wild small females; light purple) and wl (wild large females; dark purple) represent the added wild females caught during the breeding season, in a previous work. Error bars depict maximum and minimum value, boxes show lower and upper quartiles, and middle line depicts the median.

Monteiro et al. (2017a) have previously shown that, in the worm pipefish, female facial colouration and reproductive conditions are tightly correlated. They also showed that larger females tend to be more colourful (i.e. show a higher percentage of facial colouration). Thus, with no difference between female facial ornamentation at the time of the male's impregnation, we can hypothesise that females in each of our three treatments had the potential for similarly high levels of investment in reproduction.

Nevertheless, this unexpected result (lack of difference in facial colouration in females with different sizes) could have been primarily influenced by the housing environment as well as our experimental design. As fish were regularly fed, *ad libitum*, we might expect body condition to increase, thus helping to explain the similarity in colouration between female classes. In the wild, food might be harder to obtain, thus increasing the asymmetry between female colouration due to their ability to feed properly. Moreover, in our experimental setup, we kept females separated in individual enclosures, without visual contact with other pipefish. We did this explicitly to avoid overt female-to-female competition that, in the worm pipefish, sometimes results in females losing egg batches.

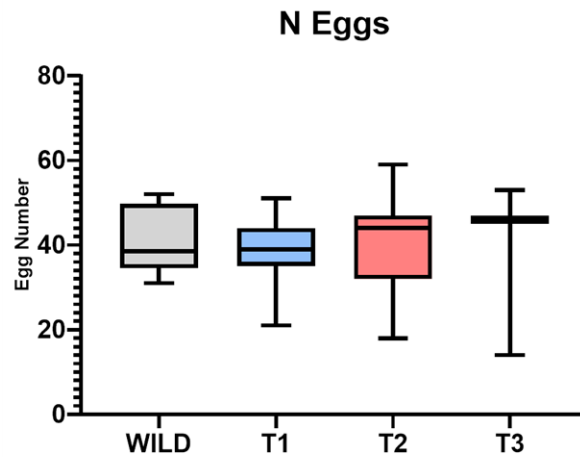


Female competition for males is one of the driving stimuli for the increase in facial ornamentation. As such, by isolating the females before pairing them, the development of these secondary sexual traits might not equate the levels seen in nature. It was to evaluate if such values were correct that the 28 wild female pipefish were added to the analysis. These wild females, unlike those used in our experiment, were collected and measured during the peak of their breeding season, ensuring a natural development of their secondary sexual characteristics. As the facial ornamentation coverage of the isolated females matched that of wild females (measured in nature during their breeding season), it suggests that the female ‘isolationism’ practised for reproduction control did not affect the percentage of facial ornamentation in the larger females. Nevertheless, it could have helped smaller females increase their colouration since they were not coerced by larger, more dominant females, as might happen in the leks observed in the wild (Monteiro et al., 2017a).

Given the observed results, it seems that female facial ornamentation does not change in the presence of a pregnant male (at least during the interval of our measurements). In treatments 1 and 3, a female whose partner was already pregnant with its offspring could have reduced colouration, especially when no other male was in the vicinity. Monteiro et al. (2017a) previously showed that females who laid eggs in a male tended to reduce colouration while the others continued to expand it. In our experiment, we could then expect that reproducing females would have lower colourations. That was not the case. Alternatively, we could also hypothesise that the “Woman in Red” females could also either expand - signalling their reproductive potential and prompting any hypothetical post-copulatory sexual selection mechanism that would free the male from its pregnancy and thus becoming available - or reduce their colouration, lowering the costs of maintaining a secondary sexual character in front of a single unavailable, already pregnant, male. Again, we were unable to see any meaningful differences in female colouration between the WR females and all the others. In summary, since female colouration did not vary between treatments, one might suggest that, in future experiments, females should perhaps be kept together, so as to not impact on intrasexual competition, which might be relevant for the expression of secondary sexual traits.

### 3.1.2 Eggs

We were unable to observe differences in the number of eggs carried by males among our treatments (one-way ANOVA:  $F(3, 25) = 0.106$ ,  $P = 0.956$ ; Figure 12).

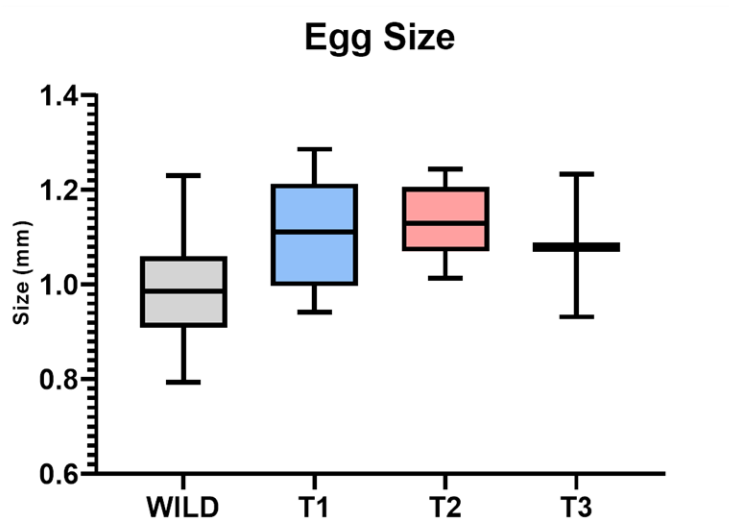


**Figure 12** - Average egg numbers in pregnant males, in our experimental treatments. Error bars depict maximum and minimum value, boxes show lower and upper quartiles, and middle line depicts the median.

Also, there was no difference in the size of the eggs when considering each treatment (one-way ANOVA;  $F = 2.120$ ;  $P = 0.124$ ; Figure 13). We were not expecting differences in egg size between treatment 1 and 2, since they were laid by females of similar size. Nevertheless, we were expecting larger egg sizes in treatment 3, since these were laid by larger females. We should note that, unfortunately, the number of pregnant males from treatment 3 was very low (as we will justify later) and, thus, the results from this treatment should not be over-emphasized.

Interestingly, the egg sizes measured in males captured in the wild while already pregnant did not significantly differ from those obtained in the lab, showing that our conditions were suitable for the production of 'normal' eggs. Although statistically similar, the average egg size from wild males was slightly smaller. We believe that this translates into the fact that males in the wild have the opportunity to mate with a vast array of females (small and large), which can have an impact on egg size. In the lab, although we used two female size classes, we should stress that even our 'smaller' females are relatively large when compared to most of the females

regularly observed in the field. They are smaller only in comparison with the very large, and hard to sample (rarest female in the wild), females used to act as “Woman in Red”.



**Figure 13** - Average egg size of the brood of males used in our experimental treatments. Error bars depict maximum and minimum value, boxes show lower and upper quartiles, and middle line depicts the median.

With some exceptions, all males gave birth to living offspring. The exceptions (N = 4; one in treatment 1 and 3, and two occasions in treatment two) were eggs that the male released nearing the one-month term completion (Figure 14). All of these embryos stopped their development at the gastrulation stage (see Monteiro et al., 2003). We could wonder if these occurrences could support the hypothesis of an abortion mechanism in *N. lumbriciformis*. However, we think it is unlikely as the few cases observed were scattered among all treatments. It is possible that these few instances of developmental arrest result from the stress induced by the repeated capture of the female that shared the male’s living space.

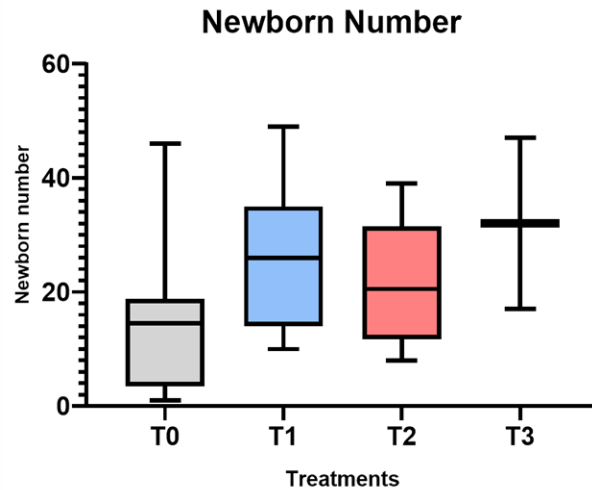


**Figure 14** - Row of unborn eggs at the end of term, that do not depict embryonic development past gastrulation stage. Right image is a close-up of two embryos.

### 3.1.3 Newborns

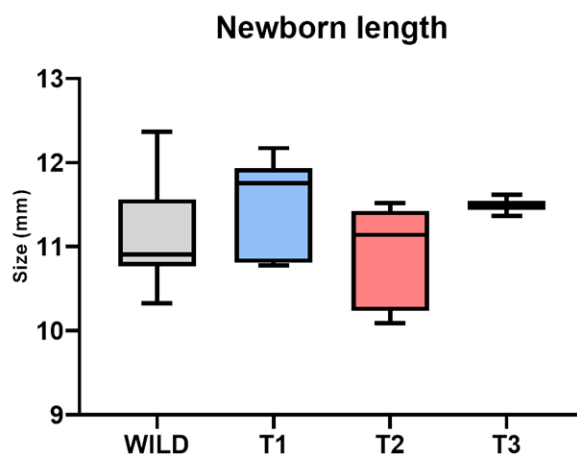
As previously mentioned, we opted to include only the offspring that could potentially survive in the wild, disregarding those presenting deformities or a lack of a completed embryonic development (Initial n<sup>o</sup> offspring-Final n<sup>o</sup> offspring, the average reduction in offspring number as a percentage; WILD: 165-123, 25%; T1: 216-186, 14%; T2: 149-130, 13%; T3: 69-64, 7%). We made this decision based on two lines of evidence: 1) the highest number of less developed offspring was observed in the wild males' group (twice as high as in our experimental groups), and 2) treatment 2 was not significantly different (proportion comparison;  $\chi^2 = 0.114$ ,  $P = 0.735$ ) from treatment 1 (which would be expected if the “Woman in Red” effect occurred in the worm pipefish in a similar way as it was described in the black-striped pipefish).

We found no significant difference among groups in the number of offspring (one-way ANOVA;  $F(3, 19) = 1.179$ ,  $P = 0.344$ ; Figure 15).



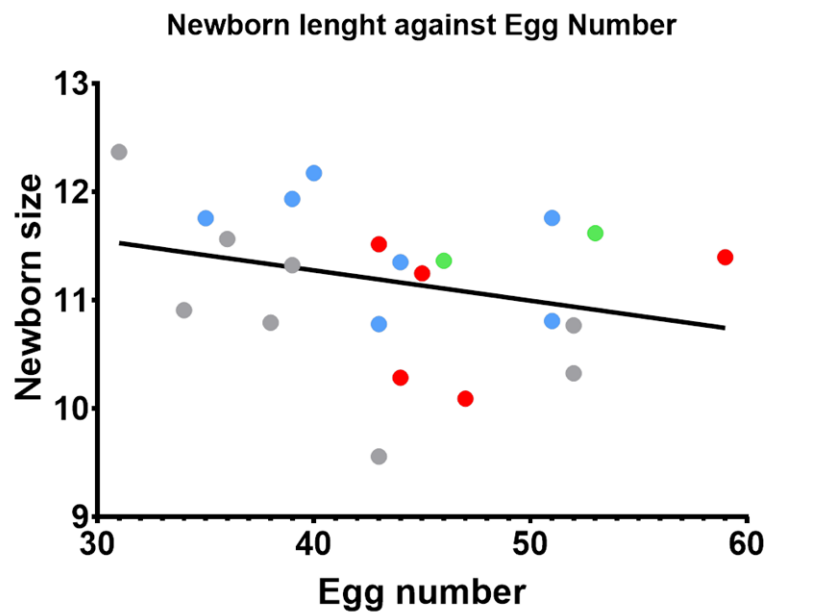
**Figure 15** – *Nerophis lumbriciformis* newborns obtained from our experimental treatments. Error bars depict maximum and minimum values, boxes show lower and upper quartiles, and middle line depicts the median.

Also, we were unable to find differences in offspring size at birth (one-way ANOVA;  $F(3, 18) = 1.208$ ;  $P = 0.335$ ; Figure 16), with the Wild males' group acting as an indication of the possible range of outcomes that could occur in nature, encompassing most treatment values within its range. Since the natural variation observed from the wild males' group (expected, as these males probably mated with a diverse array of females) could potentially interfere with our ability to discriminate more subtle differences in our experimental groups, we went a step further and tested newborn sizes between treatment 1 and 2. Again, we were unable to discern differences between the groups (Unpaired t-test with Welch's correction;  $F = 1.199$ ,  $P = 0.099$ ).



**Figure 16** - Average newborn lengths, obtained in our experimental treatments. Error bars depict maximum and minimum values, boxes show lower and upper quartiles, and middle line depicts the median.

After obtaining these results, we wondered if the newborns' length could potentially be influenced by the total number of eggs each mother laid. As resources are finite, reproducing females face the inevitable dilemma of producing more or larger, richer eggs. Figure 17 shows that, despite the slope suggesting a negative correlation between newborn size and the egg count ( $r = 0.285$ ,  $P = 0.198$ ), data variability hindered the discovery of a significant correlation between the two considered variables.

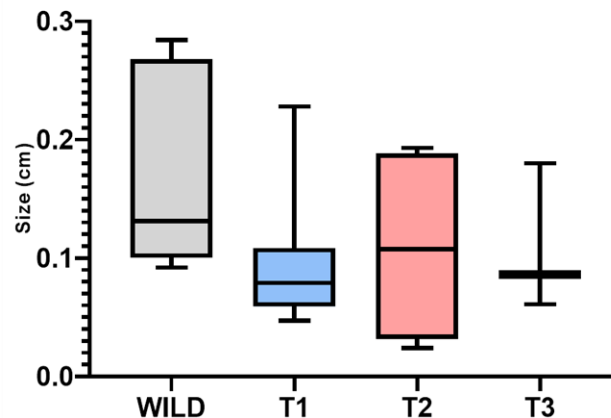


**Figure 17** - Scatterplot displaying the relation between newborn length (mm) and egg number (WILD – Grey; T1 – Blue; T2 – Red; T3 – Green;  $y = 12.39 - 0.028x$ )

We conducted an analysis of covariance (ANCOVA) using the egg count as the covariable and newborn length as the independent variable. Again, we were unable to find differences between newborns' length among our treatments, even when correcting for the egg number (ANCOVA;  $F(2,17) = 2.422$ ,  $P = 0.118$ ).

Lastly, we tested if the variation of offspring size within a brood (as observed by Cunha et al., 2018) could highlight any differences between our groups (we also used the coefficient of variation). Once more, we did not find significant differences between our groups (one-way ANOVA;  $F(3,22) = 2.399$ ,  $P = 0.095$ ), despite the wild males' group higher intra-brood offspring size variation (Figure 18).

### Brood coefficient of variation on newborn size



**Figure 18** - Average intra-brood offspring size variation (coefficient of variation) in our experimental treatments. Error bars depict maximum and minimum value, boxes show lower and upper quartiles, and middle line depicts the median.

Overall, unlike the results obtained by Cunha et al. (2018), we were unable to find any evidence of visible differences between our treatments. If the “Woman in Red” effect (or any other post-copulatory sexual selection mechanism that produces a similar effect) occurs in the worm pipefish, we were unable to detect it among our selected endpoints (many of which were identical to those selected by Cunha et al., 2018). For instance, we found no indications of brood heterogeneity in treatment 2, an expected result if the males were prone to disinvest in the current brood when faced with a higher rewarding mating prospect. Miranda et al. (2017) showed that male worm pipefish have the ability to allocate resources to a lower-quality brood, losing considerable weight in the process. This reproductive compensation (a special kind of differential allocation) shows that worm pipefish males are not passive egg carriers, but are able to interact with the developing brood intimately. Taking into consideration the natural reproductive system of this species, Miranda et al. (2017) hypothesised that this compensatory mechanism helps understand why males tend to select large and colourful females, which are more fecund and able to produce larger eggs (see Monteiro et al., 2017a). Thus, we were expecting that, similarly to *S. abaster*, worm pipefish males could also show disinvestment in the brood when exposed to a preferred female (the “Woman in Red”). A lack of investment would potentially promote offspring heterogeneity, as observed by Cunha et al. (2018). If this is the case in the worm pipefish, we were unable to detect it.

## 4. Conclusion

The “Woman in Red” effect seems to be absent in *Nerophis lumbriciformis*.

Unlike the quite evident results obtained from Cunha et al. (2018), which ultimately led to the conclusion that black-striped pipefish males were able to reduce investment in their developing broods when they encountered a potentially better mate, we were unable to observe a similar pattern of results in a different pipefish species. Even though we knew beforehand that worm pipefish males were able to impact their pregnancies (Miranda et al., 2017), an observation that insinuated the possibility of the existence of a “Woman in Red” effect, no visible disinvestment on the brood was recorded. Thus, it seems that taxonomic relatedness and male pregnancy are not enough arguments to support the idea of a family-prevalent post-copulatory sexual selection mechanism.

The offspring of males that were exposed to a large, attractive female were similar to those from males that mated with a less striking female. Moreover, the offspring were similar to those emerging from broods of males that got pregnant in the wild. All pregnancies being fundamentally alike, we might propose at least two principal, but possibly non-exclusive, interpretations of the results: 1) the occurrence of the “Woman in Red” effect is dependent on the existence of a marsupium; 2) our experimental setup, given the peculiar life history of the worm pipefish, failed to gather all the appropriate conditions to allow for the expression of a post-copulatory sexual selection mechanism equal or similar to the “Woman in Red” effect.

Despite our efforts to replicate the general conditions used by Cunha et al. (2018), there is one noticeable difference between theirs and our experiment. Unlike *Syngnathus abaster* males, *N. lumbriciformis* males do not possess a closed marsupium (see Figure 19). The evolutionary radiation of the family *Syngnathidae* seems to have been tightly connected to the diversification of structures involved in male parental care, with anatomical variations signalling differences in reproductive strategies (Monteiro et al., 2005c). Curiously, the first proposed phylogeny of the *Syngnathidae* family was based solely on brood pouch position and complexity (Herald, 1959). With the advent of genetics, new phylogenies were proposed (Hamilton, et al., 2017; Wilson et al., 2001; Wilson et al., 2003) which, without much



surprise, coincided with the initial ideas proposed by Herald (1959). Much more than a happy coincidence, these results show how the development and diversification of structures involved in paternal care (from simple ventral glueing areas to completely enclosed pouches) shaped the evolutionary pathway of the *Syngnathidae*.

A fully enclosed brood pouch likely gives the father the advantage of being far more capable of influencing its brood. This father-offspring close interaction is known to occur in many distinct ways, from aeration to nutrient exchange or immune priming (e.g. Monteiro et al., 2005; Roth et al., 2020). Thus, we might wonder whether a closer relationship between developing embryos and their father might be necessary for to the expression of the “Woman in Red” effect (or other similar post-copulatory sexual selection mechanisms).

There is, however, one line of evidence that deserves further attention: how do males exchange nutrients with their young? In a closed marsupium such as that of *S. abaster*, a highly vascularized placenta-like tissue contacts with the developing young (Watanabe et al., 1999; Partridge et al., 2007; Ripley, 2009; Carcupino et al., 1997), allowing for bidirectional transport between father and embryos (Sagebakken et al., 2010). This means that males can export nutrients to the developing embryos but also that they can sequester resources from their brood pouch, namely by reabsorbing and recycling aborted embryos. It should be noted that aborted embryos are common phenomena during pipefish pregnancies (see Cunha et al., 2018). Thus, if a male ‘wants’ to invest more heavily in its brood, it can do so at its own expense, by either sharing from his resources, or redirecting resources obtained from reabsorbed embryos. What Cunha et al. (2018) hypothesised is that the “Woman in Red” effect largely derives from *S. abaster* males stopping the reinvestment in their broods, by sequestering resources obtained from aborted embryos and keeping them for a future reproduction episode. Thus, the existence of a marsupium is integral to this strategy. In a pipefish without a closed brood pouch, like *N. lumbriciformis*, there seems to be no way of reabsorbing aborted embryos as the nutrients would be lost in the water (instead of being contained within the sealed marsupium whose inner lining is well equipped for absorption). The marsupium might just be integral to the expression of the “Woman in Red” effect.

We should stress, however, that there is still no indication that the “Woman in Red” effect is widespread in the *Syngnathidae* family. The discovery of this post-copulatory sexual selection mechanism is very recent and, although it might occur in other species (see preliminary evidence in Paczolt & Jones, 2010), it was only described in a single species (*S. abaster*). Thus, not finding this mechanism in another species also does not add much to the discussion of its actual prevalence within the family.

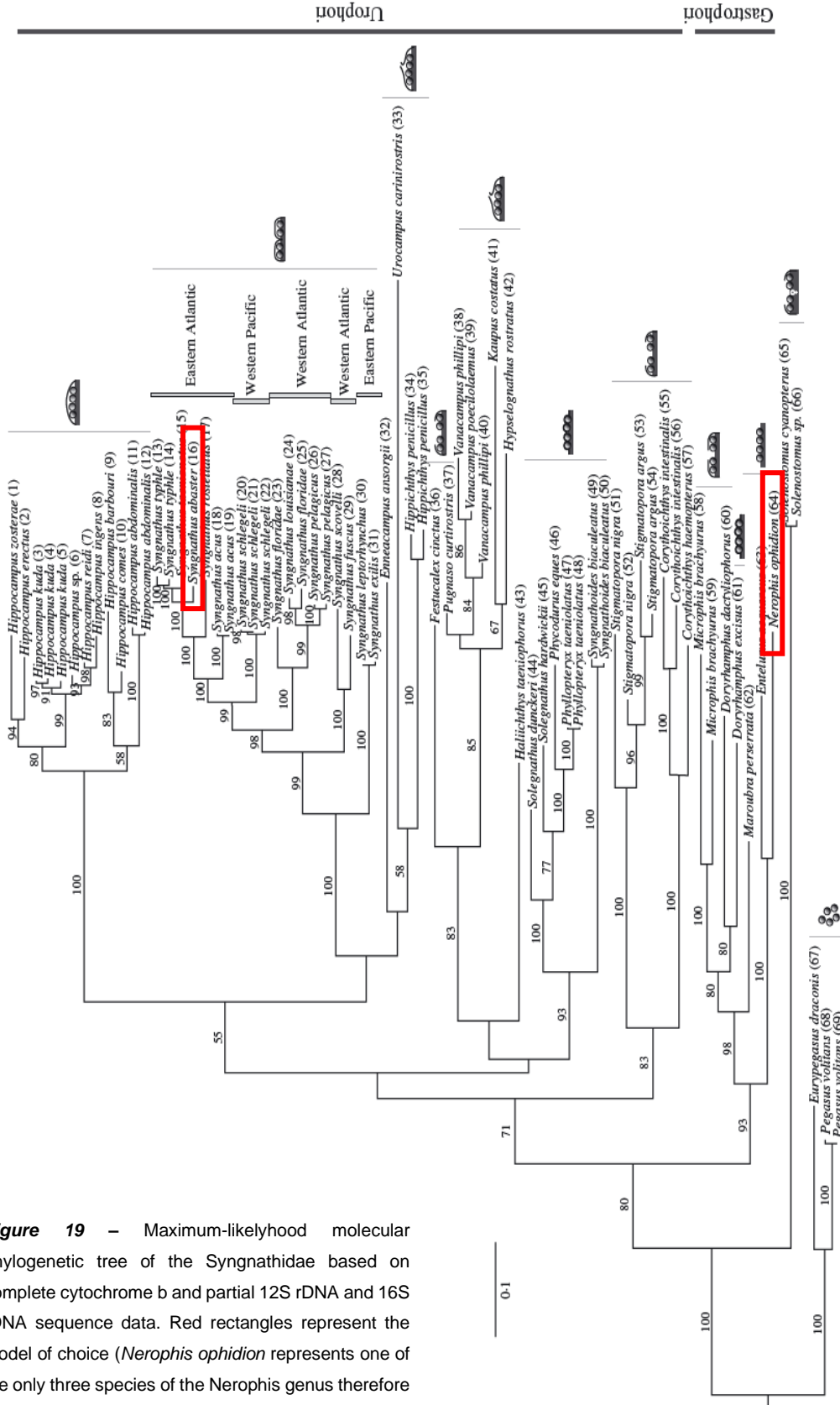
We should also take into consideration that our experimental setup might not have contributed to the potential expression of the “Woman in Red” effect. In retrospective, we must acknowledge that our experiments had clear limitations. The first was our choice of having individuals (namely females) isolated. As we discussed before, we did this in order to avoid females from competing and losing egg batches. If we were unable to detect that a female had lost a substantial portion of their reproductive ability, matching that female to a male could have potentially produced biased results. Despite our good intentions, we also acknowledge that by keeping females separated, we disallowed female-female competition, which might be integral to the expression of secondary sexual traits and egg investment strategies (see Monteiro et al., 2017a, b).

Our second, and very relevant, concern is related to the low number of replicates (quite visible in treatment three). We are aware that we should have used more pipefish to produce more replicates for our experiments. This would allow for an increased sense of security when stating that the “Woman in Red” effect seems to be absent in the worm pipefish. The reasons behind this caveat are, unfortunately, relatively straightforward. Initially, this MSc project was comprised of two main experiments: the first would evaluate if the “Woman in Red” effect was present in the worm pipefish. The second experiment, using *S. abaster*, was meant to assess which factors lead to the expression of the “Woman in Red” effect. Since the two species display different breeding seasons, the two experiments could be conducted independently and sequentially: the worm pipefish starts breeding in the winter (Monteiro et al., 2001), and the black-striped pipefish starts to reproduce in spring when waters get warmer (Silva et al., 2007). Unfortunately, shortly after the sampling period of *N. lumbriciformis* began, the COVID-19 pandemic settled in Portugal. A lockdown ensued which prevented the collection of more specimens required for new replicates. Since our aquaria are settled in an independent room

at CIBIO, we were able to keep most of the experiments going, avoiding contact with other researchers.

Nevertheless, some of the planned endpoints (e.g. offspring movement patterns) could not be completed. On top of the impact that the COVID19 had on the worm pipefish experiment, we were forced to entirely drop our goal of trying to further explore some of the mechanistic aspects of the “Woman in Red” effect, using the black-striped pipefish. Besides personal security issues, the then-current public health situation did not advise for the planning of a new experiment.

In the future, once pandemic-related constraints are lifted, experiments such as the second part planned for this project should be attempted. The “Woman in Red” effect, a Bruce-like effect, is a very new concept outside placental mammals. Hence, Syngnathids could become an even more fertile bed for innovative studies on sexual selection and intersexual conflict.



**Figure 19** – Maximum-likelihood molecular phylogenetic tree of the Syngnathidae based on complete cytochrome b and partial 12S rDNA and 16S rDNA sequence data. Red rectangles represent the model of choice (*Nerophis ophidion* represents one of the only three species of the *Nerophis* genus therefore *Nerophis lumbriciformis* would be included on a similar phylogenetic level). Image taken from Wilson & Orr 2011

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## 6. Appendix



**Figure S19** – Female (top) and male (bottom) *Nerophis lumbriciformis*. Males are typically far less ornamented than their female counterparts.