UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

Long-term seasonal changes in the abundance of *Dendrodoris* nudibranchs: a five-year survey

Ricardo Sousa Cyrne

Dissertação

Mestrado em Biologia Marinha

Trabalho efectuado sob a orientação de:

Alexandra Teodósio, PhD

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Declaração de autoria de trabalho

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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Ricardo Sousa Cyrne

INDEX:

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À minha orientadora, Professora Alexandra Teodósio, que após alguns anos de interregno me ajudou, mesmo à distância, a terminar uma etapa que ficou por concluir em 2006.

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ABSTRACT:

Researchers' interest in nudibranch species has grown during the last decades mainly due to their potential in biotechnology and neurobiology. However, a low number of ecological studies have been undertaken on this mollusk group, especially using long-term scales.

In certain intertidal and subtidal locations some species of nudibranchs suddenly appear in very large numbers and also suddenly disappear. These seasonal events are observed and explained by several theories, being the most accepted the migration theory – inshore movements to copulate and egg deposition. However, controversy still exists in whether nudibranchs migrate or not.

The aim of the present thesis is to study the migration behavior towards the intertidal zone of two nudibranch species, *Dendrodoris herytra* and *D. grandiflora* in Sado estuary, Portugal. Here it is shown that *Dendrodoris* nudibranchs start to appear in the intertidal areas in March and completely disappear around July, reaching pick density between April and May. Possible connections between *Dendrodoris* migrations with environmental parameters such as salinity, water and air temperature and water turbidity were assessed. The latter environmental factor revealed the strongest association with nudibranchs' presence in such intertidal areas; i.e. the peak in nudibranch density was consistently observed during the period of higher turbidity. Since most of the individuals were mature and ready to spawn when caught, one may argue that the increased turbidity (as a proxy of increased phytoplankton biomass) should directly enhance the survival rate of planktotrophic larvae. Other possible explanations may include food foraging, as well as predator avoidance.

During this long therm survey of five years, the density of emmerged individuals was very consistent. The environmental factors gave clues on what triggers this seasonal events, and how they induce the periodic appearance during spring months. The fact that both species were observed emmerged during low tide increases even more the interest in them and their biology.

Nevertheless the great contribution of this research to nudibranchs ecology, studies still need to be undertaken to fully understand the causes behind the occurrence of such episodic events.

KEYWORDS:
Dendrodoris herytra; Dendrodoris grandiflora; sea slug; migration; intertidal environments; emmersion.

RESUMO

Os nudibrânquios são moluscos gastrópodes delicados e coloridos. Pertencem à superordem Nudibranchia e são caracterizados por terem uma concha e cavidade no manto muito reduzidas ou completamente ausentes. Estes organismos apresentam uma distribuição global, ocupando uma grande variedade de *habitats*, desde águas tropicais marinhas, ao frio do Oceano Ártico. São componentes importante dos ecossistemas marinhos bentónicos e normalmente podem ser encontrados a alimentar-se no substrato.

Apesar da sua elevada capacidade de adaptação a diversos *habitats*, há poucos estudos ecológicos sobre este grupo, sobretudo de longa duração.

O ciclo de vida dos nudibrânquios compreende geralmente duas estratégias: i) um tempo de vida curto, quando a espécie tem mais do que uma geração cada ano; ii) um ciclo de vida anual quando as espécies desovam num período por ano. São hermafroditas e conhecidos pelo seu comportamento de agregação para a cópula, depositando as posturas em rochas ou outros substratos. Para tal, alocam grande parte da sua energia na procura de um parceiro hermafrodita, cópula, produção de massa da postura. Neste contexto há uma grande discussão global sobre se os nudibrânquios morrem imediatamente após a desova.

Em vários locais, certas espécies de nudibrânquios aparecem repentinamente em zonas intertidais em números muito grandes e desaparecem igualmente rápido. As razões por detrás destes movimentos verticais em nudibrânquios têm sido controversos e alguns estudos sugerem a teoria da migração. Esta assume que populações subtidais, migram para águas poucos profundas para copular e depositar as suas massas de ovos. Outras explicações foram propostas para estes movimentos costeiros tais como: i) influência de marés, correntes ou ondas; ii) procura de alimento; iii) orientação do campo geomagnético. Outras teorias justificam desaparecimento repentino devido à morte após a desova. Assim, há uma grande controvérsia sobre se o aparecimento sazonal esporádico em zonas intertidais é devido a uma migração. Estas diferentes justificações destacam a falta de informação sobre o ciclo de vida e ecologia geral de nudibrânquios.

Os organismos modelo da presente dissertação são as espécies *Dendrodoris grandiflora* e *D. herytra*. A espécie *D. grandiflora* tem um corpo plano, com cerca de sete centímetros. Apresenta um grande leque de cores que podem ir de cinza, creme, amarelo a castanho com manchas no corpo. Habita no ambiente subtidal até aos 25 m

podendo também ser encontrada na zona intertidal. A espécie *D. herytra*, tem um corpo plano de cerca de cinco centímetros de comprimento e três de largura. A sua coloração mais típica é o amarelo forte e uniforme. Apresenta uma distribuição muito grande e habita também zona subtital até aos 25 m podendo também ser encontrada nas zonas intertidais.

O objectivo do presente trabalho é estudar o comportamento da migração para zonas intertidais das espécies *D. herytra* e *D. grandiflora*. Dados preliminares de um estudo de quatro anos, sugeriam que as migrações podem estar relacionadas com factores ambientais. Foi feito um quinto ano de observação, a fim de obter mais dados bem como analisar as possíveis relações entre a densidade de indivíduos com vários parâmetros ambientais, tais como a salinidade, temperatura da água e ar, e a turbidez.

A observação ao longo dos 5 anos (2011-2015) dos individuos das espécies *D. herytra* e *D. grandiflora* foram efectuados por dois investigadores, num banco de ostras na Reserva Natural do Estuário do Sado (38.474857ºN, -8.775255ºW) durante as marés mais baixas do mês (< 0.8 m). Os dados de densidade foram registados e posteriormente analisada a relaçao com os parâmetros ambientais (salinidade, turbidez e temperatura do ar e água) usando uma regressão múltipla.

O aparecimento sazonal de *D. herytra* e *D. grandiflora* emersos, na zona intertidal foi observada principalmente durante os meses de primavera. A cada ocorrência, normalmente no mês de Março em baixa densidade, seguiu-se um aumento progressivo, atingindo o pico em Abril, e descendo até ao desaparecimento consistente de ambas as espécies a partir de Julho (Figura 7). Este padrão é consistente ao longo dos 5 anos de observações, com excepção do de 2011 em que o aparecimento foi mais tardio (Abril) e conincidiu com o ano em que foi registada a maior densidade de individuos. Em geral, sempre que foram registados individuos *D. herytra* e *D. grandiflora*, a espécie *D. grandiflora* surgiu sempre em maior densidade que *D. herytra*. A maioria dos indivíduos foram encontrados sozinhos, estáticos e alguns de virados para cima. Ocasionalmente os indivíduos foram observados abrigados em ostras, presumindo-se que para abrigo.

As variações dos parâmetros ambientais (salinidade, turbidez e temperatura do ar e água) durante os anos de 2011 a 2015, estão representados na Figura 8. A turbidez e a temperatura do ar e da água, apresentam padrões consistentes ao longo dos anos. Como esperado os gráficos de temperatura apresentam uma forma de sino, com o pico no verão e mínimo no inverno. A salinidade por seu lado não mostrou nenhum padrão claro.

Para efeitos estatísticos, a temperatura do ar não foi considerada, uma vez que se verificou uma correlação com outro parâmetro, a temperatura da água. O máximo de temperatura nunca foi coincidente com o máximo de densidade. Por outro lado, foi observada uma relação significativa entre a salinidade e a turbidez das duas espécies de *Dendrodoris* (Tabela 2).

Para tentar entender este comportamento, duas questões se levantam: i) serão estes eventos migrações horizontais?; ii) quais as causas que o motivam este aparecimento e desaparecimento súbito?. Neste estudo, o cenário é ainda mais controverso uma vez que os indivíduos de ambas as espécies, *D. herytra* e *D. grandiflora,* foram observados emersos, e como tal sujeitos a grande stress térmico e possível dessecação.

De acordo com as observações efectuadas, o aparecimento não deve ser justificado com a deposição de ovos uma vez que não foram encontradas massas de ovos em todas as observações, apesar de os indivíduos observados estarem em avançado estado de maturação e prontos a desovar, conforme observação em laboratório de indivíduos recolhidos no campo. Para além disso, o rápido desaparecimento não pode ser justificado pela morte após a desova, pois foi observado que os indivíduos efetuavam várias desovas ao longo do tempo em que foram mantidos para observação no laboratório.

Os resultados obtidos sugerem que as variáveis ambientais têm influência neste evento. O aparecimento sazonal foi consistente nos 5 anos observados, ocorrendo entre Março e Junho. Para além disso, o pico de densidade foi sempre atingido entre Abril e Maio, quando a temperatura da água ronda os 19 ºC em média e desaparecem em Julho quando a temperatura ronda os 23 ºC.

Dos quatro parâmetros ambientais registados, apenas foi comprovada estatisticamente a relação da salinidade e da turbidez com as densidades de *D. herytra* e *D. grandiflora*. No entanto a salinidade não parece explicar por si só a variação na densidade uma vez que não existe um padrão claro associado a este parâmetro, conforme esperado numa zona de estuário, em que há naturalmente grande flutuação de salinidade devido aos ciclos de marés, precipitação e outros. Quanto à turbidez, parece influenciar fortemente a presença de nudibrânquios na área intertidal uma vez que a sua presença coincide constantemente com um aumento e pico de turbidez. Além disso, em alturas de menor turbidez, ocorrem menores densidades de *D. herytra* e *D. grandiflora* (e.g. de primavera de 2012). A turbidez pode ser influenciada por vários fatores, tais como ondulação, re-suspensão de sedimentos, fluxo de água do rio e pressão antropogénica. Mais importante, a turbidez está associada ao aumento da biomassa de fitoplâncton, regularmente associada a *blooms* de primavera. Este será provavelmente o factor mais decisivo na ocorrência consistente de turbidez no local de amostragem durante a primavera. Estes de *blooms*, estão normalmente associados aumentos de produtividade secundária, incluíndo de zooplâncton. Uma vez que as larvas de *D. herytra* e *D. grandiflora* são provavelmente planctotróficas, estas irão beneficiar do aumento da concentração de fitoplâncton. Para além disso, a maior turbidez confere maior proteção dos predadores.

O presente estudo constitui um exemplo adicional de uma população de duas espécies, *D. herytra* e *D. grandiflora*, que aparecem sazonalmente na zona intertidal, mas emersas, e providencia um conhecimento essencial sobre a influência dos parâmetros ambientais nesses acontecimentos.

FIGURE CAPTIONS:

Figure 1 – *Dendrodoris grandiflora* egg masses in Laboratório Maritimo da Guia aquaculture holding tanks.

Figure 2 – *D. herytra* (left) and *D. grandiflora* (right) (photos adapted from Calado & Silva, 2012).

Figure 3 – Sampling site in the Sado Estuary

Figure 4 – Observers counting of nudibranchs in the oyster sandbank, located in Sado Estuary

Figure 5 – *Dendrodoris grandiflora* (top) and *D. herytra* (bottom) emerged in the oyster sandbank

Figure 6 – *Dendrodoris grandiflora* (black) and *Dendrodoris herytra* (yellow) monthly density (m^{-2}) from 2011 to June 2015.

Figure 7 – Environmental parameters from 2011 until June 2015 (water temperature, salinity, turbidity and air temperature) with density quartiles (grey)

Figure S1 – *Dendrodoris grandiflora* copulating in aquaculture holding tanks in Laboratório Maritimo da Guia.

Figure S2 - *Dendrodoris grandiflora* swimming at the surface in aquaculture holding tanks in Laboratório Marítimo da Guia.

Table 1 – Summary of the type and possible causes of horizontal movements (migrations) in sea slugs.

Table 2 – *p* values for *D. herytra* and *D. grandiflora* in regards to the environmental parameters. $p<0,05$ values highlighted in bold.

Table S1 – Full list of species described in the references used in Table 1.

1. Introduction

Nudibranchs, also called sea slugs, are delicate, colored and soft-body gastropod mollusks. These organisms belong to the superorder Nudibranch and are characterized by having a shell and mantle cavity that are either reduced or completely absent (Wägele et al. 2014). They are spread world-wide, occupying a wide range of habitats, from marine tropical waters to cold deep Artic Ocean (Dionísio et al. 2013). They constitute important components of benthic marine ecosystems and can be commonly found grazing on the substrate, in association with corals, feeding on macroalgae, or crawling over rocks or on any other substrate (Behrens and Valdés 2004).

Despite their high adaptation capacity to several habitats, there are few ecological studies on this group. In fact, much of the present ecological knowledge is occasionally found in studies not targeted to this group (Geange and Stier 2010; Rossi et al. 2012),.

1.1. Taxonomic features

Taxonomy, systematics and phylogeny of nudibranchs occupy a great part of the research done on sea slugs. Wägele et al. (2014) confirmed the most recent classification proposed by Jorger et al. (2010), in which sea slugs are considered as members of the subclass Heterobranchia, a highly diversified and successful group of marine gastropods, which includes nudibranchs in the informal group Opistobranchia (Wägele et al. 2014).

Many nudibranchs exhibit vivid body colors with unique patterns that often serve as useful characteristics for identification of species. On the other hand, considerable variation in colors and patterns also occur within a species, occasionally causing taxonomical confusion (Behrens and Valdés 2004). In fact, individuals with similar morphology but different colors/patterns can be considered a single species with color variations or as several distinct species. In such cases, radula, external morphology, internal anatomy, and larval characteristics can be crucial features for the definition of a species (Hirose et al. 2014).

1.2. Bio-ecology

1.2.1. Reproductive strategy and life cycle

Nudibranchs are hermaphrodite (Todd 1978) and are known to aggregate to copulate and spawn, laying their eggs in rocks or other substrates (Claverie and Kamenos 2008).

The life cycle of nudibranchs generally comprises two strategies which varies with different species: i) a short lifespan, when the species have more than one generation each year; ii) an annual life cycle, when species spawn in one period per year. These species are normally bigger than the first ones, showing synchronous sexual development and spawning behaviour and total mortality after reproduction (semelparous) (Aerts 1994)

Figure 1 – *Dendrodoris grandiflora* egg masses in Laboratorio Maritimo da Guia aquaculture holding tanks.

Nudibranchs allocatte most of its energy in the search for a hermaphroditic partner, copulation, egg mass production and spawning activities (Pires 2012). These requirements cause many adult nudibranchs to deplete all their energetic resources during the spawning period which possibily explains their death after spawning (Todd 1979).

In this context it is important to notice that there is a global discussion if nudibranchs experience death after spawning. From its laboratory experiments, Hecht (1895) *in* Costello (1938), found that mature sea slugs died after spawning and that non-mature individuals were easy to maintain alive. These results are in agreement with the ones obtained by Garstang (1890) *in* Costello (1938), who observed death after spawning in *Goniodoris nodosa*. However, controversy rises as Chambers (1934), pointed out that in the case of certain Atlantic coast species (*Embletonia fuscata*), spawning is not invariably followed by death. Moreover, Costello (1938) experiments resulted in relatively few nudibranchs dead in the laboratory after a period of weeks and even months. Following his results, he concluded that death probably results from an inadequate food supply. However, due to the possible disparity temperature differences, it is not inconceivable that, particularly for species from a warm-water habitat, the deposition of big size egg masses could deplete stored food supply of the body resulting in death (Costello 1938). More recently, Grant and Williamson (1985) assumed that all nudibranchs are semelparous, meaning they die immediately after reproducing and spawning. So, whenever spawning occurs, it is automatically followed by a sharp fall in the quantity of individuals in the area (Grant and Williamson 1985). However, Todd (1985) found in laboratory that not all individuals within a given population spawn at the same time, inclusively may spawn several times within the same year. This issue is particularly relevant whenever ecological studies of nudibranchs are done in the intertidal zone, particularly when considering long-term studies of what are presumed to be resident populations. Moreover, the mobility of groups of organisms needs to be considered when studying community ecology based in parameters such as abundance, biodiversity and habitat preferences.

1.2.2. Early ontogenetic development (direct or indirect)

The initial development of nudibranchs occurs in protected egg masses, which is often followed by free-swimming larva – the veliger (Robinson 2004). Then, they may undergo direct or indirect development. In direct development, they metamorphose inside the egg capsules and crawl out as fully developed juveniles, whereas in indirect development they metamorphose outside the capsules as small juveniles (Todd et al. 1998). During indirect development, larvae may develop without feed once the egg is provided with a source of nutrition (lecithotrophic development) or they may need to feed on phytoplankton for days, weeks, or even months (planktotrophic development). Although the nutritional modes of marine invertebrate larvae are typically divided between planktotrophy or lecithotrophy, nudibranchs are known to exhibit variable larval development modes within the same species, i.e. they have poecilogony as a reproductive strategy (Dionísio et al. 2013).

1.2.3. Horizontal movements (migrations)

The reasons behind horizontal movements in nudibranchs have been controversial, and some studies suggest the migration theory, which assumes that large numbers of a given species migrate from a population in subtidal to shallow waters to copulate and deposit egg masses (Pelseneer 1922; Nybakken 1978; Claverie and Kamenos 2008). Nonetheless, there is still a major controversy on whether the sporadic appearance of nudibranchs in the shore is really due to migration. Other explanations have been proposed for these inshore movements such as: i) the influence of tidal action, currents or waves (Costello 1938; Wyeth and Willows 2006a), ii) food foraging (Wyeth and Willows 2006a) , iii) geomagnetic field influence (Willows 1999) (see Table 1). Those differences and abundances of theories and justifications, highlights the lack of information on the life cycle and general ecology of nudibranchs as well as on the diverse strategies of life that they present (Crane 1972).

Such explanations as well as other observations and conclusions are detalied in table 1. As it can be seen, the majority of the studies point explanations to inshore movements rather than migrations, especially when considering the rapid dissapearance.

Table 1 – Summary of the type and possible causes of horizontal movements (migrations) in sea slugs. (*);(**);(***) see Table S1 in Annexes

Reference	Species	Location	Habitat	Migration	Plausible Causes
Crozier, 1917	*Chromodoris zebra*	Fairyland Creek, Millbrook Creek, Bermuda	Subtidal	Intertidal	Intertidal mass aggregations: diurnal movements due physical conditions, such as phototropism and response to surface turbulance.
Pelseneer, 1922	*Doris bilamellata*	Wimereux, France	Subtidal	Intertidal	Intertidal mass aggregations: migration to copulate and spawn, followed by death.
Chambers, 1934	*Embletonia fuscata*	Barnegat Bay, New Jersey, USA	Subtidal	Intertidal	Intertidal mass aggregations: not a migration situation; Fortuitous establishment (due to strong currents and waves) of colonies or rapid development of veliger to re-colonize; Disappearance as a result of environmental factors (cold, lack of food).
Costello, 1938	22 species (**)	Monterey Bay, Californa, USA	Subtidal	Intertidal	Inconclusive about migrations. Suggests the action of wave and currents as possible causes.
Miller 1962	24 species (***)	Isle of Man, UK	Subtidal	Intertidal	Not a migration situation: Sudden appearances attributed to colonization by fortuitous establishment of veligers (settlement theory); Hydroid feeding species dissapear when their food also dissapears.
Crane, 1972	*Archidoris montereyensis*	Vancouver Island, British Columbia, Canada	Intertidal	-	Fluctuation in population as a result of sporadic settlement of veliger (settlement theory); Death after spawning: Post-reproductive and due to adverse physical factors.
Nybbaken, 1978	*Triopha maculata*	Asilomar State Beach, California, USA	Intertidal	Intertidal	Fluctuations in population possibly due to change from under rock habitat to surface habitat; Death after spawning.
(Thompson 1984)	*Onchidoris bilamellata*	Wimereux, France	Subtidal	Intertidal	Not a migration situation: refutes Pelseneer (1922) claims on mass agregations;
Tween, 1987	*Onchidella celtica*	Trebarwith, Cornwall, UK	Intertidal	-	Mass aggregations to copulate; Not a migration situation;
Aerts, 1994	10 species (*)	Delta Area, Netherlands	Subtidal/Intertidal	-	Not a migration situation; No evidence of horizontal migration; Fortuitous establishment of veligers (settlement theory)
Willows, 1999	*Tritonia diomedea*	Tofino, British Columbia, Canada	Subtidal	Intertidal	Shoreward movement for food foraging, mating and others dependent on cues such geomagnetic, currents.
Knowlton & Smith, 2000	*Archidoris montereyensis*	Beach State Park, Alaska, USA	Intertidal	-	Fluctuations in population varies with prey density and successful recruitment.
Domenech, 2002	*Dendrodoris limbata*	Sant Antoni, Costa Brava, Spain	Subtidal	-	Fluctuations in population possibly due to death after spawning.
Wyeth, 2006a	*Tritonia diomedea*	North of Vargas Island, British Columbia, Canada	Subtidal/Intertidal	-	Horizontal movements for prey avoidance; Foraging; Uses sensory cues: current,flow.
Claverie & Kamenos, 2008	*Onchidoris bilamellata*	Millport, Scotland	Intertidal	Subtidal	Subtidal mass aggregations: migration associated with reproduction (mucus trail follow); Spawning occurs subtidal; Prey only found in intertidal areas.

1.2.4. Feeding ecology

Within Heterobranchia, feeding preferences are known to vary largely. As seen in section 1.2.2, nudibranch larvae may undergo direct or indirect development. As adults, some species are able to prey different types of organisms, while others display a stenophagous feeding regime (preying only one type of prey). Dietary preys are in general coelenterates, corals, tunicates and sponges (Crane 1972; Robinson 2004) and can easily be identified through molecular techniques, by analyzing DNA barcodes of nudibranchs' gut content (Robinson 2004).

1.3. Biotecnological relevance

A large number of natural products with remarkable bioactivities have been discovered during the last few decades from marine invertebrates (Leal et al. 2012). Following this trend, nudibranchs have been extremely attractive to researchers in the area of biotechnology, particularly due to the production of secondary metabolites produced as chemical defenses against predators (Leal et al. 2012).

These compounds exhibit a large variety of chemical structures and have been shown to possess ichthyotoxic, cytotoxic and feeding-deterrent properties, as well as antibacterial activity and may also act as sexual pheromones (Zhukova 2014). The origin of these natural products in nudibranchs has been attributed to the bioaccumulation or biotransformation of molecules acquired through the ingestion of their prey (Dionísio et al. 2013).

1.4. Relevance in biomedicine and ornamental trade industry

Other interest in nudibranchs comes from their potential to be used as biological models and tools for scientific research. For example, species such as *Aeolidiella stephanieae, Spurilla neapolitana* and *Elysia sp*. have been successfully used in biomedical studies and in research on symbiosis between metazoan cells and chloroplasts (Dionísio et al. 2013). More recently, sea slugs have been becoming increasingly popular as ornamental species in marine aquarium trade industry not only due to their colors and patterns but also to be used as biological weapons to control the pests in aquariums (e.g. the nudibranch

Aeolidiella stephaniaea eats exclusively the glass anemone from the genus *Aiptasia*, one of the most feared pest by marine aquarium hobbyists) (Dionísio et al. 2013).

1.5. <u>Genus *Dendrodoris* (Nudibranchia: Doridina: Porostomata: Dendrodorididae)</u>

The generic name *Dendrodoris* was introduced by Ehrenberg (1831) for two species recorded in the Red Sea (Valdès et al. 1996). Since its original description, taxonomic issues have been disputed in several *Dendrodoris* species, due to their external color variations and anatomical similarities(Hirose et al. 2014).

Dendrodoris genus has no radula and has a highly glandular anterior digestive system (Klussmann-Kolb and Brodie 1999). Because of the lack of radula, the members of this group have developed a number of anatomical transformations in the foregut as an adaptation for its stenophagous feeding regime (suctorial feeding, exclusively on sponges) (Hirose et al. 2014).

The Atlantic species of the *Dendrodoris* genus were reviewed by Valdès et al. (1996), who found considerable external variability within the nine taxa examined. After studying their external morphology and internal anatomy, reproductive system and features of the egg-mass, these authors stated that six valid species inhabit in the Atlantic Ocean and described three new *Dendrodoris* species from the Northeastern Atlantic and West Africa.

Information on these species' habitats is scarce, however it is known that direct development is significantly more prevalent than in nudibranchs worldwide (Goddard et al. 2011). Three hypothesis are given to explain such development: i) direct development has been selected as means of overcoming size constraints on post-metamorphic juveniles stemming from their lack of radula; ii) is prevalent because small adult size, which is correlated with brooding and direct development in marine invertebrates, has been selected for in many dendrodoridids and iii) as an adaptation against high larval mortality (Goddard et al. 2011).

The model organisms of the present dissertation are *D. grandiflora* and *D. herytra* which are going to be detailed in the bellow sections namely regarding their description, distribution, habitat and behavior.

Figure 2 – *D. herytra* (left) and *D. grandiflora* (right) (photos adapted from Calado & Silva, 2012).

1.5.1. Dendrodoris herytra (Valdés, 1996)

1.5.1.1. Species description

D. herytra body is flat with 4-5 cm of maximum length, and 3 cm width. Juvenile specimens are uniformly pale red, and the most typical color of adult is uniform strong yellow (Figure 2). Some specimens have a yellowish background color with red (or exceptionally pale green) spots (Valdès et al. 1996). The edge is very delicate, wavy, well developed, slightly striated and very variable in size. The rhinophores, chemosensory structures of nudibranchs, are laminated with lighter color edge (Calado and Silva 2012). The three gills are generally very short which helps to distinguish it from other similar *Dendrodoris* species and located around the anus (Valdès et al. 1996).

1.5.1.2. Distribution

Among *Dendrodoris* genus, *D. herytra* is the species that occupies the widest range in the Northern Atlantic Ocean. It can be found from the Bay of Biscay, to the Strait of Gibraltar, including in the Iberia Peninsula. It is also present from the coast of Morocco down to the coast of Mauritania and in the Macaronesia complex (Calado & Silva, 2012).

1.5.1.3. Habitat and Behavior

D. herytra vertical distribution goes from shallow waters to 25 m depth. At low depths it can be spotted on the substrate and on rocky bottoms, normally in areas exposed to strong tidal currents or wave. This species feeds on sponges during the night, and, during the day, is normally found at low depths hidden under rocks (Calado & Silva, 2012).

1.5.2. Dendrodoris grandiflora (Rapp, 1827):

1.5.2.1. Description

The body of *D. grandiflora* is flat, with around 7 cm of length. Its colour is very variable between specimens: organisms from the Mediterranean Sea possess different background colors as grey, cream, pale green, pale brown or light red, with brown or black spots on the dorsum (Figure 2); In Portugal, most specimens studied have a yellow background colour, with dark spots (brown or black) (Valdès et al. 1996). The long lobed gills (around 6-7) have the same color as the body with white tips (Calado and Silva 2012). The edge is wavy and transparent, with thin dark grooves from inside out, which help to distinguish it from other *Dendrodoris* species. Also, the shape of the male cirrus hooks and the disposition of the reproductive organs are very characteristic of this species (Valdès et al. 1996).

1.5.2.2. Distribution

This species inhabits the Mediterranean Sea, from Turkey and Israel to the Strait of Gibraltar. In the Northern Eastern Atlantic, it has been recorded from Portugal to the Cape Verde Islands (Valdès et al. 1996)

1.5.2.3. Habitat and Behavior

D. grandiflora can be found from the surface down to 25 m depth, normally hidden under rocks or near areas rich in sponges, their specific prey item (Calado & Silva, 2012).

1.6. Objectives

Benthic marine invertebrates, such as nudibranchs and their planktonic life stages are daily exposed to variable and often stressful conditions, such as wide temperature changes, dissolved oxygen concentration and salinity variations. These environmental stressors may be determinant in several traits of the life cycle of nudibranchs and can be a possible explanation for the inshore migrations often observed for these organisms.

Within this context, the aim of the present dissertation is to study the seasonal changes in the density, and possible horizontal movements, of two nudibranch species, *Dendrodoris herytra* and *Dendrodoris grandiflora* in Sado estuary, Portugal. Possible relationships between *Dendrodoris* density shifts and environmental parameters such as salinity, water and air temperature and water turbidity will be assessed.

2. Materials and methods

2.1. Study site:

Adult specimens of nudibranchs of the species *Dendrodoris herytra* and *D. grandiflora* were counted in an oyster sandbank located in Sado Estuary National Reserve, West Portugal (38.474857ºN, -8.775255ºW) (Figure 3).

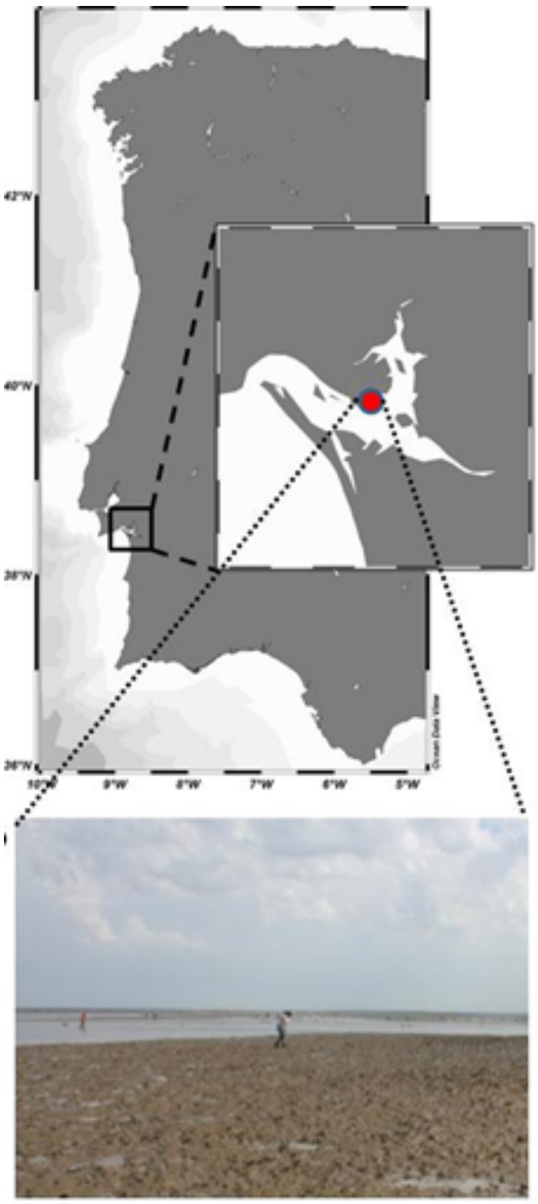

Figure 3 – Sampling site in the Sado Estuary

The oyster sandbank was emerged enough time to do the survey when the tide was lower than 0.8 m. This sandbank had a particular elevation that made it very easy to identify and limit its area. It mainly consisted of oysters shelves parts and whole shelves of cockle and also rubble, as can be seen in Figure 5. In the lower elevated area, most of the substrate was sand.

This was not the only sandbank, and a few more where found in the surroundings. However, due to the common presence of mariculture professionals, those areas are more impacted than the selected one.

2.2. Nudibranch density:

Adult specimens of *D. herytra* and *D. grandiflora* were visually counted between January 2011 and June 2015. Surveys were done by two observers in a restricted area (total area: 1050 m^2) during the lowest tides of the month, covering the entire emerged oyster sandbank (Figure 4).

Figure 4 – Observers counting of nudibranchs in the oyster sandbank, located in Sado Estuary.

Nudibranchs were mostly encountered emerged and in tide pools (Figure 5), sometimes under oyster shelves. The nudibranchs were not always easy to differentiate, since some of the *D. grandiflora* have a big range of colours. In those cases, a careful analysis and identification was made, in order to accurate counting results.

Figure 5 – *Dendrodoris grandiflora* (top) and *D. herytra* (bottom) emerged in the oyster sandbank.

2.3. Environmental data:

Environmental data was collected in order to establish a connection with the density of nudibranchs already obtained in the field, during the five-year survey. The water environmental parameters such as water temperature and turbidy were obtained with a multiparameter probe (CTD YSI 6600 V2) and salinity with a refractometer (V^2 Refractometer from Tropical Marine Centre). Air temperature data was provided by the Instituto Português do Mar e da Atmosfera (IPMA) as mean values recorded during each sampling month in Setúbal meteorological station.

2.4. Statistical analysis

In order to investigate the density of *D. herytra* and *D. grandiflora* in relation to environmental variables, a multiple regression model with the following equation was used:

$$Y = b_0 + b_i + X_i + e_i$$

where Y represents the dependent variable (species density); b_0 the model intercept; b_i the regression coefficients associated with the independent, explanatory variables (X_i); and e_i the error term, which is normally distributed around zero.

Prior to statistical analyses, a correlation matrix between environmental variables was made to test the independency of the variables. If all environmental variables are independent of each other, the regression coefficient obtained for each variable represents the total contribution to the response. However, when two or more variables are collinear to any extent, the contribution of a particular explanatory variable is mixed with other variables (Mason and Perreault 1991). The method used to choose which collinear variables should be excluded was an "all possible subsets" method of analysis, where the model was created for all possible combinations (subsets) of variables, and the subset with the greatest fit is identified as "best" using adjusted R^2 and looking into the overall goodness of fit (Mason and Perreault 1991).

The final model was created with temperature, salinity and turbidity as independent variables.

Logarithmic [log 10 (x + 1)] transformations were conducted to density data to approximate normal distributions. All the linear models were tested for statistical significance at the $\alpha = 0.05$ level using an F-test. The residuals were visually inspected for normality (Mason and Perreault 1991).

Correlation between the average air temperature and individuals density was assessed using a simple Spearman Correlation test.

Statitiscal analyses were done using Statistica software.

3. Results

3.1. Inter- and intrannual changes in nudibranch densities

The seasonal appearance (emersion) of *Dendrodoris herytra* and *D. grandiflora* specimens in the intertidal region of an oyster sandbank in Sado estuary was observed mainly during spring months and registered during five consecutive years. Each annual appearance event was followed by a consistent disappearance of the organisms of both species from July onwards (Figure 6 A,B,C,E), with exception of 2014, when no individuals were observed during June (Figure 6 D).

The first year of observations, 2011, presented the highest density observed, with a maximum o 129 ind.km^{-2} of *Dendrodoris* nudibranchs observed in April. However, that year the first emerged individuals were observed in April, in opposition to the following years. More specifically, from 2012 to 2015, nudibranchs of both species started to appear emerged in the oyster sandbank in March in very low number (*D. herytra:* <8 ind. km^{-2}; *D. grandiflora*: <8 ind. km^{-2}). The density then increased to its maximum in April (up to 62 ind. km^{-2} in *D. herytra* 67 ind. km^{-2} in *D. grandiflora*), except for 2014, where its maximum was in May (14 ind. km^{-2} in *D. herytra* 49 ind. km^{-2} in *D. grandiflora*). June was the last month where they have been reported, except for 2014. In 2014, the peak was reached later, namely in May, as seen before, and it was suddenly followed by an abrupt dissapearence in June, thus showing a different pattern observed for the other years, where after a peak, a small decrease was always observed (Figure 6 A,B,C,E).

In general, whenever *Dendrodoris* nudibranchs were emerged in the oyster sandbank, *D. grandiflora* presented a higher density than *D. herytra.* The exception was 2012, when a total of 22 ind. km^{-2} of *D. herytra* were observed, against 18 ind. km^{-2} of *D. grandiflora.* Maximum density of both species occurred with an average of 66 ind. km^{-2} in 2011, whereas the lowest density was obtained in 2012, with an average of 12 ind. km^{-2} during observation months.

Most of the individuals were found alone, static, some upside down. Occasionally individuals were found under oyster shelves. No egg masses were encountered in any of the survey.

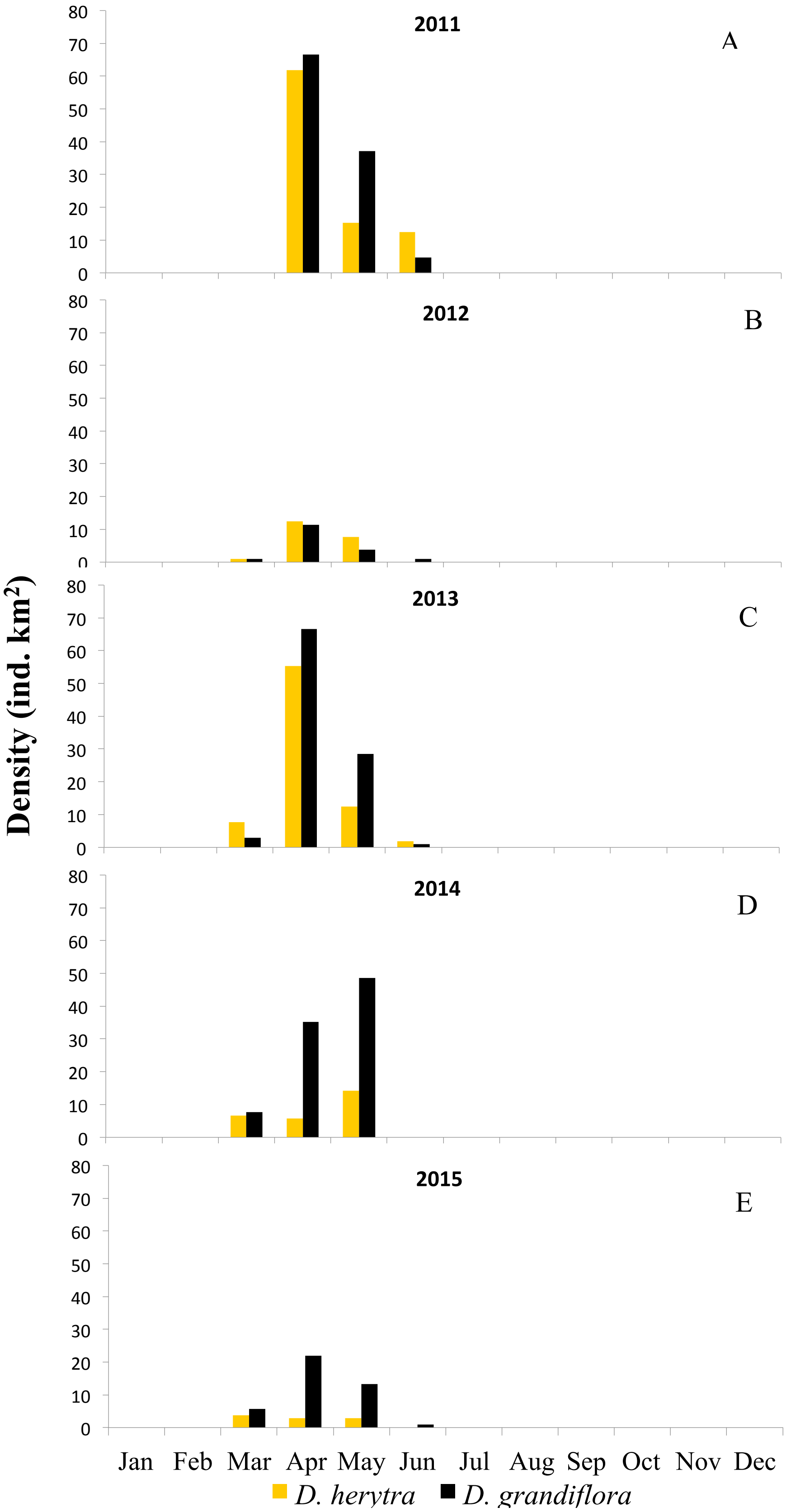

Figure 6 – *Dendrodoris grandiflora* (black) and *D. herytra* (yellow) monthly density (km^{-2}) from 2011 to June 2015.

3.2. Relationships between nudibranch densities and environmental conditions

Environmental parameters variations of air and water temperatures, salinity and turbidity in Sado estuary from January 2011 to June 2015 are presented in Figure 7. Air and water temperature as well as turbidity showed consisten patterns throughout the years. As expected, water temperature presents an increasing pattern from winter to summer months (Figure 7 A,B,C,D,E), almost like a bell-shaped graph. Maximum water temperatures occur mostly in July during the first three years of the survey. However, in 2014, June was the month where water in Sado estuary was warmer. In 2012 a different pattern also occurred, after the peak in July, the normal drop occurs, but in September, water temperature rises again. On the other hand, salinity showed no clear pattern. Turbidity consistently varied between 1 and 5 NTU during almost all the survey months excepting during March and April in which values abruptly increased (Figure 7 K,L,M,N,O).

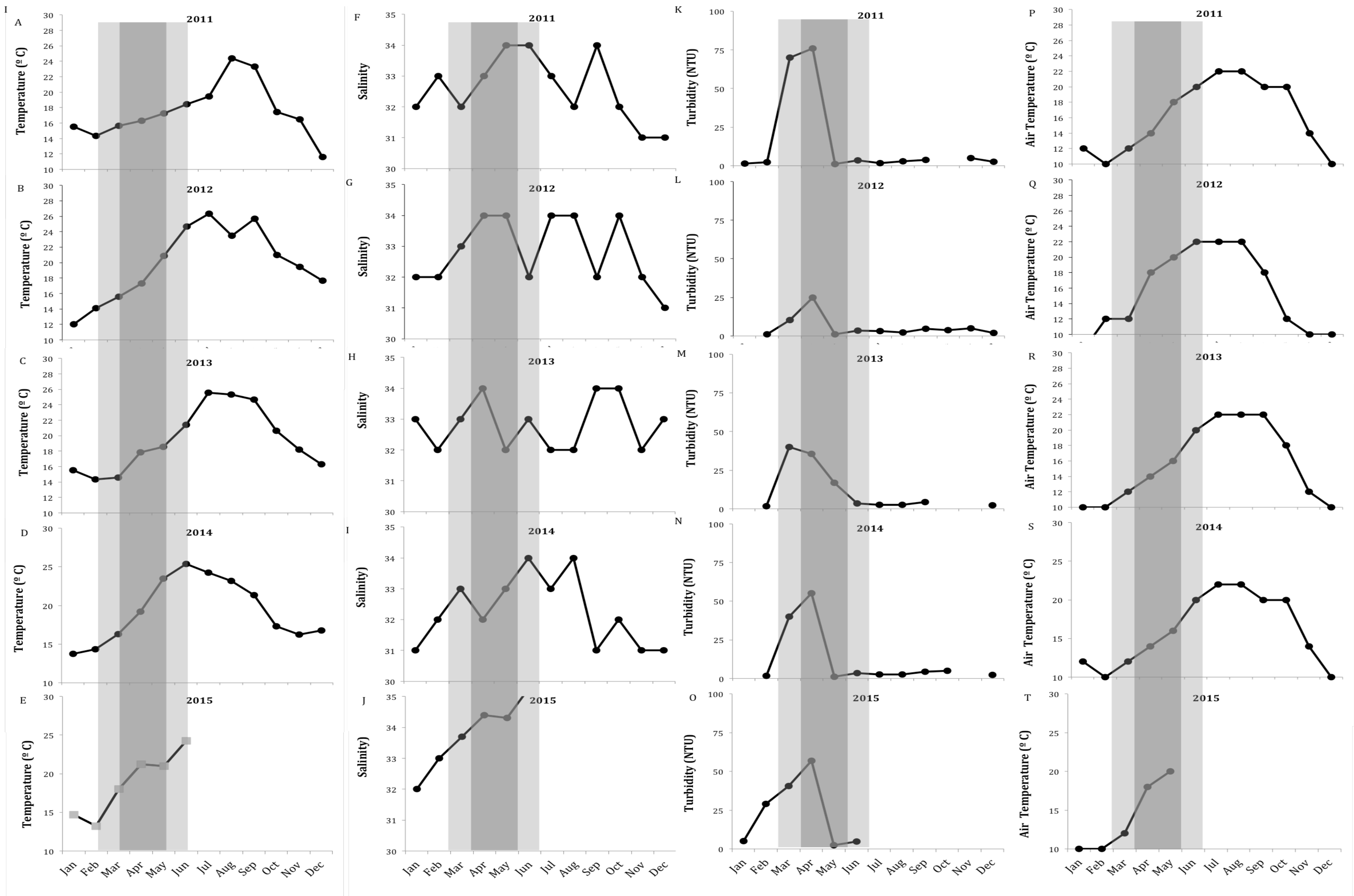

Figure 7 – Environmental parameters from 2011 until June 2015 (water temperature, salinity, turbidity and air temperature) with density quartiles (grey).

As shown in Figure 7, air and water temperature displayed a similar pattern, with no clear influence in nudibranch density. When temperature started rising, so it did the sea slugs' density. However, the temperature peak was not coincindent with the zenith of the density of nudibranchs. Thus, no significant correlation was observed between the average water temperature and species density ($p > 0.05$) (Table 2). On the other hand, salinity and turbidity were significantly related to density of both *Dendrodoris* species (Table 2). Air temperature was not considered, since there was a correlation with other parameters, namely water temperature.

Table 2 – *p* values for *D. herytra* and *D. grandiflora* in regards to the environmental parameters following a Spearman Correlation test. Significant correlation are highlighted in bold.

	D. herytra	***D. grandiflora***
	p	***p***
Temperature	0,2624	0,7555
Salinity	**0,0135**	**0,0281**
Turbidity	**0,0014**	**0,0004**

4. Discussion

Certain nudibranchs are known to sudden appear in intertidal areas in a seasonal basis (Aerts 1994; Knowlton and Highsmith 2000). Several theories try to explain these sudden events normally followed by a rapid dissapearance. In this context, two major questions arise: (i) "are these events related with horizontal migrations by nudibranchs species?"; and (ii) "what are the causes that motivate their sudden appearance/disappearance from the shore?". In our study case, the scenario was even more controversial, since *Dendrodoris herytra* and *D. grandiflora* were observed emerged, a situation that has not yet been described for this genera. This aspect is specially important due to the fact that emerged organisms are submitted to thermal stress and dessication, afecting their physiological state, growth and also survival (Teixeira et al. 2013).

Typically, migration is defined as a “seasonal movement of large numbers of animals over great distances (Gibson 2003). In the case of nudibranchs, it is accepted that they undertake migrations to spawn on the shore (Pelseneer 1922; Claverie and Kamenos 2008). However most of the authors give other explanations. For example, Nybakken (1978) justifies the “sudden presence” of nudibranchs as the emergence of individuals from under rock habitats to surface habitation. According to our observations (a five years of survey), this idea might not apply, at least to the model species, since we didn’t observe any egg mass emerged in the study site.

Although individuals were mostly seen alone, the hypothesis of being a matting behaviour should not be set aside. In order to identify the mature situation of the organisms, same individuals were brought to the lab, and there remained. During that tie they were able to copulate and spawn confirming that they were sexually mature and ready to spawn and to copulate (see Figures S1 to S3 in Annexes).

A major controversy still exists with some authors reporting death after spawning (Grant and Williamson 1985) while others contradict this information (Todd 1985) leading us to suggest that postreproductive death is a species-specific trait in nudibranchs. Moreover, some authors explain the sudden dissapearance by death following breeding (Pelseneer 1922; Nybakken 1978; Domenech et al. 2002). Some *Dendrodoris* individuals spawned not once, but twice during the period in which they were held in lab aquaria. In fact, individuals of both species lasted between four to six weeks. Since no feeding was available, the death cannot be justified as simply following spawn, but rather starvation, especially in a demanding situation such as spawning. A similar pattern was observed for *Onchidoris bilamellata*, a semelparous species that before experience death after it spawns several times, over a continuous period of weeks (Todd 1985).

Therefore, the results of the present study suggest that the sudden appearance of *D. herytra* and *D. grandiflora* in intertidal areas of Sado estuary may be due to a migration from a larger subtidal population probably to mate, where in this smaller and restricted area they will increase their chances to find a partner.

This brings us to the second question - "what are the causes that motivates their sudden appearance/disappearance from the shore?". The present long-term survey consistently showed that *Dendrodoris* nudibranchs start to appear in intertidal areas in March and completely disappear in July. They reach peak density between April and May, when the water temperature was on average 19 ºC and air temperature was on average 16 ºC. On the other hand, they disappear, when water temperature average was 23 ºC, and average air temperature was 21 ºC. Air and water temperature increased hand to hand with nudibranch's densities, but they did not explain the inter- and intrannual patterns of variation of nudibranch presence. Temperature in fact, could be considered an important factor in their behavior particularly when considering that these individuals were often emerged. For an example, *Onchidella celtica,* a common sea slug from North Atlantic region, following a two-year survey, did not emerge on days when the air temperature was 10.5 ºC or less or when the crevice temperature was 9.5 ºC or less (Tween 1987). From this investigation it was also clear that more individuals emerged on warmer days, although on some extremely warm days there were few or no foraging animals.

In the present study, while being completely emersed during low tide, individuals soon became immerged due to tide rise, with an exposure to the air of no longer than 3 hours. In the case of emerse individuals of *O. celtica* caused by low tide, they may continue emerse more than full tide (Tween 1987). The duration of the excursion is very variable in *O. celtica*; even on the same day the period of foraging of individuals ranges from a few minutes to a couple of hours. It is also stated by (Tween 1987) that it is probable that some animals may remain out for longer periods under optimal meteorological conditions.

Looking to understand the motivation of this seasonal appearances, one must look within the genus and other nudibranch species. Seasonal appearances has also been described for other *Dendrodoris* species - *Dendrodoris atramaculata*, but during the month of August. Such frugal appearance was interpreted as linked to foraging strategies, since sponge gardens (their prey) become very accessible during the low tides of that particular month (Brodie 2004*)*. Other nudibranchs such as *Acteonia senestra* and *Limapontia capitata* are common in the intertidal zone when food is abundant, namely during spring and summer months. They were also found breeding, which suggests that they invaded the intertidal zone to feed and breed (Miller 1962)

Other possible explanation to understand the behaviour our nudibranchs is the one that sea slugs are known for seeking shelter under rocks or other structures. Here, the two *Dendrodoris* species were always found within an oyster bank during the emmersion periods. Thus, it is very likely that those shells provided cryptic protection. The surroundings of the oyster bank was mostly composed by muddy and sand, thus, providing no shelter for the nudibranchs. Studies on the effects of substratum on gastropod behaviour have usually examined types of substratum, rather than texture. Exceptions are the studies conducted by: i) Jones and Boulding (1999), where emerged snails (*Littorina sitkana*) generally selected complex, rather than simple topographies and ii) Barbeau et al. (2004) where sea slugs (*Onchidoris bilamellata*) preferred rough substratum to smooth substratum, but only when they were in the dark. Under light conditions, sea slugs did not show any preference between textures.

Another possibility to take in consideration is the one Crozier (1917) also observed in a shoreward movement associated to changes in factors such as light. Intertidal benthic invertebrates generally display photonegative responses in or out of water, but more complex responses are also common. Such complex response was observed with the nudibranch *Hermissenda crassicornis* (Meadows and Campbell 1972), which preferred intermediate light intensities in the day and low intensities at night (Izja Lederhendler et al. 1980).

From the environmental parameters recorded here, both salinity and turbidity revealed to be related with the fluctuations in the densities of emerged *D. herytra* and *D. grandiflora*. Yet, there was not a clear pattern with salinity alone. Nonetheless, salinity in an estuary strongly fluctuates at a daily (e.g. due to tidal regimes (Geyer and MacCready 2014) seasonal (e.g. due to rainfall) (Hopkinson et al. 1999); and yearly (e.g. due to infrastructure changes in the estuary that affect the flow through of freshwater and intrusion of salt water (Rose 1986; Lorenz 2014). Also, low and high salinities are known to affect the reproductive success and early ontogenetic development of marine invertebrates (Allen and Pechenik 2010).

As for turbidity, it seemed to strongly influence nudibranchs' presence in the intertidal areas once their presence was consistently related with a peak in turbidity. Moreover, lower turbidity in the spring months of 2012 as compared with the other years was coincident with the year with lower *D. herytra* and *D. grandiflora* densities. High turbidity is associated to several situation such as to wind-wave sediment re-suspension, flooding tide, salinity intrusion, vertical gravitational circulation, sediment entrance from rivers and anthropogenic pressure (e.g. industry and agriculture runoffs) (Green and Coco 2014). Moreover, increasing turbidity is related with increasing phytoplankton biomass which commonly occurs during spring which is commonly referred as the spring bloom (Barlow et al. 1993). This is possible the main reason why there is a consistent maximum in turbidity in the sampling location during spring. As a result of the spring phytoplankton bloom, secondary productivity increases including zooplankton grazers (George et al. 2015). Giving the fact that *D. grandiflora* and *D. herytra* larvae are probably planktotrophic (Goddard et al. 2011) they will benefit from the increasing phytoplankton concentration.

Another benefit as a result of increasing turbidity is that an increase in suspended particulate matter concentrations leads to light attenuation (Cloern and Jassby 2012). This way, high turbidity may constitute a refuge for *D. herytra* and *D. grandiflora* by minimizing predation risk (Speckman et al. 2005), which is particularly important during reproduction season.

Summing up, distribution patterns of nudibranchs in intertidal areas raise a number of questions that still need to be answer. The present study constitutes an additional example of a population that seasonally appears emerse and more importantly, gives essential knowledge on the influence of environmental parameters on these events. However, further studies will be necessary in order to properly confirm if and how environmental factors influence these nudibranchs' life cycle. Other explanations have been proposed to explain nudibranchs' migrations (e.g. action of tides, currents or waves (Costello 1938) food foraging (Crane 1972; Wyeth and Willows 2006b; Wyeth and Willows 2006a) geomagnetic field and we not rule out that they may also explain nudibranchs' density in intertidal areas (Willows 1999).

In the future, other experimental procedures and field observatios should be conducted in order to understand in more depth the impact of emmersion, environmental parameters, and others. These experiments can be conducted under laboratory conditions. Field observations, including tagging can also apply, in order to understand the winter patterns and habitats of the individuals.

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6. Annexes (Supplementary material)

Figure S1 – *Dendrodoris grandiflora* matting in aquaculture holding tanks in Laboratório Maritimo da Guia.

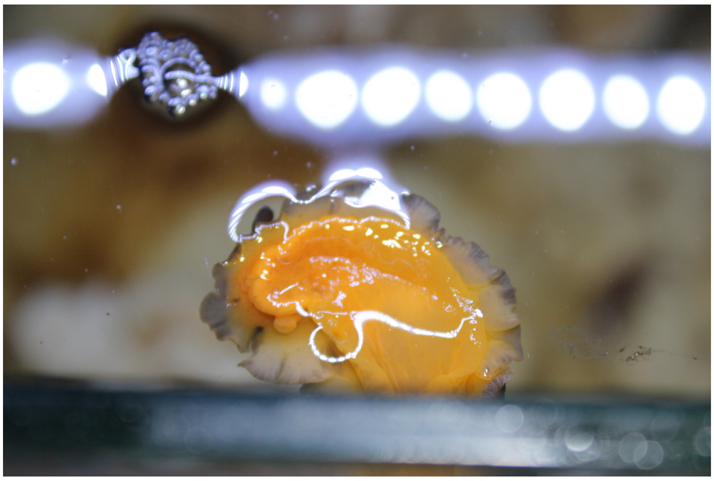

Figure S2 – *Dendrodoris grandiflora* swimming at the surface in aquaculture holding tanks in Laboratório Maritimo da Guia.

Table S1 – Full list of species described in the references used in Table 1.

(*) Aerts, 1994	(**) Costello, 1938	(***) Miller, 1962
Aeolidia papillosa	*Aegires albopuiictatuv*	*Acantodoris pilosa*
Aeolidiella glaucaa	*Aeolid (maroon-black cerata)*	*Acteonia senestra*
Coryphella gracilis	*Aeolid (orange-white cerata)*	*Aeolidia papillosa*
Cuthona amoena	*Aldisa sanguinea*	*Archidoris pseudoargus*
Cuthona gymnota	*Anisodoris nobilis*	*Coryphella lineata*
Dendronotus frondosus	*Antiopella aureocincta*	*Cuthona amoena*
Eubranchus sp.	*Archidoris montereyensis*	*Dendronotus frondosus*
Janolus cristatus	*Cadlina flaromaculata*	*Doto coronata*
Onchidoris bilamellata	*Cadlina marginata*	*Doto fragilis*
Tergipes tergipes	*Cadlina sp.*	*Elysis viridis*
	Dendrodoris fulva	*Eubranchus exigus*
	Diaulula sandiegensis	*Eubranchus pallidus*
	Discodoris heathi Mac-	*Eubranchus tricolor*
	Herrnissenda crassicornis	*Goniodoris nodosa*
	Hopkinsia rosacea	*Hero formosa*
	Laila eockerelli	*Jorunna tomentosa*
	Lateribranrhaeia festiva	*Limapontia capitata*
	Polycera atra	*Onchidoris fusca*
	Rostanga pulehra	*Onchidoris muricata*
	Triopha carpentcri	*Onchidoris pusilla*
	Triopha grandis	*Polycera quadrilineata*
	Triopha maculata	*Tergipes despectus*
		Trinchesia aurantia
		Tritonia hombergi