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| **Acronym:** | **COCKLES** |
| **Title:** | Co-Operation for Restoring CocKle SheLlfisheries and its Ecosystem Services in the Atlantic Area |
| **Contract:** | EAPA_458/2016 |

## Deliverable 4.3

# Cockle reproductive health

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| **Due date of Output** | 31/03/19 |
| **Actual submission date** | 19/06/20 |

| **Dissemination level** | | | |
|---|---|---|---|
| ☐ | **PU** Public | ☐ | **PP** Restricted to other programme participants |
| ☐ | **RE** Restricted to a group specified by the Consortium | ☐ | **CO** Confidential, only for members of the Consortium |

**Acknowledgement**

The work described in this report has been funded by the European Commission under the INTERREG-Atlantic Area Programme (EAPA_458/2016).

# Contents

# 1. EXECUTIVE SUMMARY

Deliverable 4.3 examined the reproductive health of Atlantic Area (AA, 37°N to 55°N in latitude) cockle populations, in the past and present, and at different latitudes. To manage cockle beds and guide successful culture and restoration programmes, a strong understanding of reproductive strategies and influencing factors is needed. A literature search and analysis were carried out to provide an insight into historical trends. To look at more current events, field studies in all partner countries: Ireland, France, Spain and Portugal, were completed. Firstly, a five-year survey (2007 - 2011), based on monthly sampling, was performed to characterise the gonad cycle in a highly productive shellfish bed, Lombos do Ulla, in the Ría de Arousa (Galicia, Spain). Additionally, during the same period, cockles were collected from five shellfish beds scattered along the Galician coast to estimate the size at first sexual maturity (*Survey 1*). Subsequently, a 24-month survey (March 2016 to February 2018) on the reproductive cycle and size at first sexual maturity was carried out at Ría Formosa, Portugal (*Survey 2*). Finally, in a 19-month field study (April 2018 to October 2019), cockles were sampled from five AA sites in Ireland (3), France (1) and Portugal (1) to investigate between-site and latitudinal variation in sex ratio, age and size at sexual maturation, reproductive cycle and trematode parasite prevalence (*Survey 3*).

In total, 21 historical records (1954 – 2010) pertaining to cockle reproduction (in Denmark, France, Ireland, Norway, Spain, Sweden, the Netherlands and the UK; covering latitudes 42°N to 63°N) were sourced in the literature. Comparing these, it appeared that spawning initiated earlier in the year at more southern latitudes. Furthermore, there was a potential lengthening of spawning period across time within the latitudes 50 -53°N.

In the field trials, all sites, except for Carlingford and Dundalk in Ireland, showed a balanced 1:1 sex ratio. The smallest size at first maturity ranged in length between sites, from 11 mm (wild cockles in Cork) to 22 mm (fished cockles in Dundalk). Different reproductive strategies were observed across AA sites, with latitudinal grouping (**Table 1**). It was found that gametogenesis and spawning start earlier in areas <45°N than in areas >50°N, thus the spawning period is longer in the former areas. In *Survey 1*, the general pattern of the annual gonad cycle was characterized by a resting period in autumn and two spawning peaks. Mass spawning was observed in spring to early summer, after which cockles restored and became ripe for a second spawning in which the percentage of spawning cockles was lower. There was clear interannual variation in the month of first spawning detection (February to April) and the length of spawning period (4 to 8 months). Gametogenesis in Ría Formosa (*Survey 2*) started in autumn with a highly variable interannual spawning season (timing and duration), i.e. spawning from May to June in 2016 and for an earlier and prolonged period in 2017 (April to September). The proportion of spawning individuals was much higher in 2017 (31%) compared to 2016 (17%). In *Survey 3*, differences in spawning periods and the synchronicity during gametogenesis of males and females, were observed between sites in Ireland, between countries and inter-annually. Periodic spawning cycles, peaking in spring or summer were observed at all Irish sites, while year-round spawning, with only occasional breaks, was recorded at Arcachon and Ría de Aveiro.

Environmental parameters significantly influenced gametogenic cycles. In *Survey 1*, a weak but significant, positive correlation ($r_s$= 0.324, $p < 0.05$) between the percentage of “around-spawning” cockles and the monthly mean temperature was detected, with spawning first detected each year when seawater temperature was around 13 °C. Moreover, there was a stronger positive correlation between the percentage of “around-spawning” cockles and monthly mean chlorophyll *a* concentration ($r_s$= 0.475, $p < 0.001$). In *Survey 2*, spawning periods were triggered by a rise in seawater temperature and the prolonged spawning period in 2017 coincided with atypical summer seawater temperatures in the coastal lagoon (lower maximum temperature but higher mean temperature and remaining virtually constant from June to October). Similar to *Survey 1* and *2*, seawater temperature and, potentially, food availability, i.e. primary productivity, appeared probable influencers of the variations recorded in *Survey 3*.

The correlation between trematode infection and gametogenesis was investigated in *Survey 3*. Overall, trematode prevalence was greatest at Carlingford (IRE, 81.7%), followed by Arcachon (FRA, 42.3%), Dundalk (IRE, 20.9%), Cork (IRE, 16.5%), and Ría de Aveiro (POR, 11.7%). Results suggest that cockles may be able to cope with mild infection levels but, most importantly, they showed that trematodes (metacercariae and sporocysts) have a deleterious effect on reproduction, with reduced spawning and higher numbers of indeterminate individuals (those without gonad) at trematode-infected cockle communities.

Generally, mass spawning in spring- early summer, involving most cockles, occurred at every site, as part of a conservative strategy, while the percentages of cockles involved in spawning later through the summer, in the places where it occurred, were lower. Reduced mass spawning through summer could correspond to an adaptive strategy, depending on favourable environmental conditions, which would explain the important interannual variations found in long term monitoring studies. Many of the factors impacting cockle reproduction (temperature, food availability, parasite populations, etc.) are likely to change as a result of climate change and increasing frequency of atypical weather, thus, highlighting the importance of regular monitoring to follow changes in the cycle of cockle gametogenesis. Additionally, the mean size of spawning cockles in *Survey 3* was 33.06 mm, larger than the minimum capture size at all sites (both wild and fished). This highlights the importance of considering the individual size of cockles being fished out of a population to ensure future fecundity and sustainability of those populations. Moreover, the phenomenon of “shifting” reproductive cycles (timing and duration) associated with a changing marine environment and, in particular, years of atypical weather must be considered when setting harvesting/ fishing seasons at a local level.

**Table 1**. Key findings of reproductive strategies at all sites studied for this report (Deliverable 4.3), for *Survey 1* (monthly sampling), *Survey 2* (monthly sampling) and *Survey 3* (bimonthly sampling).

| Country | Survey no. | Site | Latitude | Survey years | Fishery type | Sex ratio M:F | Spawning pattern | Spawning synchronicity | Smallest mature or spawning cockle | Percentage indeterminate | Trematode prevalence |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Ireland** | 3 | Carlingford | 54°N | 2018-2019 | Occasional hand harvesting | 1:1.7 | Spring-summer | Synchronous in 2018, females initiated in 2019 | 21.0 mm | 30.1% | 81.7% |
| **Ireland** | 3 | Dundalk | 53°N | 2018-2019 | Suction dredge | 1:1.2 | Spring-summer | Female initiated | 22.0 mm | 30.3% | 20.9% |
| **Ireland** | 3 | Cork | 51°N | 2018-2019 | None (wild) | 1:0.9 | Spring, summer, autumn | Synchronous | 11.0 mm | 15.2% | 16.5% |
| **France** | 3 | Arcachon | 44°N | 2018-2019 | Hand rake | 1:1.1 | Year round-repetitive | Male initiated in 2018, female initiated 2019 | 16.2 mm | 10.9% | 42.3% |
| **Spain** | 1 | Arousa | 43°N | 2007-2011 | Hand dredge | 1:3 (1:1.1) | Winter/ spring-summer | Synchronous or female initiated | 17 mm | 19.7% | - |
| **Portugal** | 3 | Aveiro | 40°N | 2018-2019 | Hand rake | 1:0.9 | Year round, repetitive | Female initiated | 19.0 mm | 15.2% | 11.7% |
| **Portugal** | 2 | Formosa | 37°N | 2016-2018 | Hand dredge, knife, rake | 1:0.9 | Spring-autumn | Synchronous | 21.7 mm | 8.0% | - |

## 2. INTRODUCTION

*Cerastoderma edule* is a dioecious (separate sexes) species; that is considered an *r*-strategist (i.e. short life span and many offspring; Cardoso et al., 2009). They undergo simultaneous spawning of gametes by a large number of individuals (epidemic) or repetitive spawning (Yankson et al., 1986). Populations of *C. edule* are found across a broad geographical range, stretching between Norway (60ºN) and Senegal (14ºN). This species shows a high level of genetic diversity and inevitable local adaptation resulting in notable variation of life history characteristics (Chust et al., 2013, Martínez et al, 2015). Variations in reproductive parameters develop from differences in a range of biotic and abiotic factors and have been found at a species (Yankson et al., 1986), population (Guillou et al., 1990) and individual level (Iglesias & Navarro, 1991). Considering its variability, thorough knowledge of cockle reproductive cycles is needed for appropriate management to ensure cockle population sustainability, which is crucial in many areas because of the magnitude of the ecological services provided by this species (Beukema & Dekker, 2006; Carss et al., 2020) and, in some regions, the socioeconomic relevance of its fishery (Ruano, 1997; Freire and García-Allut, 2000; West et al., 2003; Hervas et al., 2008; Parada and Molares, 2008; Crespo et al. 2010; Burdon et al. 2014; Thomas et al. 2014; Pita et al. 2019).

Reports of age and length at first spawning have varied among different studies (Hancock and Franklin 1972, Cardoso et al. 2009, Elliott et al. 2012). The minimum size at first maturity in *C. edule* is typically around 12 mm shell length (SL) for males and 14 mm SL for females (Hancock & Franklin, 1972; Seed and Brown, 1977); however, spawning has been reported in smaller individuals (Cardoso et al., 2009). The average age at maturity is 15 - 18 months (Dabouineau and Ponsero, 2009) but varies considerably with some reports of spawning during the first year in faster growing cockles (Seed & Brown, 1977). In fact, sexual maturity is more closely associated with size than with age of individuals (Handcock & Franklin, 1972; Sauriau, 1992). Hence, size, as well as age, of cockles at first maturity are considered good indicators of population health. For example, in the Mundaka Estuary, Spain, it was found that environmental conditions which strongly limited the growth potential of *C. edule* caused a postponement of maturity as well as an overall reduced reproductive effort (Iglesias and Navarro, 1991). Another good indicator of cockle population health is sex ratio, with healthy cockle populations generally exhibiting a 1:1 sex-ratio (Boyden, 1971a). Deviations from these ratios often provide evidence of harmful biological or environmental pressures such as a sex-specific mortality due to parasitism or environmental contaminants, as observed in *C. edule* (Woolmer, 2013, Lusher et al., 2017) but also in other marine invertebrate species such as *Gammarus duebeni*, impacted by microsporidia (Ironside et al. 2003).

The typical reproduction cycle of cockles begins with gametogenesis starting in spring and spawning in the summer (Longshaw and Malham, 2013). Variation is evident in this regime, however, with cockles in Trondheim, Norway (63°N) spawning for a single month (Rygg, 1970) and cockles in the French Channel (49°N) spawning throughout most of the year (Guillou et al., 1990). Furthermore, the timing of spawning and gametogenesis can differ interannually even in cockles from the same region (Navarro et al., 1989). Many factors have been proposed as the drivers of initiation of spawning and gametogenesis in cockles, including seawater temperature (Gam et al., 2010), water quality (Lusher et al., 2017), immersion time (Honkoop and van der Meer, 1998) and feeding conditions in the previous season (Navarro et al., 1989). Successful reproduction and population recruitment requires suitable environmental conditions (e.g. water temperature, salinity, water currents, turbidity and sediment type, fishing pressure), healthy adults (e.g. sufficient energy reserves, lack of parasitism, lack of harmful environmental contaminants) and resulting balanced biological factors (e.g. sex ratios, predation, mixed age classes, density). It is clear that many factors impact cockle reproduction both temporally (Yanskon, 1986, Beukema et al., 2001) and spatially and these factors can interact to confound outcomes (de Fouw et al., 2020). A particular cause for concern is the impact of changing climates on cockle reproduction. It has already been shown that warmer winters are negatively impacting cockle recruitment (e.g. smaller sized and fewer numbers of eggs being produced; Honkoop and van der Meer, 1998), while extreme cold events reduce survival (Beukema and Dekker 2020).

*Cerastoderma edule* have a wide thermal tolerance and it has been stated that temperature does not direct or coordinate reproduction in this species (Navarro et al. 1989). This suggestion is supported by the wide variability of reproductive cycles reported from different sites within a region and over time (Martinez-Castro and Vázquez, 2012). However, it has been shown that higher water temperatures can have an energetic cost on bivalves causing reduced body mass (Honkoop and Beukema, 1997) and colder (but not extreme) winters trigger improved subsequent reproductive seasons (Yankson et al., 1986; Morgan et al., 2013; Beukema and Dekker, 2020). Therefore, the role of temperature on reproductive success is likely important, even if not direct. Water quality can also influence the condition and hence reproductive success of bivalves. Cockle populations located in polluted waters have been found to display higher levels of vitellogenin-like protein levels (precursors of the egg-yolk proteins, vitellins, which provide energy reserves for embryo development) and imbalanced sex ratios which have the potential to reduce fertility and reproduction rates (Matozzo and Marin, 2007, Lusher et al., 2017). Fishing is another well-known abiotic factor with the potential to significantly affect recruitment. Fisheries have been reported to play a role in numerous local mass mortalities of cockles (Burdon et al., 2014) and can cause high post-settlement mortality of young cockles, particularly over the first winter, resulting in reduced reproductive success (Dare et al., 2004; Crespo et al., 2010). Fishery restrictions and regulations (e.g. minimum size and gear restrictions) play an important role in the overall effect of fishing. For example, there has been evidence to suggest that suction dredging can harm recruitment (Piersma et al., 2001) whereas low-level hand-racking is unlikely to have the same level of negative impact.

Cockles are hosts to a large range of parasites (Carballal et al., 2001; Longshaw and Malham, 2013) and digenean trematodes frequently dominate (Thieltges et al., 2006), with different species occurring in specific areas of the host's range (de Montaudouin et al., 2009; Correia et al., 2020). This taxonomic group impacts tissue structure and morphology due to their size relative to the host. They exhibit a complex life cycle and can have detrimental impacts on cockle health, including reproductive health (Longshaw and Malham, 2013, de Montaudouin et al., 2012). Cockles can be infected by trematodes as either primary (sporocysts) or secondary hosts (metacercariae; de Montaudouin et al., 2009). Trematodes can influence energy allocation in bivalves (Hurd, 2001), with some species (e.g. *Bucephalus minimus* and *Gymnophallus choldeochus*) causing castration (Thieltges et al., 2006) or starvation (Carballal et al., 2001) in cockles. Such effects can result in energy trade-offs and adapted life history strategies. Thereby, pathogens and parasites play an important role in defining reproductive characteristics.

This report aims to examine the reproductive health (sex ratio, size and age at first maturation, timing and duration of gametogenesis (reproductive cycle and spawning) of Atlantic Area (AA) cockle populations, beginning first with an investigation of the historical trends outlined in previously published literature. Adding to this, field surveys (*Survey 1*: 2007-2011, *Survey 2*: 2016-2017 and *Survey 3*: 2018-2019) with stakeholder involvement, were conducted to compare the current reproductive health of AA cockle populations at different latitudes (in Ireland, France, Spain and Portugal, 54°N to 37°N) with past patterns. Data from this action will feed into models in WP6, which aims to forecast cockle larval dispersal. The findings from this deliverable will prove valuable for stakeholder groups, including fishers, managers and conservationists, by providing knowledge on the status and drivers of regional patterns of AA cockle reproduction. This will support the protection of cockle populations into the future, in order to ensure conservation as well as prosperous fisheries.

# 3. MATERIALS AND METHODS

## 3.1. Literature Review & Analyses

A literature review was conducted to create a dataset from 21 records (from 15 studies carried out in Denmark, France, Ireland, Norway, Spain, Sweden, the Netherlands and the UK) detailing the historical reproductive status of AA cockle populations. These records covered a latitudinal range of 42°N to 63°N and a time period of 1954 to 2010. An extensive web-based literature search (published and grey literature) was conducted following the methods outlined in the Deliverable 4.1 report "Baseline historical survey of common cockle (*Cerastoderma edule*) populations in the Atlantic area". Dataset criteria was explicitly set out, as detailed in the report for Deliverable 4.1. Experimental data were not included. Furthermore, studies were excluded if a time period was not explicitly stated. Data were analysed qualitatively to determine potential historical trends in the reproductive status of the common cockle.

## 3.2 Study Areas

### *Survey 1* (Spain, CIMA)

Ría de Arousa (**Figure 1**) is located on the west coast of Galicia (NW Spain). It is 33 km in length and 9 km wide, on average; with a surface area of 239 km$^2$, a volume of $4.8 \times 10^9$ m$^3$ and is approximately 70 m deep at its southern mouth (Rosón et al. 1995, 1997). Tidal range varies between 0.8 and 4.1 m depending on site and tide coefficient. There is a strong upwelling, especially during the summer, caused by the intrusion of the north–east Atlantic Central Water that replaces the surface water (Fraga, 1981), that is primarily responsible for high local productivity (Prego et al., 1999). Two main rivers discharge into this ría: Ulla (79.3m$^3$ s$^{-1}$of average flow) and Umia (16.3 m$^3$ s$^{-1}$; Río-Barja and Rodríguez-Lestegás, 1996). Cockles were collected in Lombos do Ulla (42° 38'N, 8° 46'W), in the inner area of Ría de Arousa. This is a shallow (1-5 m in depth) mostly subtidal bed, strongly influenced by the Ulla River, with salinity in the top 5 m ranging between 18 and 35 (although it has been measured as low as 3 when very significant run-off events occur), while the temperature varies between 11 and 20 °C. The total bed surface is 11.35 km$^2$. Traditionally, Lombos do Ulla was one of the most productive shellfish beds of Galicia, with an annual average landing of 534 t of cockles for the period 2002 to 2011 (internal report of the Ministry of the Sea of the Regional Government of Galicia), before the cockle fishery collapsed in the whole Ría de Arousa due to marteiliosis (Villalba et al., 2014). This shellfish bed is located within a shellfish production zone classified as B (http://www.intecmar.gal/Informacion/microbio/Clasificacion.aspx).

### *Survey 2* (Portugal, IPMA)

Ría Formosa, located in the south of Portugal, includes a large intertidal zone, about 55 km long (E – W) and 6 km (N – S) at its widest point (Newton and Mudge, 2003; **Figure 1**). It is a shallow mesotidal coastal lagoon, with a wet area of 105 km$^2$ including several channels and an extensive intertidal area. The lagoon has reduced freshwater inputs and is characterized by an exchange of 40–75% of water mass during each semi-diurnal tidal cycle (Falcão and Vale, 2003). Water temperatures range from

12-27°C and salinity ranges between 13 and 36.5 (Newton and Mudge, 2003). The sediment type varies from mud and muddy-sand flats (~2500 ha) to sandy sediments (~1000 ha; Falcão et al., 2006). Ría Formosa is a Special Protection Area (SPA) designated under the EU Birds Directive, a Ramsar Reserve (Ramsar, 2005) and part of the EU Natura 2000 network. Clam farming and systematic collection of benthic organisms as bait for recreational fishing are carried out in the area. Ría Formosa is classified as Class B and C (where bivalves must be purified, relayed to a Class A area or heat treated prior to human consumption) waters by the EU Shellfish Waters Directive (2006/113/EEC). Cockle harvesting is conducted year-round, using hand dredges or harvesting knives and rakes (Leitão and Gaspar, 2007). Cockles were collected with assistance from local shellfish farmers from a sand bank in the intertidal area located near Culatra island, in the middle of Ría Formosa lagoon (36$^{o}$ 59.858’N; 7$^{o}$ 49.812’W).

### *Survey 3* (Ireland, UCC; France, UB; Portugal, UA)

Carlingford Lough, Ireland, (54°02'N, 6°10'W) is located on the Irish Sea and covers approximately 49 km$^2$ (**Figure 1**). Mean monthly seawater temperature ranges between 8.2-15$^{o}$C and salinity averages 19.8 (Copernicus, 2020). The lough is designated a Special Area of Conservation (SAC) and a SPA under the EU Habitats Directive, as well as being a Ramsar Reserve assigned by the International Convention on Wetlands (Ramsar, 2005). No active cockle fishery exists here (although occasional hand harvesting occurs), but the area is important for mussel *Mytilus edulis* and Pacific oyster *Crassostrea gigas* aquaculture (Ferreira et al., 2007). Shellfish waters in the area are classified as Class A (i.e. bivalves can be harvested and consumed by humans without treatment; SFPA, 2019). Cockles were collected with the help of local shellfish farmers from an intertidal area of an oyster farm (54°01'49"N 6°09'19"W).

Dundalk Bay, Ireland, is an exposed bay open to the Irish Sea (Clarke and Tully, 2014), covering an area of sand and mud flats of approximately 45 km$^2$ (Fahy et al., 2004; **Figure 1**). Seawater temperature ranges annually between 6-17 °C and salinity ranges between 33.4-34 (Copernicus, 2020). Dundalk Bay is a SAC (NPWS, 2014) and supports a cockle fishery (suction dredging) from July to October, as well as dredging for razor clams and the hand gathering of mussels (Marine Institute, 2016). From 2010-2015, under the Water Framework Directive, the inner and outer bay were classified as good and moderate water status respectively (EPA, 2019). With assistance from local cockle fishers, two intertidal beds were sampled: Annagassan (53°52'48"N, 6°20'06"W) in the south of the bay and Cooley (54°00'12"N, 6°17'31"W) in the north.

Cork Harbour, Ireland, is a sheltered bay located on the south coast of Ireland (**Figure 1**). It is a SPA and mainly consists of muddy substrate (NPWS, 2015). Seawater temperature ranges from 6.9 to 17.6 °C and salinity ranges from 33.1 to 34.8 (Copernicus, 2020). The harbour is heavily industrialised, with water historically containing a wide range of pollutants (Hartl et al., 2006, Kilemade et al., 2004, Minchin et al., 1996). No commercial cockle fishing occurs in the harbour. Two intertidal sites were sampled within Cork Harbour: Cuskinny (51°51'30"N, 8°15'47"W) and Ringaskiddy (51°49'54"N, 8°18'02"W).

The 25 km$^2$ Banc d'Arguin is a national reserve located in Arcachon Bay, France (**Figure 1**). The habitat is a moderately sheltered intertidal sandflat in the south of the bay (de Montaudouin and Lanceleur, 2011). Salinity ranges from 32 to 35 and the mean seawater temperature ranges between 9.5-21.1 °C (de Montaudouin and Lanceleur, 2011). Due to its designation as a marine reserve, efforts are made to protect it from anthropogenic activity (Magalhães et al., 2018) and fishing in the area is forbidden between April and August, inclusive. A classification of 'good' ecological status has been given to the water quality in the bay (EauFrance, 2020). Samples at the Banc d'Arguin were obtained from a single bed (44°35'08"N 1°14'16"W).

Ría de Aveiro is a coastal lagoon in the northwest of Portugal (**Figure 1**), covering an area of 83 km$^2$ (Lillebø et al., 2015). Seawater temperature ranges between 9.6-23.1 °C (Vaz et al., 2016) and salinity ranges from 0 (due to riverine input) to 36 (Lillebo et al., 2015). A cockle fishery exists in Ría de Aveiro year-round, and it is a SPA under the EU Birds Directive (Lillebø et al., 2015). The area is classed by the Water Framework Directive as having 'moderate' ecological status (MAMAOT; Coelho et al., 2012). The lagoon serves as a port and is also important for aquaculture (including seabass *Dicentrarchus labrax*, *C. gigas*, grooved carpet shell *Ruditapes decussatus* and *Mytilus* spp.), agriculture, fishing and industry (Lillebø et al., 2015). Samples at the Ría de Aveiro were obtained from one bed (40°38'20"N 8°44'00"W).

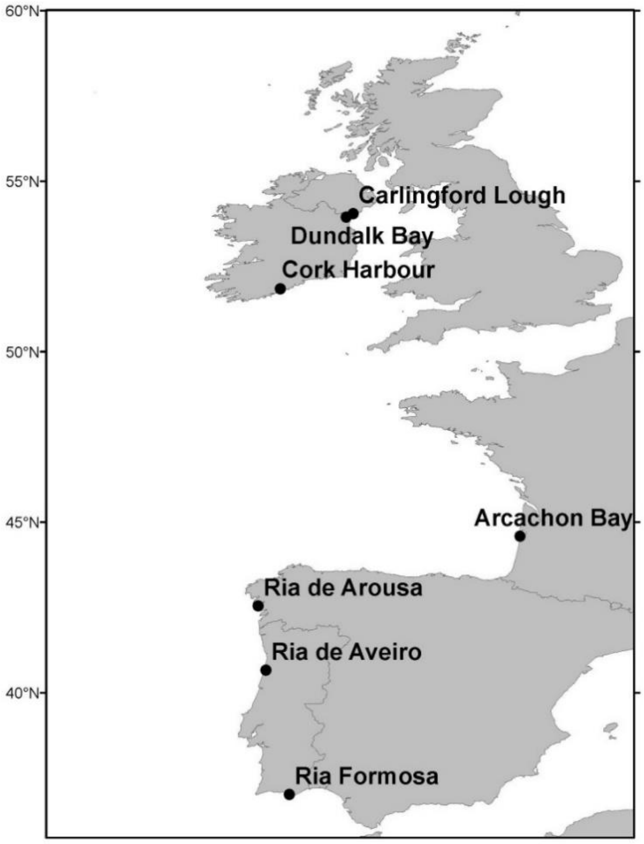

**Figure 1.** Location of study sites in Ireland, France, Spain and Portugal.

## 3.3. Stakeholder Engagement & Training

Continuous engagement with local shellfish farmers and fishermen provided researchers with information about sample collection sites and assistance with the collection of cockle samples. Training in gonad stage identification by histology was provided to Universidade de Aveiro (UA) by University College Cork (UCC).

## 3.4 Sample Collection

### *Survey 1*

In order to thoroughly characterise the gonad cycle of cockles in the shellfish bed of Lombos do Ulla (Ría de Arousa), 1,770 cockles were collected and screened over five years between February 2007 and December 2011. Approximately 30 adult cockles (mean length: 28.9 ± 0.1 mm, range: 19 – 39 mm) were collected monthly using a traditional hand dredge (70 cm width, 15 cm long teeth, 17 mm mesh size) operated from a boat (**Table 2**). The specimens were transported to the lab in cool conditions.

**Table 2.** The number of cockles analysed in *Survey 1* (Lombos do Ulla, Ría de Arousa, Spain) each month between February 2007 and December 2011.

| Month | 2007 | 2008 | 2009 | 2010 | 2011 |
|---|---|---|---|---|---|
| January | 30[a] | 30 | 30 | 29 | 30 |
| February | 30 | 30 | 30 | 30 | 28 |
| March | 30 | 29 | 30 | 29 | 30 |
| April | 15 | 30 | 30 | 29 | 30 |
| May | 30 | 30 | 30 | 30 | 30 |
| June | 30 | 27 | 30 | 30 | 29 |
| July | 30 | 30 | 30 | 30 | 30 |
| August | 30 | 30 | 30 | 30 | 30 |
| September | 30 | 30 | 30 | 30 | 30 |
| October | 30 | 30 | 30 | 30 | 27 |
| November | 30 | 30 | 30 | 30 | 29 |
| December | 30 | 30 | 30 | 29 | 30 |

[a] The sampling corresponding to January 2007 was actually collected on 8th February 2007.

Additionally, in order to calculate age and size at first maturity, trying to cover a broader geographical range representing the Galician coast, histological sections from 665 cockles that had been collected between 1999 and 2003 from five shellfish beds: Lombos do Ulla, Camaxe (Ría de Arousa, 42° 33’N, 8° 51’W), Sarrido (Ría de Arousa, 42° 30’N, 8° 49’W), Misela (Ría de Noia, 42° 47’N, 8° 55’W) and Villabril (Ría do Barqueiro, 43° 44’N, 7° 42’W) were analysed. Juveniles and adult cockles (range: 10 - 36 mm in length) that had been taken during the periods of the highest reproductive activity (between March and August, depending on the bed) were used for this purpose.

### *Survey 2*

In *Survey 2*, 450 cockles were sampled and screened over two years between March 2016 and February 2018 (**Table 3**). Approximately 20 individuals were collected monthly by raking for buried cockles. An additional sample of 130 cockles of broad size range (14.5 - 34.2 mm in length) was collected in April 2018, during the maturation peak, to estimate the size at first sexual maturity.

**Table 3.** The number of cockles analysed in *Survey 2* (Ría Formosa, Portugal) each month between March 2016 and February 2018.

| **Ría Formosa** | |
|---|---|
| Mar-16 | 11 |
| Apr-16 | 11 |
| May-16 | 15 |
| Jun-16 | 22 |
| Jul-16 | 19 |
| Aug-16 | 12 |
| Sep-16 | 20 |
| Oct-16 | 19 |
| Nov-16 | 20 |
| Dec-16 | 22 |
| Jan-17 | 20 |
| Feb-17 | 20 |
| Mar-17 | 20 |
| Apr-17 | 20 |
| May-17 | 20 |
| Jun-17 | 20 |
| Jul-17 | 20 |
| Aug-17 | 20 |
| Sep-17 | 20 |
| Oct-17 | 20 |
| Nov-17 | 20 |
| Dec-17 | 19 |
| Jan-18 | 20 |
| Feb-18 | 20 |
| **Total** | **450** |

### *Survey 3*

In *Survey 3*, 1,636 cockles were sampled and screened (1,115 from the three Irish sites, 239 from the French site and 282 from the Portuguese site). The aim was to collect 30 samples every second month, beginning in April 2018 until October 2019 (19-month time period). However, some deviation from this sampling regime occurred due to difficulty in locating cockles, as well as occasional issues with fixation for histology (**Table 4**). At two of the Irish sites (Carlingford and Cork) that had sediment composed of a larger grain size (i.e. pebbles, rocks, etc.), surfaced cockles were gathered due to sediment and tidal constraints. At these sites, because of the rocks present in the substratum, cockles could not fully bury, as has also been found previously (Barnes, 1973), and raking was impractical. At the remainder of the sites, which were sandy and muddy, both surfaced and buried cockles were sampled. It is acknowledged that previous studies have reported differences in cockle condition, infection levels and reproduction characteristics between buried and surfaced cockles (Zwarts, 1991, Desclaux et al., 2002, Morgan et al., 2012, Diaz et al., 2016). However, to reduce any possible impact or bias, sampling at Cork and Carlingford was conducted just before or after low tide (when healthy cockles move towards the surface to feed in the covering water), the majority of cockles collected were still partially submerged and, moribund individuals were avoided.

Bi-monthly sampling was deemed appropriate in *Survey 3* following the slow development of cockles in the monthly sampling regime of Morgan et al. (2013). Furthermore, when spent individuals were recorded, it was surmised that spawning had occurred in the previous weeks.

**Table 4.** Sample sizes of cockles analysed histologically in *Survey 3* from each bed at the five sites examined.

| | **Carlingford** | **Dundalk Bay** | | **Cork Harbour** | | **Arcachon** | **Ría de Aveiro** |
|---|---|---|---|---|---|---|---|
| | Oyster Farm | Annagassan | Cooley | Cuskinny | Ringaskiddy | Arguin | Aveiro |
| Apr-18 | 30 | 0 | 0 | 30 | 25 | 30 | 30 |
| Jun-18 | 30 | 0 | 0 | 30 | 19 | 30 | 30 |
| Jul-18 | 0 | 30 | 30 | 0 | 0 | 30 | 0 |
| Aug-18 | 29 | 30 | 30 | 28 | 14 | 0 | 29 |
| Oct-18 | 15 | 30 | 30 | 28 | 23 | 30 | 30 |
| Dec-18 | 30 | 31 | 30 | 5 | 9 | 30 | 28 |
| Feb-19 | 24 | 30 | 30 | 24 | 15 | 30 | 30 |
| Apr-19 | 30 | 29 | 29 | 30 | 29 | 30 | 30 |
| Jun-19 | 28 | 0 | 0 | 28 | 8 | 29 | 30 |
| Aug-19 | 0 | 30 | 30 | 27 | 16 | 0 | 28 |
| Oct-19 | 13 | 30 | 30 | 10 | 9 | 0 | 17 |
| **Total** | **229** | **240** | **239** | **240** | **167** | **239** | **282** |

## 3.5. Cockle processing

### 3.5.1 Morphometrics

Prior to histology, the whole wet weight (shell and tissue) of each cockle was recorded before dissection. Cockle morphometric measurements were gathered (**Figure 2**). Growth rings were not analysed in *Survey 2*. Growth rings in cockles are set down each winter (Orton 1926); although sometimes, lines may be hard to distinguish due to warm winters or short cold spells (Ponsero et al., 2009). Therefore, only easily distinguishable growth rings were counted to determine an estimation of cockle age.

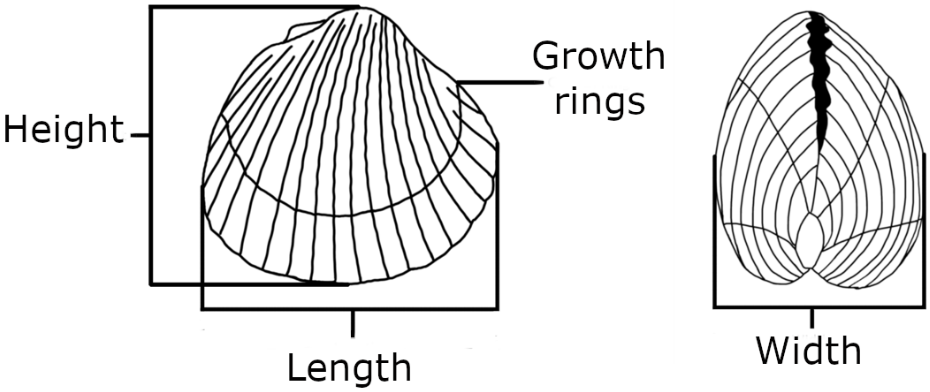

**Figure 2.** Measurements taken for cockle morphometrics.

### 3.5.2 Histology analysis for determining sex and maturity stage

Small sized cockles were placed intact into a histocasette and cross sections, including hinge, gills, mantle lobes, visceral mass and foot, were obtained from larger individuals. The tissues were fixed in Davidson's solution for 24 - 48 hours (Shaw and Battle, 1957). They were then prepared for embedding in wax, by a 20-hour cycle through graded volumes of ethanol for dehydration (adapted from Howard and Smith 1983). The samples were sectioned to at least 5 µm (3 µm if possible) before haematoxylin and eosin staining (Humason, 1979).

Samples were viewed under a light microscope at 4X, 10X and 40X to determine whether gonad was present and to determine the sex and maturity stage of each individual, as appropriate. In *Survey 1*, each cockle was assigned to one stage from the seven-stage scale used by Iglesias (2006) and Martínez-Castro & Vázquez (2012): 0 = resting gonad; 1 = early gametogenesis, 2 = advanced gametogenesis, 3 = ripe gonad, 4A = post-spawning gonad, 4B = gonad restoration and 5 = reabsorbing gonad. In *Survey 2*, cockle reproductive maturity was categorized into six stages using the gametogenic scale proposed by Gaspar and Monteiro (1998) and the first three stages were the same as in *Survey 1*: 0 = inactive, 1 = early active, 2 = late active, 3 = ripe, 4 = partially spawned and 5 = spent. In *Survey 3*, gonad staging was conducted according to the scale described by Xie and Burnell (1994) and Drummond et al. (2006) for clams and later by Morgan et al. (2013) for cockles. When one individual exhibited multiple stages, or an intermediate between two stages, the dominant stage was assigned. Cockles that did not have identifiable gonad were classed as indeterminate.

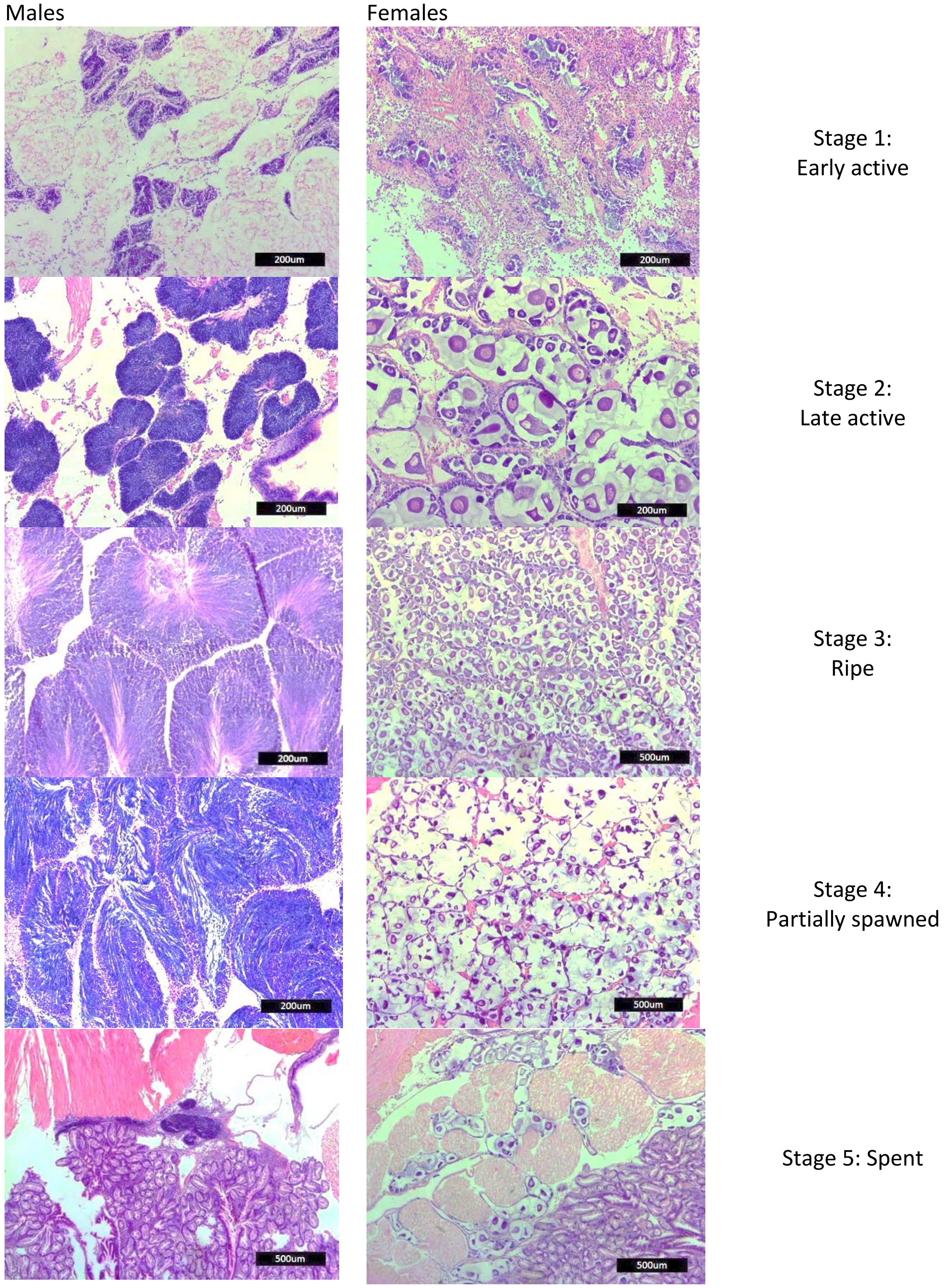

**Figure 3**. Light micrographs of gonad developmental stages in *Cerastoderma edule*, identified in the surveys and labelled according to the system used in *Survey 2* and, partly *Survey 1* (stages 1 – 3 only).

## 3.6. Estimation of sex ratio

The sex of the cockles was established as male, female or indeterminate through light microscopy examination of histological sections. Pearson's Chi-square tests with Yate's continuity corrections were performed to assess whether sex ratios (M:F) differed from 1:1 in each location.

## 3.7. Estimation of the size at first sexual maturity

To determine the size at first sexual maturity ($SL_{50}$), defined as the length at which 50% of the population is mature, the gonad stage of each cockle was determined. The individuals in gonad stages 3, 4A and 4B (*Survey 1*) and in stages 3 and 4 (*Survey 2*) were considered physiologically mature. Those cockles affected by trematode sporocysts and heavy disseminated neoplasia were discarded in *Survey 1* because both conditions affect cockle gametogenesis (Iglesias, 2006). Individual lengths were grouped in 1 mm size classes and the proportion of mature individuals in each size class was fitted to the logistic model (maturity ogive) using non-linear least squares:

$$P_i = \frac{1}{1 + e^{-r(SL_i - SL_{50})}}$$

where $P_i$ is the proportion of mature individuals; $SL_i$ the size class; r is the slope of the curve; and $SL_{50}$, the size class at which 50% of the individuals in the population are mature (King 1995).

In *Survey 3*, pairwise differences in spawning length, spawning age and proportion of indeterminate individuals between sites were analysed with Kruskall Wallis tests followed by a Dunn Test with a Bonferonni correction.

## 3.8. Gonad cycles and environmental parameters

The gonad cycle characterised at each site was compared with the variation of the local seawater temperature.

In *Survey 1*, weekly records of surface (0 to 5 m depth) seawater temperature and chlorophyll *a* concentration values, between January 2007 and December 2011, corresponding to the oceanographic sampling station A3 (42° 36.6´ N, 8° 49.9´W; 5.2 km away from Lombos do Ulla) of the *Instituto Tecnolóxico para o Control do Medio Mariño* (INTECMAR) were downloaded from their website (http://www.intecmar.gal/Ctd/Default.aspx). Temperature and salinity were measured using a CTD, while chlorophyll *a* concentration was measured using spectrofluorometric methods. A monthly mean was calculated for each environmental parameter. Spearman's rank correlation coefficients were calculated between the percentage of "around-spawning" cockles (stages 3, 4A and 4B) and seawater monthly mean temperature and monthly mean chlorophyll *a* concentration to assess the association between these two environmental variables and the percentage of cockles involved in spawning (i.e. cockles in stages immediately before and after gamete releasing).

In *Survey 2,* the mean gonadal index (GI) was calculated using the following equation:

$$GI = \frac{\Sigma\ individuals\ each\ stage\ \times stage\ ranking}{total\ individuals\ each\ month}$$

where numerical values were assigned to each stage (stage ranking) in a way to represent the ripeness of gonad as follows: (0 = less important; 5 = the most important) as follows: inactive = 0, early active = 2, late active = 4, ripe = 5, partially spawned = 3, spent = 1.

Seawater temperature data was derived from Tunipex (www.tunipex.eu/information_oceanic.php). Changes in monthly GI and seawater temperature were plotted together for comparison.

In *Survey 3*, seawater temperature data was derived from the Atlantic-Iberian Bay Irish-Ocean Physics Analysis and Forecast (Copernicus, 2020). ANCOVA analysis was conducted to determine if there was a correlation between the percentage of individuals spawning and seawater temperature.

## 3.9. Association of gonad condition and trematode infection

In *Survey 3*, histological analyses were used to determine presence or absence of trematodes (either sporocysts or metacercaria stages or coinfection of both) in cockles from each site.

Pearson's Chi-squared tests with Yate's continuity corrections were employed to examine whether the proportion of trematode infection varied across sites and whether the percentage of individuals exhibiting each stage of gametogenesis varied according to trematode infection status. As appropriate, a post-hoc analysis, comparing the adjusted critical value with the adjusted residuals was conducted to determine which variables were significant.

Data was found to be non-parametric (not following a normal distribution) and were therefore submitted to Kruskal-Wallis tests followed by a Dunn Test with a Bonferroni correction to examine pairwise differences in length and age of trematode infected individuals.

## 3.10. Statistical analyses

Minitab 17 software package was used for the statistical analyses in *Survey 1*. All data analyses for *Survey 2* were conducted using Microsoft Excel and statistical analyses for *Survey 3* were conducted in R, version 3.6.2, (R Core Team, 2019). In the case where multiple beds were studied at one site in *Survey 3*, results of these beds were pooled prior to analysis. Significance level was determined at the 95% probability level for all tests.

# 4. RESULTS

## 4.1. Historical review of cockle reproduction

Spawning times were the principle parameter reported in the historical literature. Following the application of exclusion criteria (due to differences in reporting of data, lack of information or dubious records), 21 records of cockle spawning times were obtained. These ranged in latitude from Trondheim, Norway (63°N, 10°E; Rygg, 1970) to the Ría de Vigo, Spain (42°N, 8°W; Martínez-Castro and Vázquez, 2012). The earliest record of spawning was reported in 1954 in the UK (Creek, 1960). Spawning time tended to occur within the summer season (May to September) and did not appear to vary over time, however, fewer records were obtained from the 1950s to the 1960s (**Figure 4**).

The duration of spawning periods varied greatly, with the longest spawning periods recorded from the 1980s onwards (**Figure 4**). Data showed a temporal increase in spawning periods at latitudes between 50 – 55°N. Spawning was observed for most of the year in the case of cockles in the French Channel in 1987 (Guillou et al., 1990), when sea temperatures were cooler (**Figure 4**). One of the shortest spawning periods was noted in the northernmost record, with Trondheim cockles spawning during the month of July only (Rygg, 1970), in a year when sea temperatures were warmer (**Figure 4**). A month-long spawning was also observed in south Wales in 1958 (Hancock and Franklin, 1972).

Patterns and timing of spawning also varied, as demonstrated by Yankson et al., (1986). Cockles in south Wales spawned completely in the spring and summer of 1982, yet following a severe winter the next year, they exhibited partial repetitive spawning between summer and autumn.

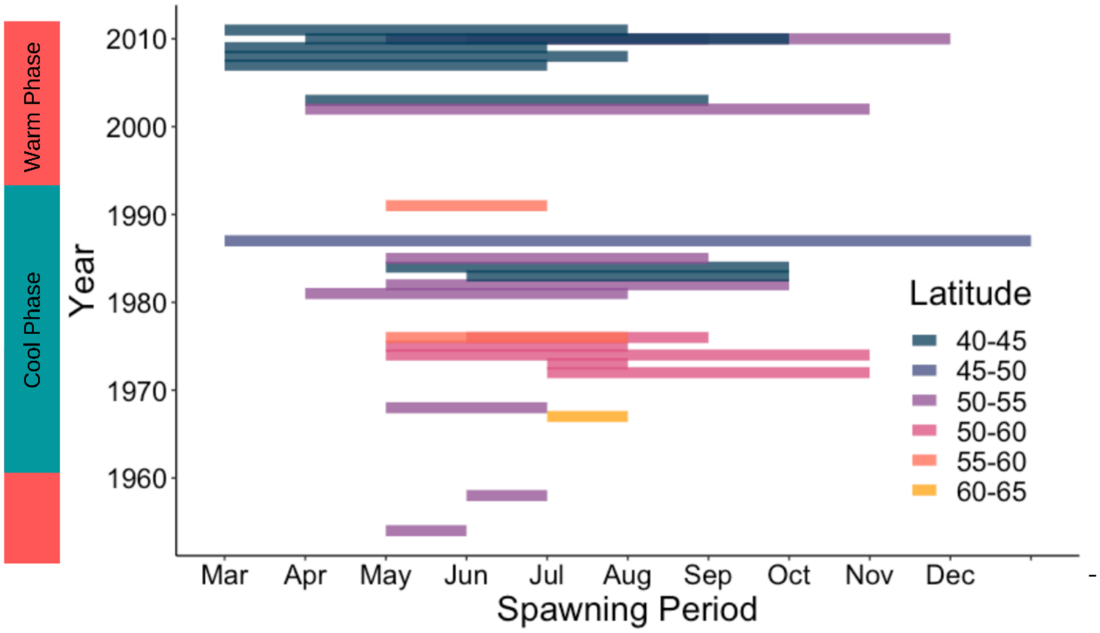

**Figure 4.** Historic spawning period of *Cerastoderma edule* across various latitudes in Europe. Cool and warm years of the Atlantic Multidecadal Oscillation (AMO) Index indicated to the left of the graph.

## 4.2 Sex Ratio

### *Survey 1*

In total, 1,770 cockles from Lombos do Ulla were examined through histology, of which 617 were male (34.3 %), 803 were female (44.8 %), 349 were indeterminate (19.7 %) and one specimen was hermaphroditic (0.1 %). The overall sex ratio (males: females, excluding indeterminate individuals) was 1:1.3, significantly different from the balanced 1:1 sex ratio ($\chi^2$ = 24.71, df = 1, $p < 0.05$). Nevertheless, when the sex ratio was computed by only including the gametogenic active periods (i.e. those samples with no cockles with indeterminate sex), the sex ratio did not statistically differ from 1:1 (375 males, 392 females, 1:1.05; $\chi^2$ = 0.38, df = 1, $p > 0.05$; **Table 5**). The reason to additionally estimate sex ratio excluding cockles with indeterminate sex was due to the fact that females are more easily distinguished than males in the early gametogenesis stage (Boyden, 1971b), thus a proportion of male cockles at this stage could be underestimated and classified as indeterminate individuals.

### *Survey 2*

Overall, 450 cockles were analysed histologically, comprising a total of 216 males, 198 females and 36 indeterminate (**Table 5**). Accordingly, the sex-ratio (males: females) was statistically balanced (1:0.9), without any significant deviation from the parity (1: 1) sex-ratio ($\chi^2$ = 0.785, df = 1, $p > 0.05$).

### *Survey 3*

In total, 1,636 cockles were examined histologically, of which 587 were male, 686 were female and 363 were indeterminate. The overall sex ratio (males: females, excluding indeterminate individuals) was 1: 1.2. However, the only sites where there was a significant deviation from the expected 1: 1 ratio was Carlingford, where more females were present than males (1: 1.7;$\chi^2$ = 12.1, df = 1, $p < 0.001$; **Table 5**) and Dundalk (1: 1.3; $\chi^2$ = 9.8129, df = 1, $p < 0.001$; **Table 5**). These sites also had high levels of indeterminate individuals (Carlingford: 30.1%, Dundalk: 30.3%).

Indeterminate individuals were found at all sites. The proportion of indeterminate individuals varied across sites ($\chi^2$ = 13.699, df = 4, $p < 0.01$, **Appendix 2**). Higher proportions of indeterminate individuals were observed at Carlingford, compared with Ría de Aveiro ($p < 0.05$) and Arcachon ($p < 0.05$). Proportions of indeterminate individuals appeared to vary over time, with peaks of indeterminate individuals in the winter months (**Figure 5**). Arcachon, particularly, had a shortened period (during the summer) when most cockles were indeterminate, possibly indicating a resting stage. However, due to a lack of sampling in August, it is unknown if this resting stage continued into the end of the summer. Resting periods appeared longer in the Irish sites during 2018 (**Figure 5**).

**Table 5.** Sex distribution, size and age range of all *Cerastoderma edule* examined in *Survey 1* from Ría de Arousa, *Survey 2* from Ría Formosa and *Survey 3* from three Irish sites (Carlingford, Dundalk and Cork)) and, one French (Arcachon) and one Portuguese (Ría de Aveiro) site. Significant differences in Chi Square Tests for Sex Ratio highlighted in bold.

| | n | Male (%) | Female (%) | Indeterminate (%) | Sex Ratio (M: F) | $\chi^2$ | df | p | Length (mm) | | No. of Growth Rings | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | Range | Mean | Range | Mean |
| **Ría de Arousa** | 617 | 34.3 | 44.8 | 19.7 | 1:1.3 | 24.71 | 1 | **<0.05** | 19 - 39 | 28.9 | - | - |
| **Ría Formosa** | 450 | 48.0 | 44.0 | 8.0 | 1:0.9 | 0.79 | 1 | >0.05 | 20 – 34 | 25.1 | - | - |
| **Carlingford** | 229 | 25.3 | 44.5 | 30.1 | 1:1.7 | 12.1 | 1 | **<0.05** | 21 – 45.8 | 33.8 | 1 – 11 | 4.2 |
| **Dundalk** | 479 | 25.7 | 33.1 | 30.3 | 1:1.3 | 5.06 | 1 | **<0.05** | 18.1 – 49.1 | 32.7 | 0 – 9 | 2.9 |
| **Cork** | 407 | 41.3 | 43.5 | 15.2 | 1:0.9 | 0.23 | 1 | 0.63 | 9.5 – 49.9 | 32.9 | 0 – 13 | 3.6 |
| **Arcachon** | 239 | 41.8 | 47.3 | 10.9 | 1:1.1 | 0.8 | 1 | 0.37 | 16.2 – 40 | 28.6 | 1- 7 | 3.7 |
| **Ría de Aveiro** | 282 | 43.6 | 41.1 | 15.2 | 1:0.9 | 0.21 | 1 | 0.65 | 7 – 36 | 25.8 | - | - |

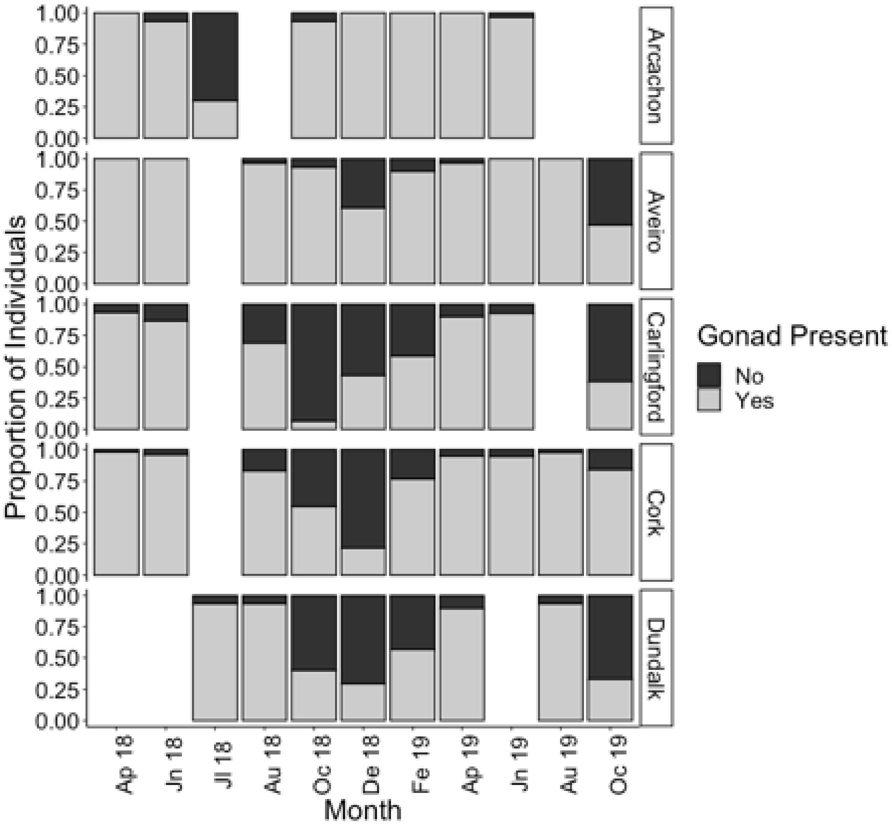

**Figure 5.** Proportion of *Cerastoderma edule* with and without evident gonad at each site in *Survey 3*. Note that months without a column indicate sampling was not conducted. Months with no bar indicate that no sampling was conducted on this month at a particular site.

## 4.3 Age and size at first maturity

### *Survey 1*

A total of 665 cockles ranging in size from 10 to 36 mm (**Table 6**) were used to fit the maturity ogive. The smallest size class with mature cockles was 17 mm. The size at which cockles first reach maturity in Galicia was estimated to be 19.5 mm (**Figure 6**). Nevertheless, from a precautionary approach, in terms of fisheries regulation it is better to use the $L_{95}$ (length of mass maturity - at which 95% of the population is mature) to define minimum capture size. The $L_{95}$ of Galician cockles was 24.1 mm.

Von Bertalanffy growth models derived from different shellfish beds suggest that Galician cockles reach first maturity during their first year of life, while first mass maturation would be reached between 10 and 20 months of life (**Figure 7**).

**Table 6**. Number of cockles and number of mature individuals in each size class used to fit the maturity ogive of Galician cockles.

| Shell length (mm) | No. cockles | No. mature cockles | Mature cockles (%) |
|---|---|---|---|
| 10 | 1 | 0 | 0.00 |
| 11 | 3 | 0 | 0.00 |
| 12 | 4 | 0 | 0.00 |
| 13 | 7 | 0 | 0.00 |
| 14 | 3 | 0 | 0.00 |
| 15 | 2 | 0 | 0.00 |
| 16 | 6 | 0 | 0.00 |
| 17 | 7 | 2 | 0.29 |
| 18 | 23 | 11 | 0.48 |
| 19 | 11 | 2 | 0.18 |
| 20 | 11 | 6 | 0.55 |
| 21 | 12 | 10 | 0.83 |
| 22 | 26 | 24 | 0.92 |
| 23 | 39 | 35 | 0.90 |
| 24 | 53 | 46 | 0.87 |
| 25 | 42 | 40 | 0.95 |
| 26 | 67 | 65 | 0.97 |
| 27 | 71 | 66 | 0.93 |
| 28 | 70 | 63 | 0.90 |
| 29 | 59 | 51 | 0.86 |
| 30 | 35 | 29 | 0.83 |
| 31 | 41 | 40 | 0.98 |
| 32 | 28 | 27 | 0.96 |
| 33 | 18 | 16 | 0.89 |
| 34 | 15 | 13 | 0.87 |
| 35 | 9 | 8 | 0.89 |
| 36 | 2 | 2 | 1.00 |
| **Total** | **665** | **556** | - |

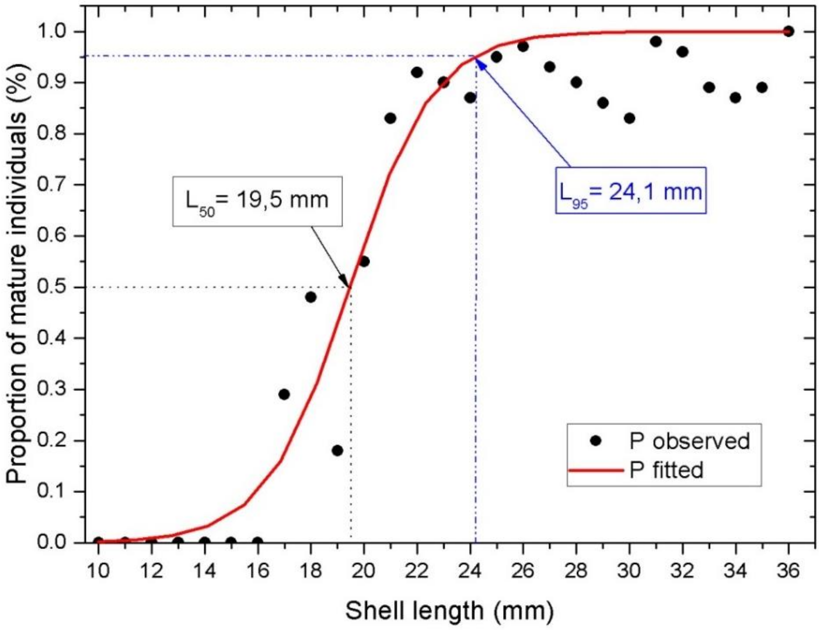

**Figure 6**. Size at first sexual maturity ($L_{50}$) and size of mass maturity ($L_{95}$) for cockles from Galicia (Spain) based on the proportion of mature individuals (stages 3, 4A and 4B) as a function of shell length (grouped into 1 mm size classes).

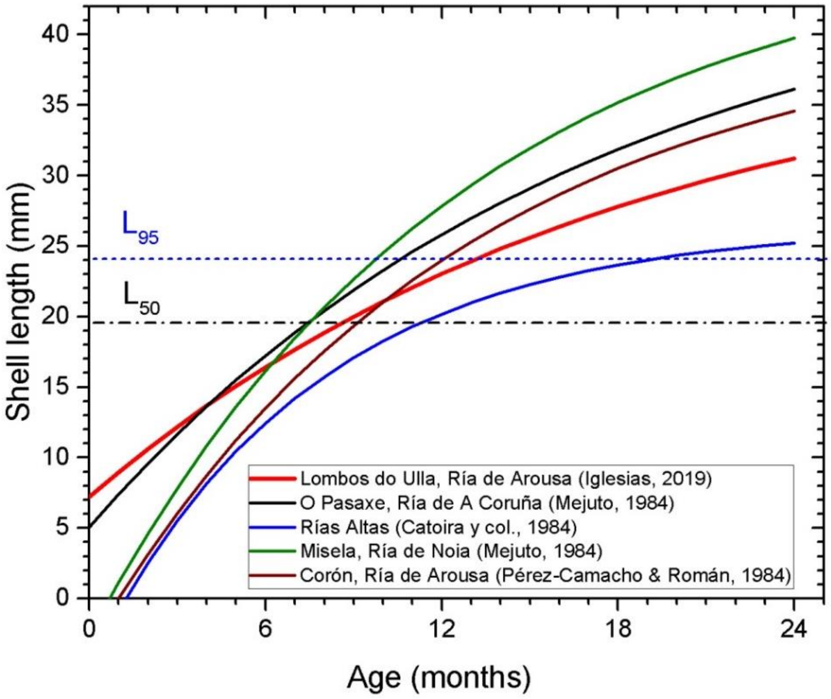

**Figure 7.** Age of first sexual maturity ($L_{50}$) and mass maturity ($L_{95}$) estimated from von Bertalanffy growth models derived from different Galician shellfish beds.

### *Survey 2*

A total of 123 cockles with size ranging from 14.5 to 34.2 mm were collected in April 2018. The smallest mature individuals, with gonadal maturation assigned to Stage 3, were a male of 22.0 mm and a female of 21.7 mm. The fitted maturity ogive (R = 0.9772; **Figure 8**) revealed that the size at first sexual maturity (shell length, $SL_{50}$) of the male and female cockles from Ría Formosa was reached at 22.9 mm.

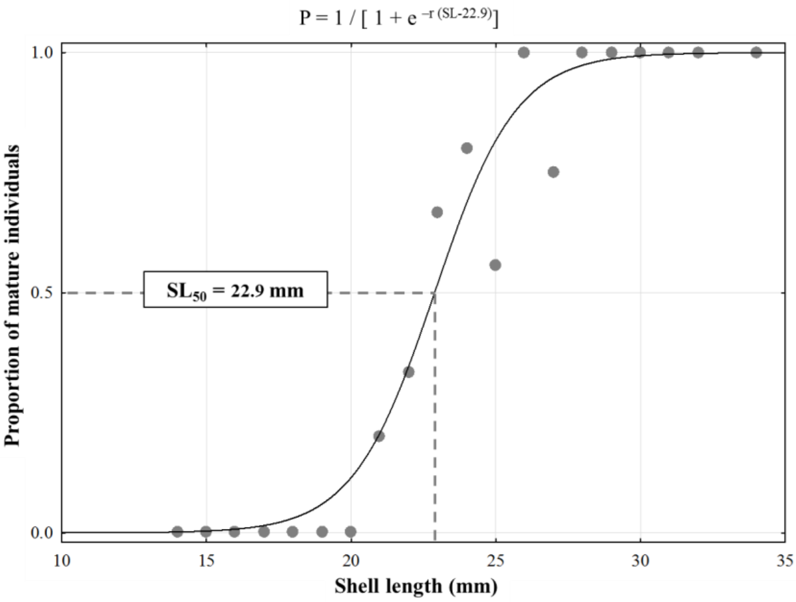

**Figure 8.** Size at first sexual maturity (shell length, $SL_{50}$) for cockles from Ría Formosa, based on the proportion of mature individuals (Stages 3 and 4) as a function of shell length (grouped into 1 mm size classes).

### *Survey 3*

The minimum and mean lengths of cockles measured at Ría de Aveiro and Arcachon were smaller than those measured at the northern sites in Ireland (**Table 5**). The largest size range of cockles captured was observed at Cork, an unharvested site. The highest mean number of growth rings (i.e. older cockles) was also observed in cockles at Cork along with the largest range (**Table 5**). The smallest spawning individual (16.16 mm) was recorded at Arcachon in June 2019 and the largest individual recorded (49.94 mm) at Cork in August 2018 was ripe.

A Kruskal-Wallis test showed that mean length at spawning differed significantly between sites ($\chi^2$ = 143.31, df = 4, $p < 0.001$; **Figure 9A, Appendix 3**). Post-hoc analysis showed that cockles spawning at Arcachon and Ría de Aveiro were significantly smaller than all other sites ($p < 0.001$ in all cases). Considering the sites for which information on the number of growing rings was available, a Kruskal-Wallis test showed that the number of growth rings (age) of spawning individuals also varied significantly by site ($\chi^2$ = 73.615, df = 3, $p < 0.001$; **Figure 9B**, **Appendix 4**), with Carlingford cockles being the oldest followed by cockles at Cork, Arcachon and Dundalk, respectively (age was not examined at Ría de Aveiro).

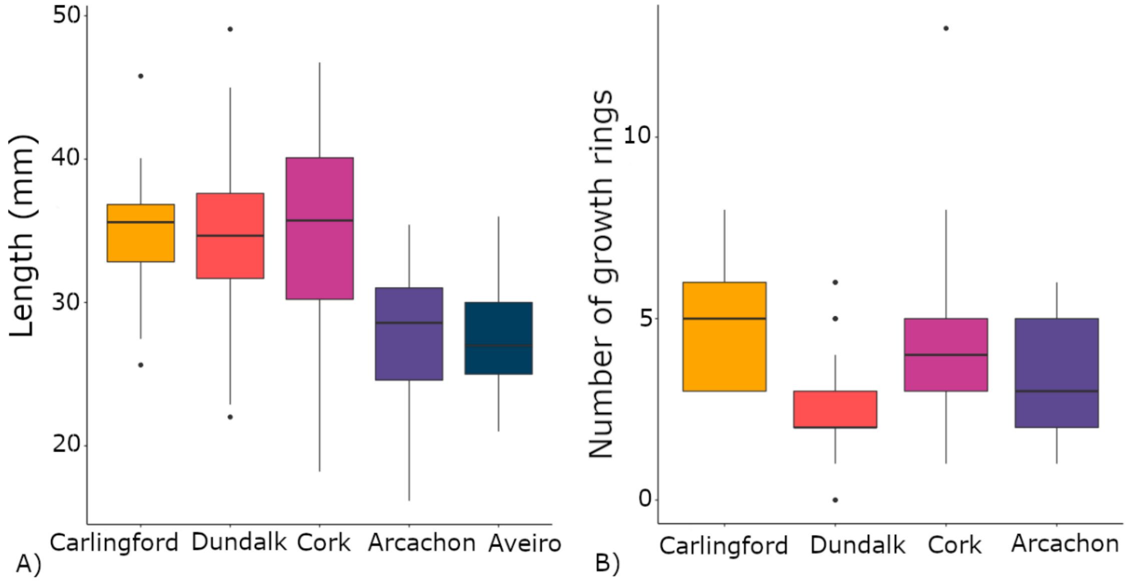

**Figure 9. A)** Length and **B)** number of growth rings, of spawning cockles. Lower and upper box boundaries indicate 25th and 75th percentiles, respectively, line inside box indicates median, error lines indicate 1.5 times the interquartile range and filled circles show data falling outside these values. No growth ring data was available for cockles from Ría de Aveiro.

## 4.4. Gametogenesis and reproductive cycles

### *Survey 1*

The analysis of histological slides suggests that the onset of gametogenesis was detected slightly early in females than in males. This observation probably reflects that oogonia are more easily identified than spermatogonia in early gonad development and therefore both sexes were considered together in the gametogenic cycle description. Even with interannual variations, an annual reproductive cycle could be identified in the cockles of Lombos do Ulla during the studied period (**Figure 10**). The general pattern was characterized by an extended period of gonad activity from winter to summer and a resting period, in which most or all individuals showed reabsorbing or resting gonads, in autumn. The activation of the gonads began in early winter and the gametogenesis progressed during January and February, when most cockles showed gonad activity. Once gonad ripeness was attained, mass spawning was observed in spring early-summer, with cockles restoring after spawning to achieve ripeness and then spawning again, although the percentage of spawning cockles declined through the summer. There was clear interannual variation in the month of first spawning detection and the length of spawning period, *i.e.* the period with cockles showing "around-spawning" gonad stages (3, 4A or 4B), the shortest one from March to June (2009) and from April to July (2007), while the longest spawning period went from February to September (2008), with intermediate lengths in the other years, March to September (2010) and February to August (2011). Thus, April, May and June were the months included in the spawning period all those years (**Figure 11**).

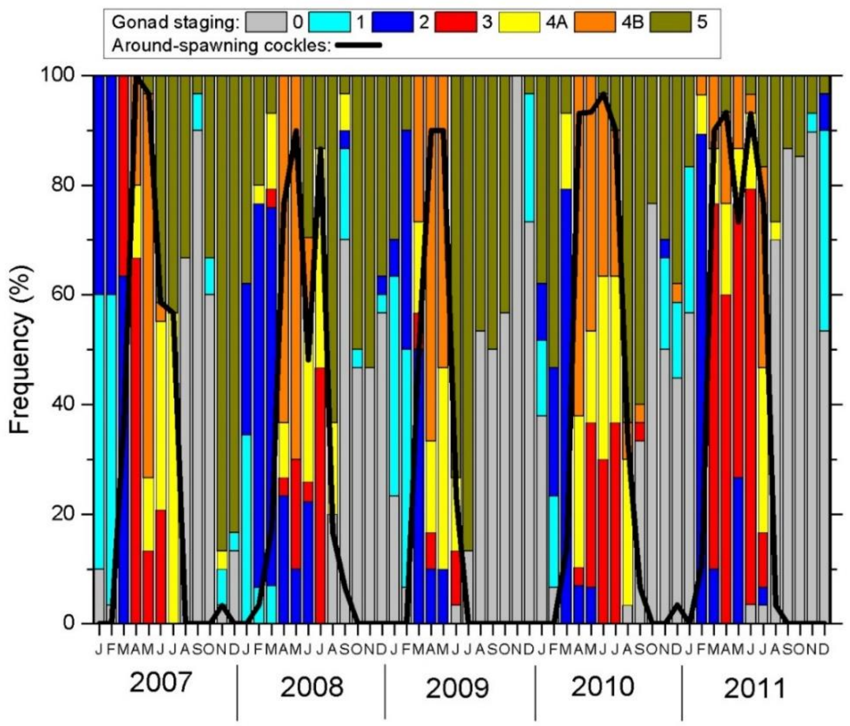

**Figure 10.** Monthly variation of the distribution of cockles in gonad stages (bars) and variation of the percentage of "around-spawning" (stages 3 + 4A + 4B) cockles (black line) in Lombos do Ulla. Gonad stages are distinguished by colours, as shown above the graph.

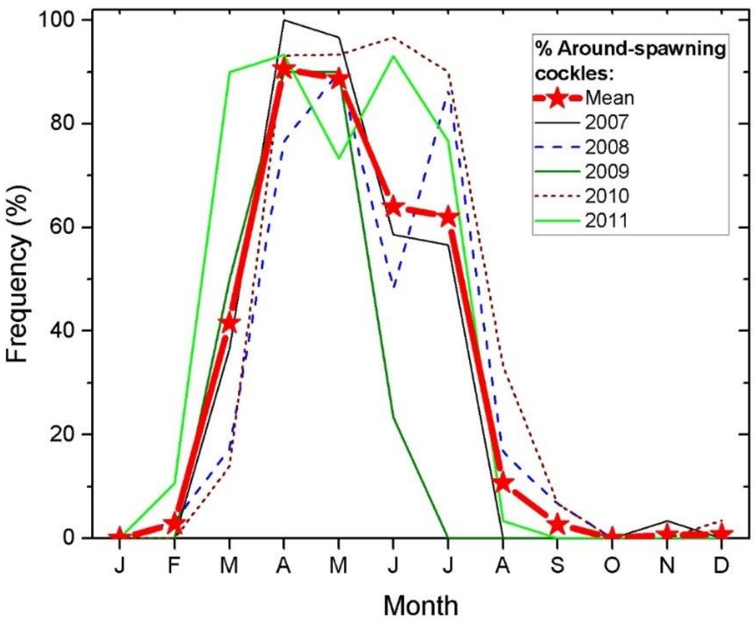

**Figure 11.** Modeled annual cycle of the percentage of "around-spawning" (stages 3 + 4A + 4B) cockles in Lombos do Ulla.

The monthly mean temperature during the study period was 14.9 ± 0.1 °C (mean ± S.E.), and ranged from 12.1 ± 0.3 °C between December and January, to 17.4 ± 0.5 °C, between July and September. A weak but significant positive correlation ($r_s$= 0.324, $p < 0.05$) between the percentage of "around-spawning" (stages 3 + 4A + 4B) cockles and the monthly mean temperature was detected. "Around-spawning" cockles were first detected every year when seawater temperature was approximately 13 °C, and the maximum percentage of "around-spawning" individuals coincided every year with the sharp temperature increase in spring (**Figure 12**). There was also a stronger, significant positive correlation between the percentage of "around-spawning" cockles and monthly mean chlorophyll *a* concentration ($r_s$= 0.475, $p < 0.001$); the maximum values of the percentage of "around-spawning" individuals temporally coincided with maxima of monthly mean chlorophyll *a* concentration (**Figure 13**).

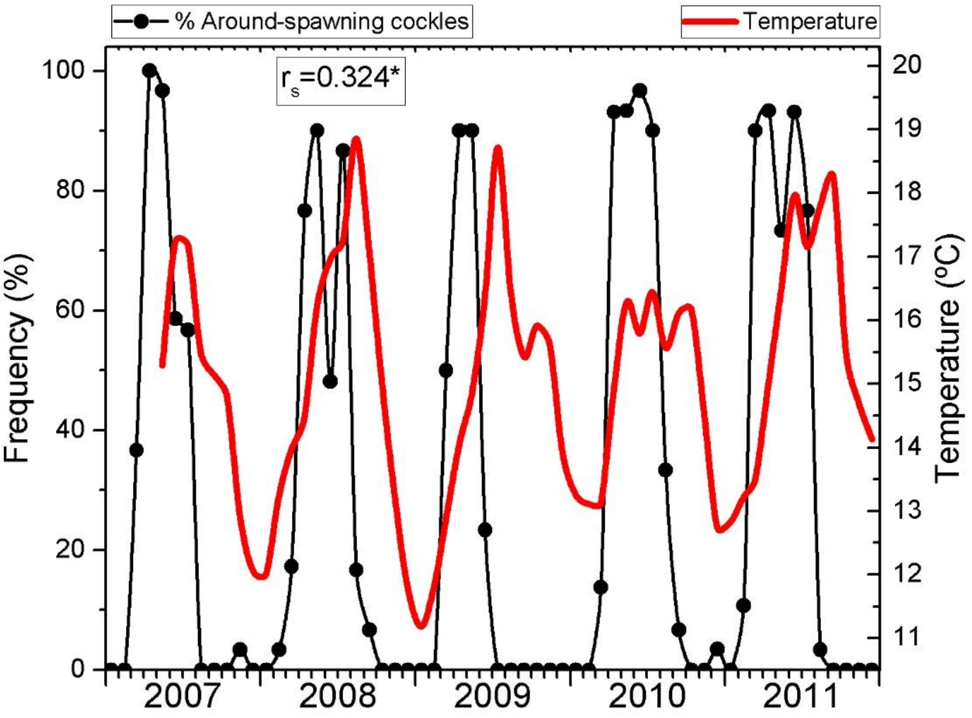

**Figure 12.** Relationship between the variation of the seawater temperature (monthly average) and that of the percentage of "around-spawning" (stages 3 + 4A + 4B) cockles at Lombos do Ulla.

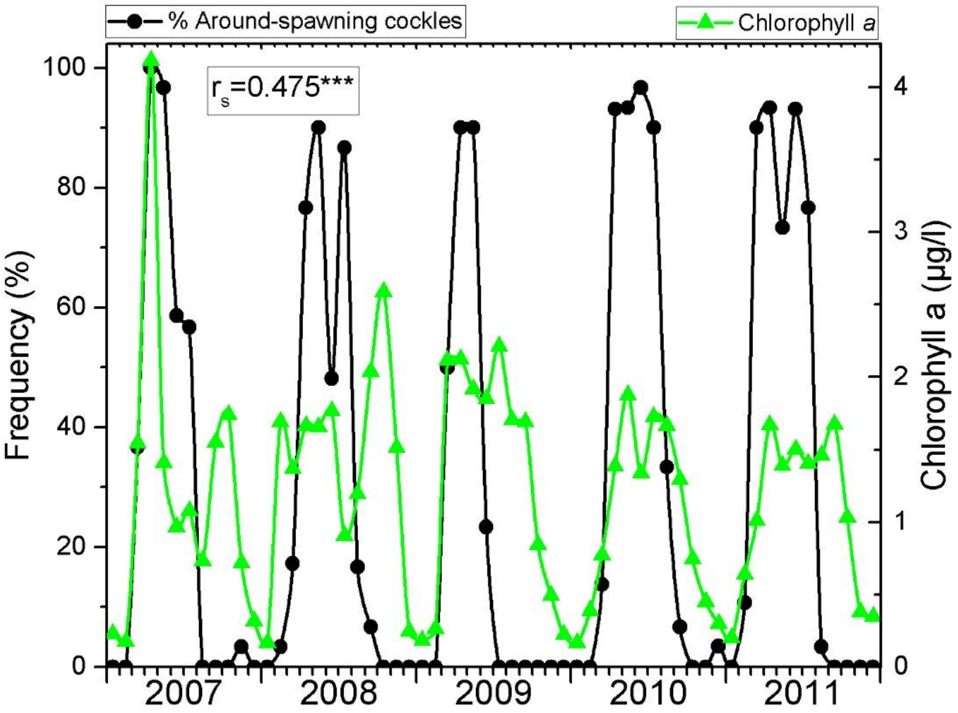

**Figure 13.** Relationship between the variation of chlorophyll *a* concentration in seawater (monthly average) and that of the percentage of "around-spawning" (stages 3 + 4A + 4B) cockles at Lombos do Ulla.

### *Survey 2*

The reproductive cycle was analysed independently of the sex (synchronous gametogenic development between males and females). Clear gonad cycles were apparent across both years, with short resting periods (Stage 0 - inactive) mainly during late summer and early autumn (**Figure 14**). Gametogenesis started in autumn with the main spawning season (Stage 4 – partially spawned) varying significantly between the two years: May – June in 2016 vs April – September in 2017. Co-occurrence and overlapping of three maturation stages: Stage 5 - spent, Stage 0 – inactive and Stage 1 - early active, was observed.

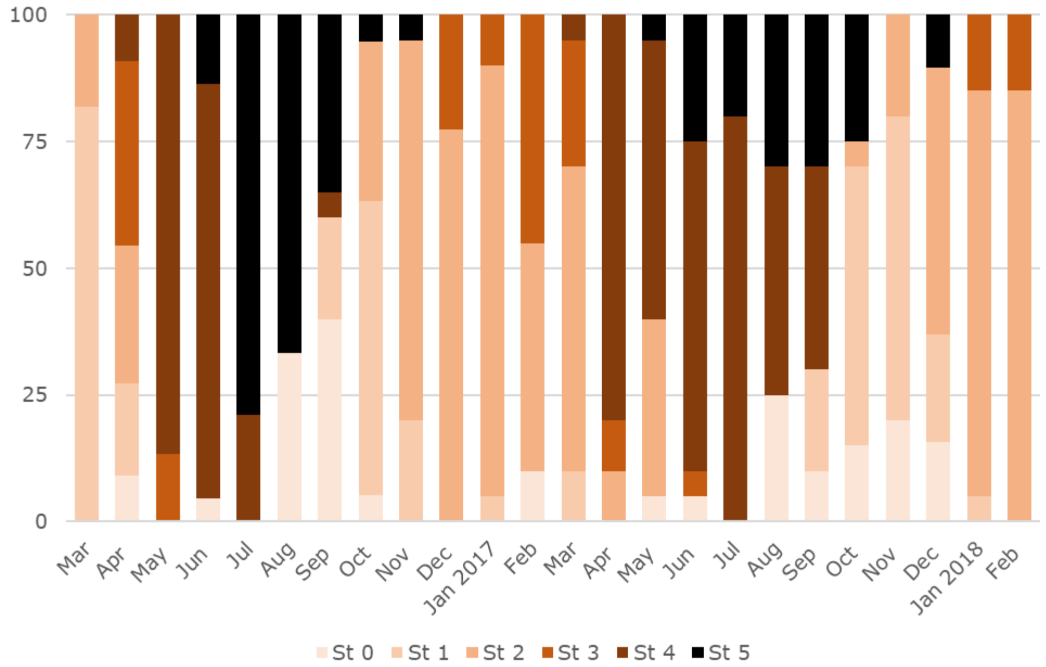

**Figure 14.** Monthly variation of gonad maturation stages in *Cerastoderma edule* during the 2-years study period at Ría Formosa for *Survey 2*. St 0 = inactive, St 1 = early active, St 2 = late active, St 3 = ripe, St 4 = partially spawned and St 5 = spent.

During the study period for *Survey 2*, the water temperature varied from 13.2 ºC to 23.7 ºC, averaging at 18.7 ºC in 2016 and 18.4 ºC in 2017. Atypical water temperature development was recorded in summer 2017, remaining almost constant from June to October that year. In April 2016, water temperature was 15.3 ºC, but in the same month in 2017, it reached 2 ºC higher (**Figure 15**). This irregular variation of temperature in 2017 was reflected in gametogenic development. Gamete release (Stage 4) started earlier (April) and ended later (September) in 2017 (**Figure 14**). Another remarkable interannual difference was the occurrence of ripe cockles (Stage 3) from Dec 2016 to March 2017 before spawning started, while there was no ripe cockle in March 2016, before commencement of spawning that year (**Figure 14**). GI reflects the main dynamics of the reproductive cycle: spawning periods triggered (when GI values decrease) by rising seawater temperature, with spawning events beginning when temperature is around 17 ºC (**Figure 15**). Furthermore, **Figure 15** shows how spawning in 2016 was regular and continuous, as the temperature increased. In 2017, however, water temperature was atypical (almost constant and lower on average over an unusually long period, June to October) and the GI during this time shows several small peaks, corresponding to intermittent spawning events.

**Figure 15.** Relationship between water temperature and mean gonadal index (GI) of *Cerastoderma edule* during the 2-years study period of *Survey 2* at Ría de Formosa.

### *Survey 3*

In April 2018, spawning had initiated at all sampled sites. However, at Carlingford, at that time, no males were spawning. At Cork, spawning had started in both sexes (27.6% and 24% for males and females respectively). At Arcachon, four stages of gametogenesis were evident in females (38.9% late developing; 50% ripe; 5.6% spawning; 5.6% spent) and three stages in males (16.7% ripe; 75% spawning; and 8.3% spent), indicating that spawning had already commenced before the start of the sampling period. Similarly, at Ría de Aveiro, spawning had commenced in both sexes (14.3% males and 68.8% females), although a proportion of cockles were still in early or late development (28.6% males and 31.5% females). At Dundalk, sampling was not initiated until July 2018, at which point the majority of males (56.5%) and females (48.5%) were spawning.

In 2019, at Dundalk and Carlingford, spawning had likely commenced by March, as spent individuals were observed in April. At Cork, males likely spawned in January, with spawning and spent individuals noted in February. Females at Cork spawned later, with spawning and spent individuals noted during sampling in April 2019. In Ría de Aveiro, spawning was observed in all sampled months (except December 2018 and October 2019) and was likely to have been occurring also in the interim months, with spent individuals observed in most samples. Similarly, at Arcachon, spawning was observed during all sampling months (except April 2019).

Consecutive monthly sampling was conducted at some sites, allowing for a comparison of speed of gametogenesis (**Figure 17**). At Dundalk, cockles were sampled in both July and August 2018, and similar proportions of spent and spawning individuals were noted in both months. Gametogenesis also appeared to progress at a similar rate at the other Irish sites and at Ría de Aveiro (**Figure 10**). For example, at Cork in April 2018, a large proportion of individuals were in late development in April (32% female, 17.2% male), and some were still in late development in June (4.2% female and 4.4% male). At Arcachon, however, development was more accelerated, as evidenced by the consecutive sampling in June and July 2018, where large changes in stage was observed (e.g. ripe individuals observed in June, but not in July). At Ría de Aveiro, the southernmost site in *Survey 3*, gametogenesis did not progress as quickly as at Arcachon. For example, similar proportions of spawning individuals were reported in April 2018 (68.8% female and 57.1% male), as in June 2018 (47.4% female and 54.6% male).

Spawning periods varied between sites in Ireland, between countries and inter-annually. In the Carlingford cockle population, only one spawning period was observed in each year, ceasing by October of both years, with a large proportion of indeterminate individuals observed (93.3% in 2018 and 61.5% in 2019). In 2018, Dundalk cockles spawned continuously until August for females and October for males, with development recommencing by October 2018 in both sexes (**Figure 17**). A second, smaller spawning in 2018 was observed in males (20%) in December but did not continue throughout the winter, with no spent males noted in February 2019. In Cork cockles, in 2018, a single spawning event was noted, ceasing during the winter. In 2019, spawning was observed in all months sampled, until the end of the sampling period (October). In the southern sites on mainland Europe, resting periods appeared shorter (**Figure 16, 17**). A potential spawning peak was observed in Ría de Aveiro in April and again in June 2019 (**Figure 17**). In 2018, Ría de Aveiro cockles spawned until at least October but ceased prior to October in 2019.

Differences in the synchronicity of gametogenesis was observed between sites. At Carlingford spawning generally appeared synchronous between sexes (**Figure 17**). At Cork, it appeared that more females were spawning in 2018 but the opposite was observed in 2019. However, as many males were ripe during the sampling period, male spawning may have occurred in the interim months. At Arcachon, more males were spawning in early 2018 (**Figure 17**) and at the southernmost site, Ría de Aveiro, spawning of both sexes was less synchronous in 2018 than in 2019, where similar numbers in both sexes reached a spawning peak by June (**Figure 16**). Spawning was generally initiated by females at all sites, except at Carlingford and Dundalk in 2018 (**Figure 17**).

At the Irish sites, synergy was observed between temperature and spawning time (**Figure 15**). Mean seawater temperatures at Carlingford ranged from 8.2°C to 13.6°C, with an average of 11°C. At Dundalk seawater temperature ranged from 6.5°C to 17.35°C, with a mean of 12.4°C. Seawater temperatures at Cork ranged from 8.5°C to 15.4°C, with a mean of 12.2°C. The mean seawater temperature at Arcachon was the highest of all sites studied (15.6°C) and ranged from 11.1°C to 21.5°C. Temperatures at Ría de Aveiro had the narrowest range, from 12.6°C to 16.4°C, with a mean of 14.8°C. At Dundalk, there was a positive correlation between percentage of individuals spawning and temperature ($F = 14.2$, df = 1, $p < 0.01$). At Cork, there was a similar trend ($F = 3.994$, df = 1, $p = 0.06$). No significant correlation between spawning and temperature was observed at the other sites, despite a potential trend appearing, as seen in **Figure 17**.

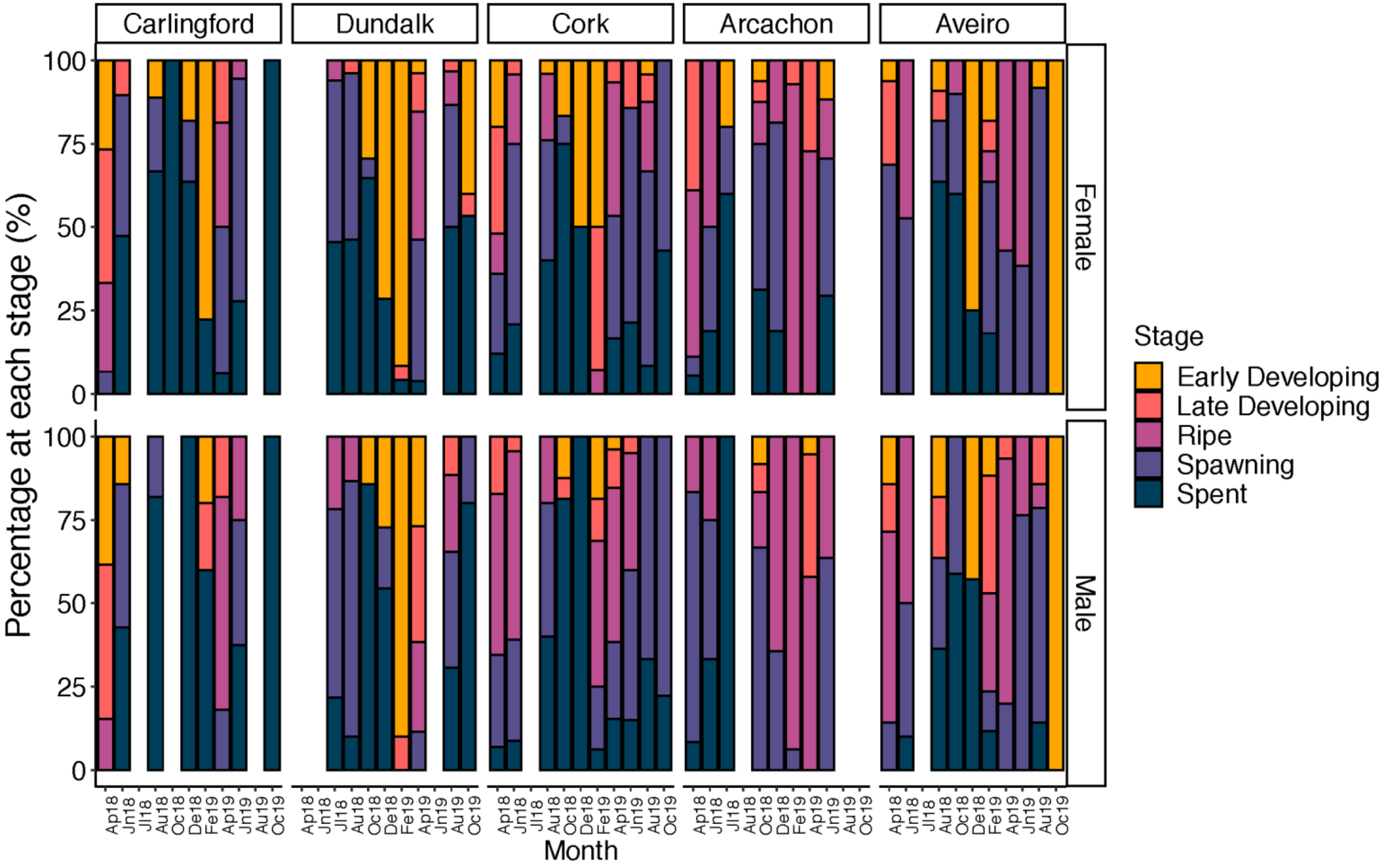

**Figure 16.** Percentage of *Cerastoderma edule* at each stage of gonadal development at one French site, one Portuguese site and three Irish sites in *Survey 3*. Sampling commenced in April 2018 (Ap 18) and was completed in October 2019 (Oc 19). Indeterminate individuals were omitted, see **Figure 5.** Missing bars in a given month indicates that no sampling was conducted.

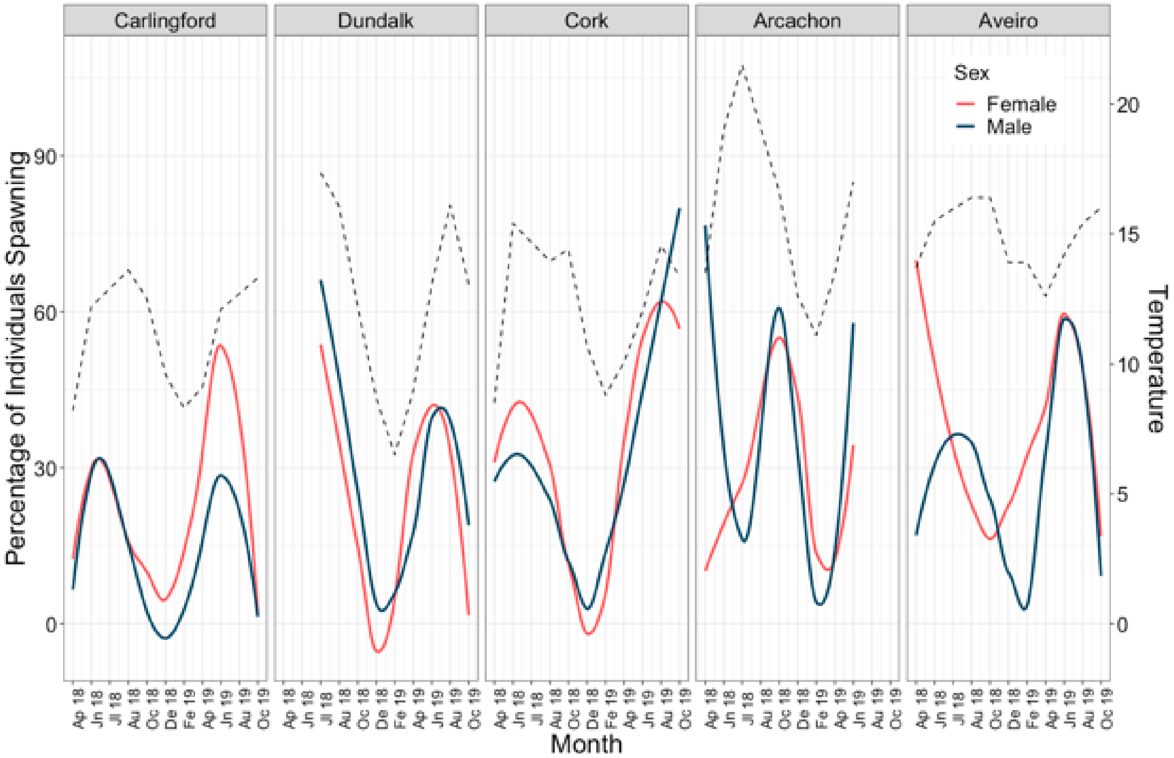

**Figure 17.** Percentage of males and females spawning during each month of *Survey 3*, compared with sea water temperature (dotted line), at each site.

## 4.5. Impact of Trematodes on Gametogenesis (*Survey 3* exclusively)

Trematode infection varied spatially, with both sporocysts and metacercariae present at all sites. Metacercariae were the most prevalent and frequently observed life stage at all sites. Sporocyst prevalence was highest at Arcachon Bay (7.5%) and metacercariae prevalence was highest at Carlingford (76.8%). Prevalence of both stages were lowest at Ría de Aveiro (sporocysts = 2.1%, metacercariae = 9.6%). Prevalence of these stages varied temporally across all sites. For sporocysts, the only apparent trend was for higher infection at Arcachon Bay (23.33%) subsequent to temperatures dropping to 11.2°C. Metacercariae prevalence at Arcachon Bay (mean water temperature = 16.3°C) decreased gradually over the summer months, when the temperature reached a monthly mean of 22.2°C in July 2019, exceeding that of all other sites, and peaked in December at 60% infection (**Figure 18**; mean temperature = 12.6°C). At Ría de Aveiro, the peak of metacercariae infection was earlier in 2018 than 2019 (13.3% at 15.5 °C in June 2018 vs 17.7% at 16 °C in October 2019), when temperatures peaked later in 2019. Infection was consistently high at Carlingford, peaking at 91.7% in February 2019, when mean seawater temperature was 8.3°C (**Figure 18**).

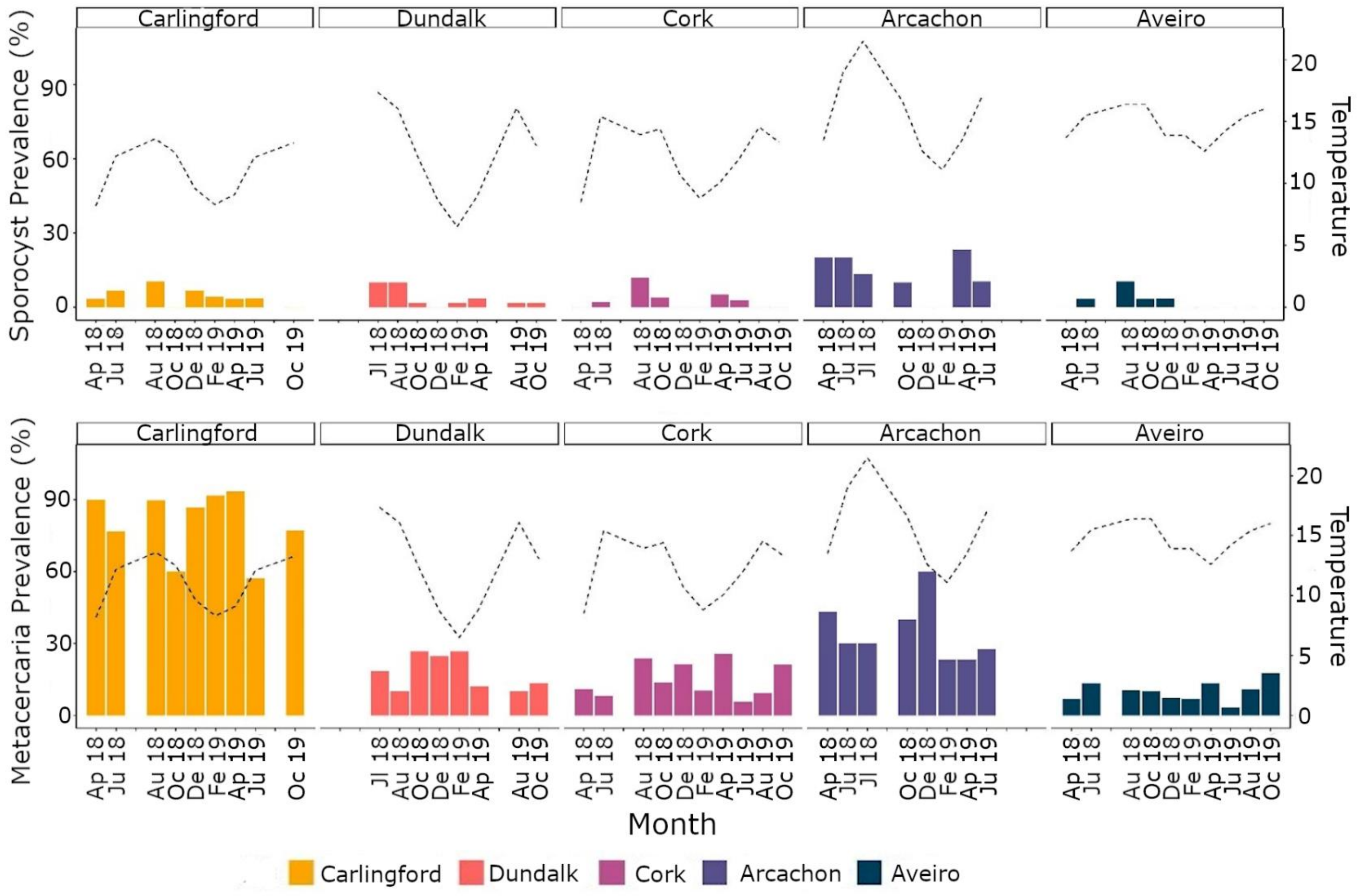

**Figure 18**. Time series of trematode prevalence (metacercariae and sporocysts) recorded in *Cerastoderma edule* at all study sites, compared with sea water temperature (dashed line). Individuals co-infected with metacercariae and sporocysts were included in both graphs.

Uninfected cockles were generally smaller (χ2 = 43.3, df = 3, $p < 0.001$) and younger (χ2 = 64.433, df = 3, $p < 0.001$) than individuals infected with metacercariae (**Table 7**). While there was no difference between the size of metacercariae and sporocysts infected individuals, sporocyst infected individuals were significantly younger than those infected with metacercariae ($p < 0.01$).

**Table 7.** Mean Length ± SD (mm) and number of growth rings ± SD for cockles infected with trematodes at all study sites. Note: Growth ring data is absent for Ría de Aveiro.

| Site | Metacercariae | | Sporocysts | | All Trematodes | | All Cockles | |
|---|---|---|---|---|---|---|---|---|
| | Length | No. of growth rings | Length | No. of growth rings | Length | No. of growth rings | Length | No.of growth rings |
| Carlingford | 33.7 ± 3.9 | 4.2 ± 1.6 | 33.6 ± 4.0 | 4.1 ± 2.3 | 33.7 ± 3.9 | 4.2 ± 1.6 | 33.8 ± 3.9 | 4.2 ± 1.6 |
| Dundalk | 33.5 ± 5.8 | 3.4 ± 1.6 | 35.1 ± 5.2 | 3.1 ± 1.1 | 33.7 ± 5.4 | 2.9 ± 1.5 | 32.7 ± 5.7 | 2.9 ± 1.5 |
| Cork | 35.5 ± 7.8 | 4.2 ± 2.0 | 36.9 ± 4.0 | 3.3 ± 1.1 | 35.5 ± 8.4 | 3.6 ± 1.8 | 32.9 ± 8.3 | 3.6 ± 1.8 |
| Arcachon | 29.4 ± 4.3 | 3.9 ± 1.2 | 30.3 ± 4.1 | 3.7 ± 1.5 | 29.5 ± 4.3 | 3.7 ± 1.4 | 28.6 ± 4.5 | 3.7 ± 1.4 |
| Ria de Aveiro | 26.2 ± 6.9 | - | 29.3 ± 3.6 | - | 26.8 ± 6.5 | - | 25.8 ± 5.3 | - |

Individuals coinfected with both sporocysts and metacercariae were significantly more likely to be indeterminate, rather than ripe or spawning ($p < 0.05$ in both cases; **Figure 19**). Furthermore, a trend was observed for metacercariae infected individuals to be indeterminate, rather than ripe or spawning ($p = 0.08$ in both cases, **Figure 19**).

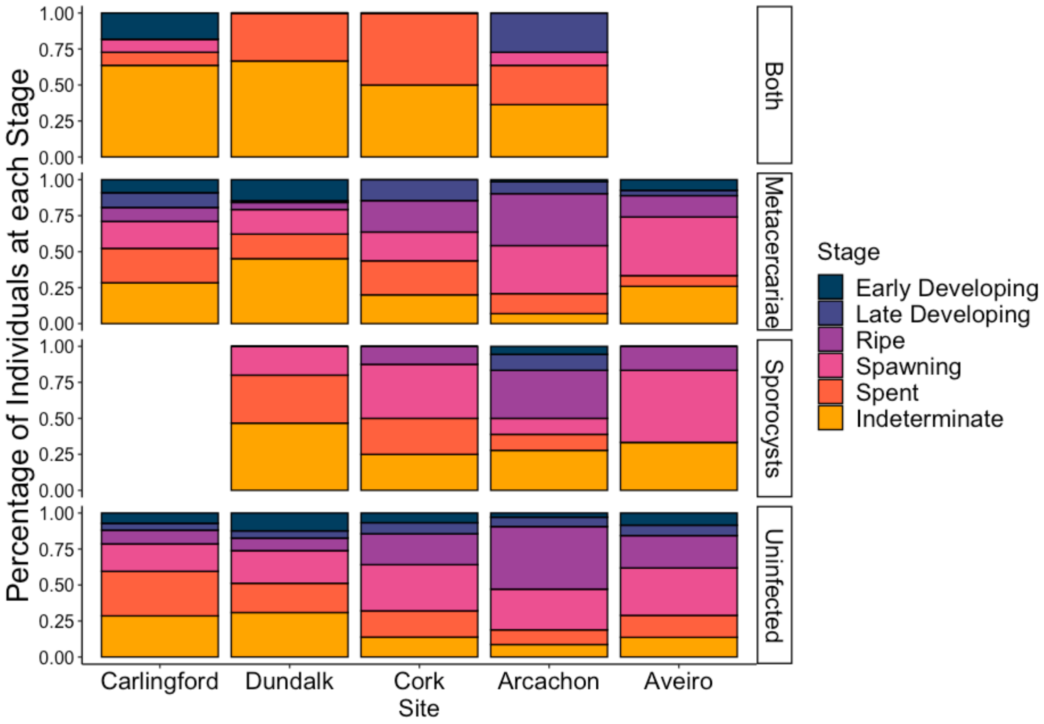

**Figure 19.** Percentage of *Cerastoderma edule* at each stage of gametogenesis for individuals infected and uninfected by trematodes. Infection state is indicated on the right of the graph and “Both” indicates that an individual was co-infected by sporocysts and metacercariae.

## 4.6. Comparison of current and historic spawning times

There was no overlap in sites from which historical reproduction data was collected with the sites in the more recent *Surveys 1, 2* and *3*; however, there was an overlap in latitudes.

No historic reproduction studies examining spawning times had been conducted as far south as either Ría de Aveiro or Ría Formosa (**Table 8**). The nearest site to Portugal, and Arcachon, where cockle reproduction has been studied was the Ria de Vigo (approximately 200 km north of Ría de Aveiro, 600 km north of Ría Formosa and 650 km southwest of Arcachon, with a latitude difference of approximately 2°, 5° and 2°, respectively). Comparisons of cockle reproduction at these sites are addressed in the discussion. The nearest site to Cork reported in the historical survey, Flaxfort Strand, reported peak spawning between May and August 2011 (Morgan et al., 2013), which were comparable with the spawning season at Cork in 2018. However, spawning at Cork peaked later in 2019 (October). Similarly, at Strangford Lough (approximately 60 km north east of Carlingford Lough and 70 km north of Dundalk), spawning was studied between 1972 and 1976, and occurred during the summer, with only slight fluctuations in start and finishing dates. While no studies were conducted in the interim, making inferences difficult, it appeared that similar spawning times were being observed at Carlingford Lough even with a 40+ year time difference between the two studies.

**Table 8.** A comparison between spawning times at sites examined in this study, as well as previous studies at the same/nearby latitudes (*Survey 1, 2* and 3 sites in bold).

| Location | Country | Year | Spawning | Latitude (°N) | Source |
|---|---|---|---|---|---|
| **Formosa** | **Portugal** | **2017** | **April-July, September** | **37** | **1** |
| **Formosa** | **Portugal** | **2018** | **March-September** | **37** | **1** |
| **Aveiro** | **Portugal** | **2018** | **April-October** | **40** | **1** |
| **Aveiro** | **Portugal** | **2019** | **February-August/September** | **40** | **1** |
| **Ria de Vigo** | **Spain** | **2003/4** | **April-August, October** | **42** | **2** |
| **Lombos do Ulla** | **Spain** | **2007** | **April-July** | **42** | **3** |
| **Lombos do Ulla** | **Spain** | **2008** | **February-September** | **42** | **3** |
| **Lombos do Ulla** | **Spain** | **2009** | **March-June** | **42** | **3** |
| **Lombos do Ulla** | **Spain** | **2010** | **March-September** | **42** | **3** |
| **Lombos do Ulla** | **Spain** | **2011** | **February-August** | **42** | **3** |
| **Arcachon** | **France** | **2018** | **April (or before)-December** | **44** | **1** |
| **Cork** | **Ireland** | **2018** | **April (or before)-October/September** | **51** | **1** |
| **Cork** | **Ireland** | **2019** | **February-October /September** | **51** | **1** |
| South Wales | Wales | 1958 | May-June | 51 | 4 |
| Burry Inlet | Wales | 1981 | April-July | 51 | 5 |
| Burry Inlet | Wales | 1982 | May-September | 51 | 5 |
| Oosterschelde | The Netherlands | 1985 | May-August | 51 | 6 |
| Flaxfort Strand | Ireland | 2010 | May-August | 51 | 7 |
| **Dundalk** | **Ireland** | **2018** | **Before July-December** | **53** | **1** |
| **Dundalk** | **Ireland** | **2019** | **March/April-October** | **53** | **1** |
| **Carlingford** | **Ireland** | **2018** | **June-August, December** | **54** | **1** |
| **Carlingford** | **Ireland** | **2019** | **April-June** | **54** | **1** |
| Strangford Lough | Northern Ireland | 1972 | June-October | 54 | 7 |
| Strangford Lough | Northern Ireland | 1973 | July-August | 54 | 7 |
| Strangford Lough | Northern Ireland | 1974 | May-October | 54 | 7 |
| Strangford Lough | Northern Ireland | 1975 | May-July | 54 | 7 |
| Strangford Lough | Northern Ireland | 1976 | June-August | 54 | 7 |

1 This Study
2 Martínez-Castro and Vázquez, 2012
3 Hancock and Franklin 1972
4 Yankson et al., 1986
5 Hummell and Bogaards, 1989 as read in Smaal et al., 1997
6 Morgan et al., 2013
7 Seed and Brown, 1977

# 5. DISCUSSION

This report, consisting of a historical literature study and three recent field surveys, provides information on the current status of the reproductive health (sex ratio, age and size at first maturity, gametogenesis, seasonality, recruitment success and site influence) of cockle populations in the AA. The results clearly provide further evidence of the variable nature of reproduction in cockle populations, both temporally and spatially. These temporal and spatial differences are influenced by abiotic (temperature, latitude) and biotic (food availability, trematodes) drivers, which act associatively to impact the reproductive characteristics of cockles.

As part of Deliverable 4.1 10,878 records dating from between 1859 and 2019 were analysed for a historical survey of AA cockle populations. Out of these records, only 21 records contained information or data on cockle reproduction. However, due to the large distance and likely varying environmental factors, it is difficult to derive a conclusion regarding the differences in reproduction found between cockle populations in these records. From the limited data in the historical records, it appeared that latitude influenced spawning, with initiation of spawning occurring later at higher latitudes. However, spawning duration does appear to increase across time at 51°N and 53°N (Ireland, Wales, The Netherlands). This observation was also noted by Morgan et al. (2013) who reported spawning starting earlier and continuing later into autumn in more recent studies. However, as these studies were only conducted over a short period it is difficult to deduce if this trend is in fact occurring. Changes in climate, and a general warming of seawater temperatures could be a factor driving this perceived lengthening of spawning season at the higher latitudes (Heath et al., 2012). At sites between 40°N and 42°N there were only records covering a 16-year time difference so temporal changes could not be fully explored. However, the results from recent field sampling in *Survey 3* (prolonged spawning at Ría de Aveiro and Arcachon where average seawater temperature was higher) supports the hypothetical correlation between seawater temperature and longer spawning periods. Contrastingly, average water temperature was highest in Ría Formosa (*Survey 2*), where cockles had distinct spawning periods. Despite this, such hypotheses are worthy of further investigation to be fully understood.

The field data in this study showed sex ratios largely followed the expected 1:1 ratio, which is a good indication of health (Malham et al., 2012). In *Survey 1*, the M:F ratio was 1:1.3, significantly different from the balanced 1:1 sex ratio, when all the sampled cockles were included in the calculation; however, when the sex ratio was computed, more appropriately, by only including the gametogenic active periods (to avoid underestimation of males in the very early gametogenic stages; Boyden 1971a), the sex ratio (1:1.05) was not statistically different from 1:1. In *Survey 2*, the sex-ratio was statistically balanced (1M: 0.9F), without any significant deviation from the parity (1M: 1F) sex-ratio. In *Survey 3*, however, the cockles at northerly nearby sites of Carlingford and Dundalk deviated from the expected 1:1 ratio. A higher number of females were found at this site, a phenomenon also seen in other areas (Boyden 1970, Martínez-Castro and Vázquez, 2012) and a few possibilities may be suggested to explain this. First, deviation from a 1:1 sex ratio can result from sex specific mortality (Longshaw and Malham, 2013). This can occur from reduced energy availability, however, in this situation female mortality would be more likely (Brokordt and Guderley, 2004). High trematode infection levels may also have played a role in altering the sex ratio at Carlingford. A previous study reported higher trematode infections in males

(52.2% vs 47.7% in females) but this trend was not found to be statistically significant on that occasion ($p = 0.06$; Morgan et al., 2012). It is possible that males have an increased susceptibility; however, further analysis of this relationship at other AA sites would be required for these hypotheses to be conclusive. The interaction of trematode parasites and cockles at Carlingford is discussed further below.

Within the whole study, the smallest cockles recorded spawning were 16.16 mm in length, from Arcachon (*Survey 3*), which was larger than that found in other studies (Morgan et al., 2013). In *Survey 2,* to measure the sexual maturity of cockles only individuals up to 34.2 mm were recorded, at which length they were all sexually mature. The size at first maturation was estimated to be 19.5 mm and 22.9 mm, in *Surveys 1* and *2*, respectively. The latter value was slightly lower than the present minimum capture size legally established for the species (25 mm) in Ría Formosa. The largest cockle found in *Survey 3* was 49.9 mm, the maximum size cockles have been noted to reach (Hayward and Ryland, 1995). In *Survey 3*, the oldest and largest spawning cockles were found in Cork as might be expected as a site free of any cockle fishing activity. Cockles in Carlingford, a site also free of large-scale commercial fishing, reached similar ages to the Cork cockles. Ría de Aveiro and Arcachon (fished sites) had smaller spawning sizes than Irish sites, including Dundalk Bay, which is also exploited. At these southern sites, the cockles appear to allocate more energy towards reproduction, i.e. longer spawning periods, rather than growth. Such variations in reproductive and life history strategies, through differing energy allocation, have been found in other shellfish (Egerton et al., 2020) and marine invertebrates (Darling et al. 2012). However, it would be beneficial to further investigate the impact of temperature, food availability and water quality at these sites.

Similar to the historical data collected, latitude was not a clear primary driver in the reproductive cycle of cockles. However, the northern most sites in *Survey 3* did exhibit similar timing of spawning peaks. This study shows that cockles have the capacity to periodically spawn throughout most of the year, with spawning occurring almost year-round in both Arcachon and Ría de Aveiro. In line with the southern sites in *Survey 3*, in *Surveys 1* and *2* the population at Lombos do Ulla and the Ría Formosa lagoon also presented only short resting periods, mainly during late summer and early autumn, and overlap of spent and early developing individuals, indicating a continuity of the cycle within the population. These results, together, potentially indicate some latitudinal similarities, and latitudinal variation has been reported elsewhere (Tarnowska et al., 2009), though not supported in all studies (Boyden 1970; Guillou et al. 1990).

As many other marine organisms with planktonic larval stages, cockles try to adjust the spawning time to match the optimal environmental conditions for larval development and dispersion. Thus, food availability (quantity and quality) and temperature, which are crucial environmental factors influencing larval survival, may influence cockle gonad cycle and spawning time, while other environmental factors, varying in association with those previously mentioned, such as photoperiod, could also influence cockle gonad cycle. Regarding the influence of seawater temperature, two of the three Irish sites showed positive correlations between seawater temperature and spawning time. Comparison of water temperature with gametogenesis in *Survey 2* provides results that suggest that seawater temperature ranging from 17 to 20 $^{o}$C is optimum for cockles to spawn. These high temperatures (> 17$^{o}$C) were not reached at all the study sites but spawning still occurred, suggesting that it may be an increase in temperature, not its absolute value, that influences the onset of gametogenesis (Dabouineau and Ponsero 2009). In *Survey 1*, the association of the gonad cycle with temperature was also clear, with first detection of spawning when seawater reached 13°C and mass spawning was coupled with a sharp temperature increase in the spring. In *Survey 2*, considerable differences were observed in the sexual cycles in 2016 and 2017. In 2016 the spawning lasted about 2 months (May-June), while in 2017 the spawning occurred in a more temporally fragmented way and extended until later (approximately until September). This atypical spawning cycle coincided with atypical seawater temperatures, which are hypothesized to have driven the temporal evolution of the cockle gametogenic cycle. These results demonstrate that seawater temperature represents one of the most important environmental parameters in bivalve reproduction. It is also important to note that while bimonthly sampling was sufficient at the Irish sites and Ría de Aveiro (cooler sites), more frequent sampling may be necessary at Arcachon (and likely other warmer sites), to examine the more accelerated gametogenesis here.

While the observed spawning peaks were seasonal, reproductive stage may be influenced by other factors including genetics, food availability and immersion time. Gametogenesis is stimulated when sufficient energy resources are available (Hummel and Bogaards, 1989), and it is probable that the sexual cycle is connected to the local plankton blooms (Tarnowska et al., 2009). Indeed, in *Survey 1*, there was a significant positive correlation between the percentage of "around-spawning" cockles and the concentration of chlorophyll *a* in the seawater, supporting the importance of food availability as a driver of cockle reproduction. There is information on the influence of sun light, specifically the photoperiod, in the timing of reproductive cycle of some bivalve species, such as scallops, oysters, clams and mussels (Couturier and Aiken, 1989; Villalejo-Fuerte and Ochoa-Báez, 1993; Saout et al., 1999; Silva et al., 2002; Fabioux et al., 2005; Domínguez et al., 2010), but this is not the case for cockles. Thus, it is an issue to be studied in the near future.

Significant variation in duration and number of annual spawning events were recorded between the different sites. Cockles at Ría Formosa in 2016 (*Survey 2*) and at Carlingford (*Survey 3*) differed from the other sites, undergoing a single annual spawning event. The cockle populations at Arcachon and Ría de Aveiro (*Survey 3*) and at Ría Formosa in 2017 (*Survey 2*) spawned almost continuously. However, it is important to note that this continuous spawning was not always at a large scale (i.e. among all individuals), as resting periods were observed in large proportions of cockles. As a general trend, the spawning in spring and early summer involved most cockles in the populations, while the percentages of cockles involved in spawning later through the summer, in the places where it occurred, were lower. The spring-early summer mass spawning could correspond to a conservative strategy and the less massive spawning through summer could correspond to an adaptive strategy, depending on favourable environmental conditions, which would explain the important interannual variations found in long monitoring studies (*Survey 1*; Seed and Brown 1977). The long-term effects of these strategies are yet to be determined. Long, continuous periods of gametogenesis reduce the rest periods between cycles and may be detrimental on the quality of recruitment (Philippart et al., 2003). However, it also provides a greater window in which female and male gametogenesis can synchronise.

In line with the findings on the reproductive cycle, high levels of indeterminate individuals were mostly found at all sites from autumn to winter. Some cockles may go through an over-wintering stage when their gonad is undifferentiated (Boyden, 1970). Although various stages of gametogenesis were observed in a proportion of cockles over the winter, others may be undergoing a resting period, even within the same site, which may partially explain this scenario. It must also be noted that many species of trematodes can result in castration (Carballal et al., 2001, Thieltges et al., 2006) and in *Survey 3* it was found that the infection with metacercariae increased the probability of having resting gonad (indeterminate sex). Even if castration is not occurring, the lower numbers observed in active gonad stages among infected individuals may possibly be a result of faster gametogenesis, as was seen by Lusher et al. (2017) in individuals impacted by another stressor - pollution. It is generally believed that parasites infecting as sporocysts (proliferative stage) are likely to cause more damage than those infecting as metacercariae (encystment stage; Wegeberg and Jensen 1999). However, it was evident in this study that both metacercariae and sporocysts have a deleterious effect on reproduction, with reduced spawning and higher numbers of indeterminate individuals in trematode infected cockle communities. Trematode infection can also cause starvation (Dubois et al. 2009), which may result in less energy being expended on gametogenesis. It is important to note that histology is not an appropriate procedure to diagnose metacercariae because its sensitivity is very low (too many false negatives). Nevertheless, if only heavy infections with metacercariae were detected with histology, the effects of metacercariae on gonad condition could be restricted to the heavy infection cases.

Age is a determinant of parasite intensity with a positive correlation reported in many invertebrates, including cockles, where older specimens accumulate more infections over time (de Montaudouin et al., 2000). Trematode infection levels varied across sites, with the highest prevalence detected at Carlingford. Similar to previous observations (de Montaudouin, Kisielewski, Bachelet, & Desclaux, 2000), at Carlingford there was a high metacercaria prevalence, as well as a high abundance of old cockles (as well as a higher proportion of females). However, when tested, the age distribution at this site did not differ significantly from that of the other Irish sites, which had significantly lower levels of parasite infection. Cockles gathered in Carlingford were partially surfaced, possibly due to impeded burrowing by trematodes (Desclaux et al., 2002). However, as lower trematode infection levels were found in the partially surfaced cockles at Cork, it is unlikely that this is the sole explanation of high trematodes at Carlingford. Metacercariae intensity was not examined and so, it may in fact have been low due to the dilution effect of sympatric *Crassostrea gigas,* which are cultured in the area (Krakau, Thieltges, & Reise, 2006). Another driver of high infection levels at this site may be the presence of intermediate and final host species (Byers, Blakeslee, Linder, Cooper, & Maguire, 2008; de Montaudouin & Lanceleur, 2011; Hechinger & Lafferty, 2005; Thieltges & Reise, 2006, 2007). At Carlingford, birds may not be the factor driving prevalence, due to high human activity on the oyster farm, but it would worth examining abundance and trematode infection in gastropod species here (Longshaw & Malham, 2013). Interestingly, high numbers of barnacles were observed fouling cockles at Carlingford. These fouling organisms potentially keep cockle shells from closing entirely, thus increasing the potential for trematode infection, but conversely can predate cercariae (Welsh, Liddell, Van Der Meer, & Thieltges, 2017). It is important to note that not all trematodes have a negative impact on cockles, with *Himasthla interrupta* being harmless under normal conditions (Desclaux et al., 2004). It may be worthwhile for further studies to carry out a molecular analysis of trematode species, as identification is not possible via histology. It would also be beneficial to conduct quantitative diagnostics to reveal infection intensities.

Cockle populations are variable, particularly so in their reproduction. While the limited historical data did not support it, some similarities between sites at the same latitude were observed in this study. Seawater temperature and seasonal food availability are factors that likely control the reproductive characteristics of *C. edule*. Spawning season was prolonged at the southern sites, potentially causing a re-allocation of energy resources away from individual growth (indicated by smaller size), denoting varying life history strategies amongst different families, and sites. However, the smaller size could also be a result of differing fishing parameters at the different areas. This study also supports the general guide that balanced sex ratios indicate good health within cockle communities. This was particularly evident at the Carlingford site where an imbalanced sex ratio was recorded, along with high levels of trematode infection and high numbers of indeterminate individuals. Gametogenesis and spawning are impacted by trematodes, as shown in this study, as well as by a range of biotic and abiotic factors including pollution (Lusher et al., 2017) and energy reserves (Hummel and Bogaards, 1989) highlighting the importance of regular monitoring to follow changes in the cycle of cockle gametogenesis.

The findings of this study lead to some important recommendations. First, it is necessary to conduct fine scale surveys, even if cockles are located nearby (e.g. large differences between Carlingford and Dundalk, despite proximity). Second, it would be beneficial to use molecular analysis to identify trematode species in the future, due to the varying distribution of trematodes spatially (de Montaudouin et al., 2009) and the range of effects they can inflict on their hosts. Third, minimum capture size should be set at a local scale, rather than regionally or nationally, due to the evident variations in spawning size spatially. Finally, it is important to consider the phenomenon of “shifting” reproductive cycles (timing and duration) associated with a changing marine environment and atypical years. Following these recommendations will be required in the decision-making process to ensure that (a) the spatial variability of cockles is accounted for, (b) sufficient numbers of broodstock remain to contribute to the future fecundity and sustainability of those populations and that (c) the harvesting/fishing season does not significantly reduce reproductive input.

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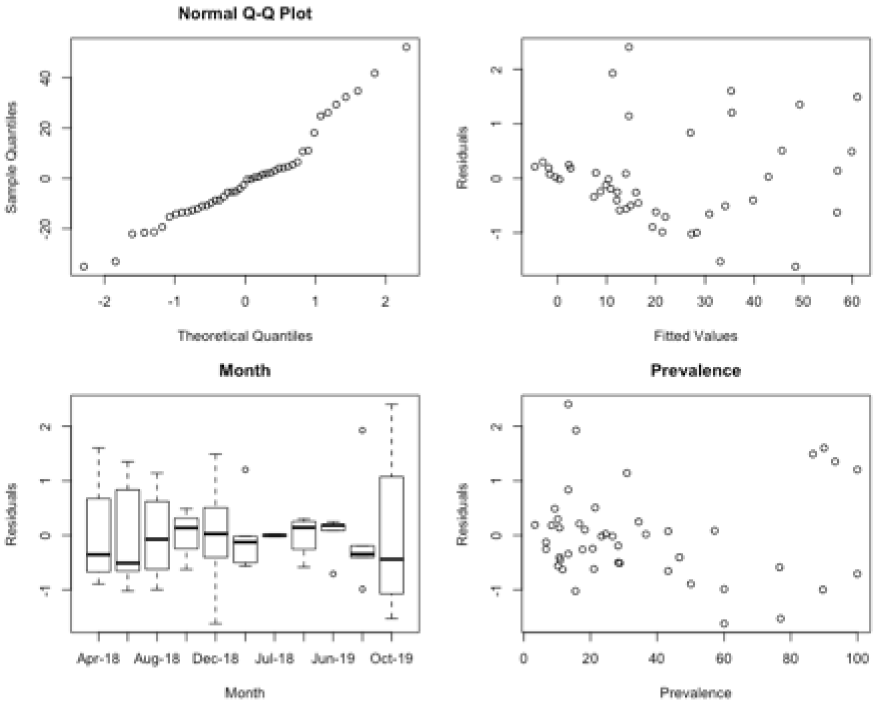
# 7. SUPPLEMENTARY MATERIAL

**Appendix 1.** Assumptions for a mixed effects model examining the relationship between percentage of indeterminate individuals and, site and trematode prevalence.



**Appendix 2.** Result of Dunn Tests with Bonferroni correction to determine if the percentage of indeterminate individuals differed between sites

| | Arcachon | Aveiro | Carlingford | Cork |
|---|---|---|---|---|
| Aveiro | 0.99 | - | | |
| Carlingford | **0.026** | **0.04** | - | - |
| Cork | 0.66 | 0.94 | 0.80 | - |
| Dundalk | 0.06 | 0.08 | 0.99 | 0.99 |

**Appendix 3.** Result of Dunn Tests with Bonferroni correction to determine if mean length at spawning differed between sites.

| | Arcachon | Aveiro | Carlingford | Cork |
|---|---|---|---|---|
| Aveiro | 0.99 | - | | |
| Carlingford | **<0.001** | **<0.001** | - | - |
| Cork | **<0.001** | **<0.001** | 0.99 | - |
| Dundalk | **<0.001** | **<0.001** | 0.99 | 0.99 |

**Appendix 4.** Results of Dunn Tests with Bonferroni correction to determine if number of growth rings of spawning individuals differed across sites.

| | Arcachon | Carlingford | Cork |
|---|---|---|---|
| Carlingford | **0.003** | - | - |
| Cork | 0.488 | **0.021** | - |
| Dundalk | **0.003** | **<0.001** | **<0.001** |

**Appendix 5.** Results of from a chi square test examining if the proportions of metacercariae infected individuals varied across sites

| Comparison | Adjusted Chi Square p value |
|---|---|
| Arcachon vs Aveiro | **< 0.001** |
| Arcachon vs Carlingford | **< 0.001** |
| Arcachon vs Cork | **< 0.001** |
| Arcachon vs Dundalk | **< 0.001** |
| Aveiro vs Carlingford | **< 0.001** |
| Aveiro vs Cork | **< 0.001** |
| Aveiro vs Dundalk | **< 0.001** |
| Carlingford vs Cork | **< 0.001** |
| Carlingford vs Dundalk | **< 0.001** |
| Cork vs Dundalk | **< 0.001** |

**Appendix 6.** Results of from a chi square test examining if the proportions of sporocyst infected individuals varied across sites

| Comparison | Adjusted Chi Square p value |
|---|---|
| Arcachon vs Aveiro | **< 0.05** |
| Arcachon vs Carlingford | **< 0.001** |
| Arcachon vs Cork | **< 0.05** |
| Arcachon vs Dundalk | **< 0.05** |
| Aveiro vs Carlingford | > 0.05 |
| Aveiro vs Cork | > 0.05 |
| Aveiro vs Dundalk | > 0.05 |
| Carlingford vs Cork | > 0.05 |
| Carlingford vs Dundalk | **< 0.05** |
| Cork vs Dundalk | > 0.05 |

**Appendix 7.** Results of chi square test examining if the proportions of metacercariae infected individuals differed at each stage of gametogenesis.

| Comparison | Adjusted Chi Square p value |
|---|---|
| Indeterminate vs Early Developing | > 0.05 |
| Indeterminate vs Late Developing | > 0.05 |
| Indeterminate vs Ripe | 0.08 |
| Indeterminate vs Spawning | 0.08 |
| Indeterminate vs Spent | > 0.05 |
| Early Developing vs Late Developing | > 0.05 |
| Early Developing vs Ripe | > 0.05 |
| Early Developing vs Spawning | > 0.05 |
| Early Developing vs Spent | > 0.05 |
| Late Developing vs Ripe | > 0.05 |
| Late Developing vs Spawning | > 0.05 |
| Late Developing vs Spent | > 0.05 |
| Ripe vs Spawning | > 0.05 |
| Ripe vs Spent | > 0.05 |
| Spawning vs Spent | > 0.05 |

**Appendix 8.** Results of chi square test examining if the proportions of metacercariae and sporocyst co-infected individuals differed at each stage of gametogenesis.

| Comparison | Adjusted Chi Square p value |
|---|---|
| Indeterminate vs Early Developing | > 0.05 |
| Indeterminate vs Late Developing | > 0.05 |
| Indeterminate vs Ripe | **<0.05** |
| Indeterminate vs Spawning | **<0.05** |
| Indeterminate vs Spent | > 0.05 |
| Early Developing vs Late Developing | > 0.05 |
| Early Developing vs Ripe | > 0.05 |
| Early Developing vs Spawning | > 0.05 |
| Early Developing vs Spent | > 0.05 |
| Late Developing vs Ripe | > 0.05 |
| Late Developing vs Spawning | > 0.05 |
| Late Developing vs Spent | > 0.05 |
| Ripe vs Spawning | > 0.05 |
| Ripe vs Spent | > 0.05 |
| Spawning vs Spent | > 0.05 |