UNIVERSITY OF SOUTHAMPTON

FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCES

School of Ocean and Earth Sciences

The Autecology of *Tapes philippinarum* (Adams and Reeve, 1850) in Southampton Water, UK

by

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ABSTRACT

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THE AUTECOLOGY OF *TAPES PHILIPPINARUM* (ADAMS AND REEVE, 1850) IN SOUTHAMPTON WATER, UK

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The non-indigenous venerupid clam *Tapes philippinarum* (Manila clam) was probably introduced to the Solent and Southampton Water in 2005. Since then clams have been intensively harvested by the local fishermen because of high economic value. Two current measures are in force to regulate the commercial exploitation, (i) the EU minimum landing size regulation (35 mm shell length) and (ii) the district byelaw prohibiting boats from over 12 m fishing within SIFCA district. This thesis presents an investigation the autecology of *T. philippinarum* in Southampton Water. The knowledge obtained would not only to provide base-line information for the species in the Solent system but also to establish the appropriate fishery management for the sustainability of local stock.

Histology revealed that the sex ratio of *T. philippinarum* was approximately 1:1. Gametogenesis started in February and the gonads matured from May onwards. Spawning was observed between May and September with a single peak from June to August. *T. philippinarum* in Southampton Water seems to have adapted to the lower water temperature (compared to its native range) of the UK because the gonad was active when the seawater temperature was much lower (4.8°C) than the minimum temperature reported elsewhere to initiate gonadal activity (10°C). The day degree rate required for successful spawning was more rapid (64.27 D°) than reported in the literature. Two peaks of bivalve larval density in June and August possibly indicated the successful spawning and development into larvae of the clam. The minimum size at sexual maturity of the clam was about 20 mm shell length, only clams larger than 25 mm showed a high percentage of the maturity (87.50%-100%). In addition, the oocyte diameter of the clams significant related to the shell length and time. Therefore, recent suggestions that the current 35 mm MLS could be reduced to say, 20 mm are not supported by this study. On the contrary, these results indicate that the MLS should not drop below 34 mm. The majority of the *T. philippinarum* population was within the 30-35 and 35-40 mm shell length size classes. The low proportion of the larger size

class (> 40 mm) might reflect the use of fishing gear developed during the period when the EU MLS was 40 mm. The environmental conditions in Southampton Water seem to provide *T. philippinarum* with a favourable habitat as indicated by its annual growth rate (2.93±1.23 mm in shell length) and normal immunocompetence. Winter and the spawning period were the stressors for *T. philippinarum* revealed by the lower haemocyte count, the thining of digestive epithelial thickness, and the increase in mean luminal radius/mean epithelial thickness. However, the immunocompetence was stabilised by the consistency of phagocytosis. An examination of the synergistic effect of temperature and reproductive development under food deficiency revealed that the winter food levels combined with temperature of 'warm' winter could result in a negative energy balance in *T. philippinarum* due to insufficient energy input to meet the energy demand for gonad development. The normal growth, good health status and completion of life history indicated that *T. philippinarum* has successfully naturalised in Southampton Water. Additionally, this bivalve species has great potential to extend its temperature tolerance below published observations.

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DECLARATION OF AUTHORSHIP

I, Wanwiwa Tumnoi

declare that the thesis entitled

The autecology of *Tapes philippinarum* (Adams and Reeve, 1850) in Southampton Water, UK

and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- none of this work has been published before submission.

Signed: ……

Date:……

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Chapter 1 General Introduction

Tapes philippinarum (Adams and Reeve, 1850) (Manila clam), is a commercially important venerupid clam, native to the western Pacific Ocean (Derrick, 1992; Goulletquer, 1997; Skarlato, 1981). The species was introduced into the UK in the 1980s for aquaculture (Britton, 1991; Utting and Spencer, 1990). During the introduction of *T. philippinarum* in the UK, a concern was raised by some marine nature conservationists that the species would naturalise and reproduce successfully. The available evidence at the time suggested that the minimum conditions needed for a successful spawning were between 460-600 day degrees; D° (number of days x degrees above a critical minimum threshold of 10°C) which were unlikely to be achieved in the UK (Beninger and Lucas, 1984; Kersuzan, 1989; Lovatelli, 1985; Utting and Doyou, 1992). Nevertheless, the first record of the naturalisation of *T. philippinarum* in the UK appeared from Poole Harbour. The clams in Poole Harbour did naturalise and produce viable spat (Grisley, 2003; Jensen *et al*., 2005; Jensen *et al*., 2004). *T. philippinarum* has subsequently been found in the Solent System, including Southampton Water since about 2005, possibly introduced either by commercial fishing interests (the Southern Inshore and Fisheries Conservation Authority (SIFCA), pers. comm.) or by larval dispersal from Poole Harbour (41.8 km west of Southampton Water) (Herbert *et al*., 2012). It has been intensely harvested following its presence in the area because of its high market value (up to £5 kg^{-1} 1st sale value; SIFCA, pers. comm.). Currently (October 2012), only two management measures are in force to regulate the commercial exploitation of the clams. These are the EU Minimum Landing Size (MLS) legislation (35 mm shell length) and the general prohibition of commercial fishing vessels over 12 m in overall length fishing within SIFCA district. The exploitation of the fishery would be greatly increased if a proposal from the local fishing industry of a reduction of the MLS from 35 to 20 mm shell length was accepted.

The aim of the present study was to provide a quantitative study of the reproductive cycle of *T. philippinarum* in Southampton Water. Histological staging of gonad development and size at sexual maturity were examined over one year period so that the potential impact of an MLS of 20 mm could be evaluated. In a wider context the information including immunological responses, bioenergetics, population structure, growth rate and bivalve larval abundance would contribute to not only an understanding of the spread of *T. philippinarum* in northern European Waters, but also to the establishment of a more targeted science-based fishery management regime for this species in Southampton Water and The Solent.

The principal objectives of this thesis are;

(1) To investigate the reproductive cycle of *Tapes philippinarum* in Southampton Water and compare the result with the populations from Poole Harbour.
(2) To study population structure of the clam and bivalve larval abundance in Southampton Water.
(3) To reveal the clam's adaptation to Northern European Waters through its growth and immunological responses to environmental changes.
(4) To investigate the relationship between environmental parameters and biology of the clam.
(5) To examine the effect of cold and warm winters on the spring mortality of the clam using gametogenesis and scope for growth as indicators.

1.1 Nomenclature and Taxonomy

Tapes philippinarum (Manila clam, Japanese littleneck clam, Short neck clam, Carpet shell) belongs to the family Veneridae. The clam is known by various scientific names, for example, *T. semidecussatus, T. philippinarum, Venerupis philippinarum, V. semidecussatus, V. semidecussata* (Howson and Picton, 1997). According to the international taxonomy database CLENAM (Check List of European Marine Mollusc), the genus *Tapes* is mainly recognized according to the following historical chronical (Goulletquer, 1997).

Genus:	*Tapes*	Von Mühlfeldt (1811)
	= *Parambola*	Roemer (1857)
	= *Tanis*	Weyenbergh (1875)
	= *Ruditapes*	Chiamenti (1900)
	= *Amygdata*	Roemer (1857, non Gray J.E. 1825)

Fischer-Piette et Métivier (1971) revised the taxonomy of Tapetinae (Veneridae), nevertheless the synonymous of this bivalve species in particular *Tapes philippinarum* and *Ruditapes philippinarum* have been continuously used depending on the publication state origin (Goulletquer, 1997). *R. philippinarum* is used in most European countries and in Asia whilst *T. philippinarum* is the most common name in the Anglo-Saxon literature (e.g., USA, EN) (Goulletquer, 1997). According to the Aquaculture compendium (CABI) and a bibliography by Goulletquer (1997), *Tapes philippinarum* (Adam and Reeve, 1850) or Manila clam are the names most frequently used and will be adopted for this study. The taxonomy of *T. philippinarum* was given by Jones *et al*. (1993).

Phylum: Mollusca
Class: Bivalvia
Superorder: Heterodonta
Order: Veneroida
Family: Veneridae
Genus: *Tapes* or *Ruditapes*

1.2 Morphology

The shell shape of *Tapes philippinarum* is generally a rounded triangle and the shell length is greater than the shell height (Fig. 1a). The valves are thick, heavy, equivalve and inequilateral. The beak is in the anterior half, somewhat broadly oval in outline. The inset ligament (unconcealed) is a thick brown elliptical arched body extending almost half-way back to the posterior margin (Quayle and Bourne, 1972). The elongated heart shaped lunule is clear, even though it is not well defined, with light and dark fine radiating ridges. The escutcheon reduced to a mere border of the posterior region of the ligament. The external shell has radiating ribs and concentric ridges. The shell colour varies from greyish-white, through yellowish-buff to brown, often with geometric pattern of black and white in the young (Quayle and Bourne, 1972). Each valve has three cardinal teeth. The left valve has a central tooth and the right valve has central and posterior teeth. No lateral teeth are seen. The internal surface of the valve is smooth and yellowish-white with deep purple colour at the posterior end (Bourne, 1982). The pallial sinus is relatively deep but not extending beyond the middle of the shell. The internal ventral margin of the shell is smooth, distinct from the crenulated margin of one of the morphologically similar native British species, Palourde, *Tapes decussatus*. The siphons of *T. philippinarum* fuse together for almost their entire length and the tips of the siphons are brown in colour (Fig. 1b), but those of *T. decussatus* completely separate (Fig. 1c). The siphon of *T. philippinarum* is short in comparison to some other clams reflecting that it lives only a shallow distance under the substrate surface (Quayle and Bourne, 1972).

Figure 1 *Tapes philippinarum* (Manila clam) and *T. decussatus* (Palourde) (a) The external shell of *T. philippinarum*; left hand side row and *T. decussatus*; right hand side row (b) The siphons of *T. philippinarum* split at the tip (c) The siphons of *T. decussatus* completely separate (Figure courtesy of Dr. Antony C. Jensen)

1.3 Geographic distribution and Introduction to the UK

Tapes philippinarum is widely recognised as a native species of the subtropical to low boreal of the Western Pacific (Goulletquer, 1997). The natural distribution of *T. philippinarum* is between the latitudes of 25˚ and 45˚ North and they can be found in the Philippines, the South and East China Seas, Yellow Sea, Sea of Japan, the Sea of Okhotsk, and around the Southern Kuril Islands. The present worldwide distribution of *T. philippinarum* is shown in Fig. 2 and Table 1.

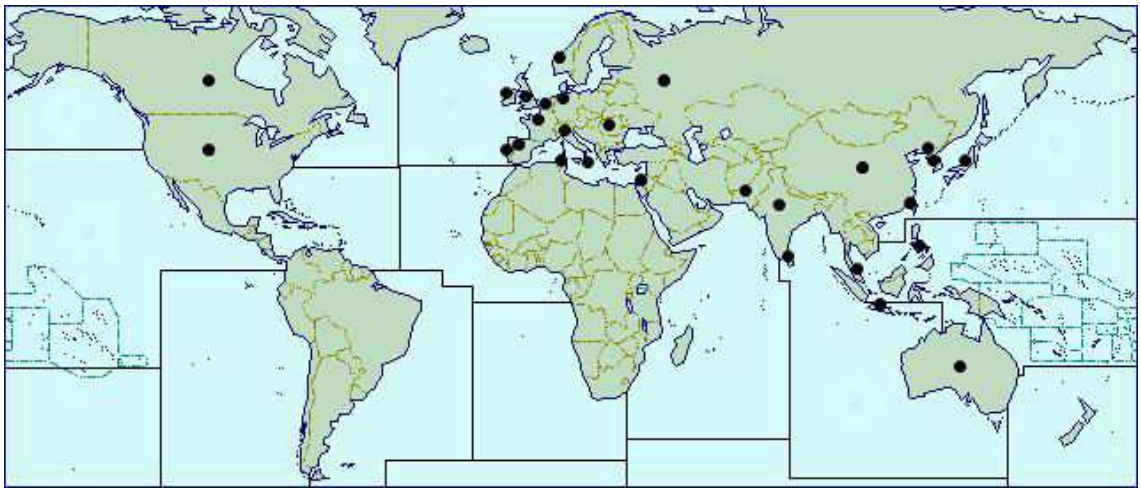

Figure 2 Present worldwide distribution of both native and introduced *Tapes philippinarum*. Black dots indicate the countries recorded the presence of *Tapes philippinarum* (modified from Invasive Species Compendium, 2012).

Table 1 List of countries recorded the presence of *Tapes philippinarum* (modified from Invasive Species Compendium, 2012).

Region	Origin	Country/Sea areas
Asia	Native	China, India, Indonesia, Japan, Korea DPR, Republic of Korea, Malaysia, Pakistan, Philippines, Sri Lanka, Taiwan
	Introduced	Israel
Africa	Introduced	Tunisia
North America	Introduced	Canada (British Columbia), USA (Alaska, California, Hawaii, Oregon, Washington)
Europe	Introduced	Belgium, France, Germany, Ireland, Italy, Norway, Portugal, Romania, Russian Federation, Spain, UK
Oceania	Introduced	Australia
Sea areas	Native	Pacific (Northwest, Western Central)
	Introduced	Arctic Sea, Atlantic (Northeast), Indian Ocean (Eastern), Mediterranean and Black Sea, Pacific (Eastern Central, Northeast)

T. philippinarum was accidentally introduced into the Pacific coast of North America in 1936 along with Pacific oyster seed imported from Japan (Jones *et al*., 1993). The Pacific coast from California to British Columbia was then colonized by the species, and it became the most commercially important clam in this geographical location (Anderson *et al*., 1982). The clam production from Washington State, USA is currently up to 6400 metric tons per year (Cheney *et al*., 2012). The SATMAR (Société ATlantique de MARiculture) corporation intentionally introduced the clam into France for aquaculture purposes between 1972 and 1975 because of its high market value (Flassch and Leborgne, 1990), these subsequently naturalised in the Bay of Arcachon (Robert *et al*., 1993). *T. philippinarum* is now found widely throughout Europe, from the Norwegian Atlantic coast to the Adriatic and Aegean seas (Goulletquer, 1997; Jensen *et al*., 2005; Jensen *et al*., 2004). Clams are exploited both in aquaculture and as a naturalised fishery in France, Spain and Italy (Goulletquer, 1997). In Ireland (introduced in 1982; Britton, 1991) and Norway (introduced in 1987; Mortensen and

Strand, 2000) *T. philippinarum* have not naturalized (Britton, 1991; Drummond *et al*., 2006; Mortensen and Strand, 2000).

T. philippinarum was imported into the UK from Washington State, North America in 1980 and kept in quarantine at the MAFF Fisheries Laboratory, Conwy, North Wales (Laing *et al*., 1990). Descendants of this population were laid in Poole Harbour (41.8 km west of Southampton Water) on the South coast of England by Othneil Shellfish Ltd. for farming in 1988 (Utting and Spencer, 1990). Prior to this, concerns were raised by some marine nature conservationists that the recruitment of this species could be successful in UK waters and that could possibly pose a serious threat to native marine fauna by displacement. The available evidence in the 1980s showed that this animal was unlikely to successfully recruit as the summer seawater temperature in the UK did not typically rise above the minimum requirement for gametogenesis, spawning, successfully larval settlement and survival of this species for long enough. The minimum temperatures that can trigger the gonadal activity is 10°C (Millican and Williams, 1985); maturing and spawning appeared above 14°C (Holland and Chew, 1974; Ohba, 1959). Furthermore, the minimum conditions needed for a successful spawning in *T. philippinarum* were between 460 and 600 D° (Beninger and Lucas, 1984; Kersuzan, 1989; Lovatelli, 1985; Utting and Doyou, 1992). Nevertheless, the first naturalisation of *T. philippinarum* in the UK was reported in Poole Harbour in the early 1990s (Jensen, pers.comm.) showing that the *T. philippinarum* was able to adapt to the UK conditions (Grisley, 2003; Jensen *et al*., 2005; Jensen *et al*., 2004).

Until 1994, the exploitation of this naturalised population in Poole Harbour was by local fishermen and wading birds (Humphreys *et al*., 2007). The Southern Sea Fisheries Committee (SSFC; now SIFCA) licensed the *T. philippinarum* fishery in 1997 within the Poole Harbour Regulatory Order (1915, revised 1985). The SSFC established a closed season between October and January (an 8-10 week season) and the regulation of fishing techniques. The minimum landing size (MLS) which was 40 mm shell length (now 35 mm) was already in force through EU legislation (to manage existing fisheries in Italy and France) (Jensen *et al*., 2004). The licensed fishery originally supported 31 local fishermen, landing approximately 250 tons of clams in 2002. The 'pump scoop' (a pole mounted 460 x 460 x 300 mm mesh basket through which seawater is pumped as the scoop is dragged through the sediment; Fig. 3) is widely used for harvesting the clam in Poole Harbour (Jensen *et al*., 2004). The landings over three years (2005-2007) were between 250 and 500 tons, nonetheless the landings of illegal clam fishing activity are unclear. Though the *T. philippinarum* has been protected through a number of fishery regulations, the concerns of Natural England regarding the impact of the fishery on the infauna providing food for overwintering birds had not been

completely allayed. No-dredge zones were introduced in order to provide low disturbance areas for overwintering birds. Illegal fishing activity, now widely spread throughout Poole Harbour, creates additional fishing pressure on the clam population throughout the year (Humphreys *et al.*, 2007).

Figure 3 The 'pump scoop' for harvesting *T. philippinarum* in Poole Harbour (Figure courtesy of Dr. Antony C. Jensen)

T. philippinarum appeared in the Solent within Southampton Water in about 2005, allegedly transferred by commercial fishing interests (SIFCA, pers. comm.) and is now subject to an intensive fishery. Nevertheless, the model of larval behaviour from Poole Harbour investigated by Herbert *et al*. (2012) illustrated that theoretically the larvae of

T. philippinarum could be moved out of Poole Harbour by residual currents and spread along the English south coast. The two regulations controlling exploitation in Southampton Water are the EU minimum landing size regulation (MLS) of 35 mm shell length and the SIFCA byelaw prohibiting commercial fishing boats over 12 m total length fishing within the SIFCA district. Fishermen in Southampton are lobbying for a MLS reduction to 20 mm.

1.4 Risks associated with the introduction of *Tapes philippinarum*

There are a number of non-native marine animals that have been either intentionally (e.g. *T. philippinarum* into Poole Harbour) or unintentionally (e.g. *Crepidula fornicatae* to Southern England) introduced into a new habitat. The spread of a non-native or 'alien' species mostly causes some ecological impacts (Savini *et al*., 2010). Five categories of environmental risks have been proposed by Kohler (1992) including habitat alteration, trophic alteration, spatial changes, gene pool deterioration and introduction of diseases. *T. philippinarum* has shown an ability to naturalise in many of the areas where it has been introduced around the world such as Pacific coast of USA, France and south coast of the UK (Anderson *et al*., 1982; Flassch and Leborgne, 1990; Goulletquer, 1997; Grisley, 2003; Jensen *et al*., 2005; Jensen *et al*., 2004).

The ecological impacts of *T. philippinarum* are ambiguous because both negative and no effects have been recorded. A negative impact, habitat modification, was observed by Sgro *et al*. (2005); sediment erosion and re-suspension rates significantly increased because of the clam's feeding and burrowing behaviour. Additionally, *T. philippinarum* farming activity significantly affected biotic and physical characteristics at the sediment-water interface (Castel, 1984). The competition between *T. philippinarum* and other species for food or habitat was observed by Toba *et al*. (1992), however, the clam became a new food item for a native species. The preference for *T. philippinarum* by the predatory crab *Cancer productus* was 1.7 times higher than the co-familial bivalve *Protothaca staminea* (Byers, 2005). According to Figueras *et al*. (1996), *T. philippinarum* can be a disease carrier, for examples the clam imported from Gore, Italy into a depuration plant located in the Ria de Vigo, Spain showed signs of brown ring disease. Furthermore, the benthic community structure might be changed by the presence of *T. philippinarum* as a decline of nematodes caused by the activity of the clam was noted by Castel (1984). Gerard (1978) showed that hybridisation was impossible between *T. philippinarum* and the native species *T. decussatus* on the grounds of a reproductive mating barrier. However, recent genetic and cytological evidence from clams in NW Spain revealed the occurrence of hybridization between

female *T. decussatus* and male *T. philippinarum* (Hurtado *et al*., 2011). This might cause the disruption of local genetic adaptation and loss of genetic integrity of native species. According to Hurtado *et a*l. (2011), the hybrids of *T. decussatus* and *T. philippinarum* share some intermediate characteristics including the striation pattern and the morphology of the lunule, but the both types of siphon (fused and separated) are found in the hybrid specimens.

In spite of the many negative impacts, some areas report no negative impact of *T. philippinarum* e.g. no long-term effects on the environment or benthic community were observed close to an area of *T. philippinarum* cultivation in Kent, south-east England (Kaiser *et al.,* 1996). The co-familial bivalve *Protothaca staminea* were not affected by *T. philippinarum* even at high density (88-222 individuals m^{-2}) (Byers, 2005). In addition, Caldow *et al*. (2007) revealed the benefit of an introduction of *T. philippinarum* into Poole Harbour for the Eurasian oystercatcher *Haenotopus ostralegus ostralegus*. In spite of the low density of the clams (5 individuals m^{-2}), the over-winter mortality of oystercatchers predicted using a model reduced from 4.1% to 1.1% of the existing wintering population of oystercatchers.

1.5 Life history

1.5.1 Reproduction

Tapes philippinarum is a gonochoric organism (separate sex) and there are usually equal numbers of males and females in a population (Grisley, 2003; Cooke, 2009; Drummond *et al*., 2006; Ponurovsky and Yakovlev, 1992). Some studies nevertheless revealed the occurrence of hermaphrodites, but these are rare (Drummond *et al*., 2006; Ponurovsky, 2008; Ponurovsky and Yakovlev, 1992; Holland and Chew, 1974). The size at sexual maturity of *T. philippinarum* is varied. Although some mature clams ranging between 5 and 10 mm in shell length were recorded (Holland and Chew, 1974; Ponurovsky and Yakovlev, 1992), most previous observations revealed that the clams mature at shell length larger than 15 mm (Cooke, 2009; Grisley, 2003; Holland and Chew, 1974; Ko, 1957; Ponurovsky and Yakovlev, 1992; Yap, 1977). Gender is impossible to differentiate using external characteristics. The standard method involving gonad tissue fixing, a histological processing and light microscope examination is employed to distinguish the sexes (Gosling, 2003). The gonad of clams is situated at the base of the foot and covers the outer surface of the intestine within the mantle cavity. During the resting stage, the gonad is shrunken and totally filled with connective tissue which is similar to body tissue leading to a difficulty in distinguishing gender (Gosling, 2003). Gametogenesis is the process that produces

gametes within the gonad. A species reproducing sexually has different forms of gametogenesis named as spermatogenesis and oogenesis in males and females, respectively. Gametogenesis in clams can be divided into several stages for instance early active, developing, late active, mature, spawning, and spent stages (Gosling, 2003).

Reproduction is mainly influenced by the interaction between intrinsic (endogenous or internal) and extrinsic (exogenous or external) factors. The intrinsic factors include genotypes and physiological condition whereas food availability, temperature, diseases, etc. play the role of the extrinsic factor. These factors can affect the gametogenesis period and reproduction pattern (e.g. single spawning or several spawning events) (Adiyodi and Adiyodi, 1983; Giese, 1959; Sastry, 1975). Spawning in *Tapes philippinarum* can occur either once or twice a year or even continuously throughout a year depending on geographical and environmental factors (Ponurovsky and Yakovlev, 1992). The populations in northern areas generally spawn once a year, whereas some populations in further south spawn twice. For examples, *T. philippinarum* from Vostok Bay, Sea of Japan (42° 52' N) released the gametes once per year between July and October (Ponurovsky and Yakovlev, 1992). But the gametogenesis in the clam from Tokyo Bay (35° 25' N) showed two annual peaks of spawning from April to June and from August to October (Toba *et al*., 1993). The prevalence of spawning at low level throughout the year was observed in the clam population in Kaneobe Bay, Honolulu Island (21° 27' N) with a peak between January and February (Yap, 1977).

Similar reproductive pattern and timing of *T. philippinarum* are seen from several areas in North Europe e.g. NW Ireland (54° 33'-88' N) (Drummond *et al*., 2006), Poole Harbour, England (48° 41'-42' N) (Grisley, 2003), South and Western Brittany, France (47° 30'-40' N and 48° 23' N) (Laruella *et al*. 1994), and the Venice lagoon (45° 23' N) (Meneghett *et al*., 2004). These recorded a single spawning between May and September or October. The reproductive cycle of *T. philippinarum* can also be determined by other environmental factors for example food availability. A distinction was seen in *T. philippinarum* from two adjacent lagoons in Po River Delta, Adriatic Sea where the clam in the Sacca Del Canarin lagoon spawned once a year from May to October, but the clam living in the Sacca Degli Scardovari lagoon spawned twice a year between June-July and September-October (the latter spawning was greater) (Sbrenna and Campioni, 1994). The trophic resource is considered as the trigger of semiannual spawning in *T. philippinarum* at Sacca Degli Scardovari (Sbrenna and Campioni, 1994). This is because no differences in temperature and salinity regime between these two lagoons, however the biochemical reserved of the clam in Sacca Del Canarin is higher

than those in Sacca Degli Scardovari from late winter to early spring (Sbrenna and Campioni, 1994). In addition, Po River Delta is acknowledged as the intrinsically eutrophic water body which emphasizes the environmental stresses on the benthic community (Ceccherelli *et al.*, 1994; Sei, 1998).

1.5.2 Eggs and Fecundity

The egg size of *Tapes philippinarum* varies depending on the nature of the habitat of the adults (Cooke, 2009; Grisley, 2003; Laruelle *et al.*, 1994; Meneghetti *et al.*, 2004; Xie and Burnell, 1994). The average oocyte diameters of clams from S. Angelo and Chioggio, Venice lagoon, Italy were 35.8 µm and 41.6 µm, respectively (Meneghetti *et al.*, 2004), whilst *T. philippinarum* in Brittany, France produced oocytes ranging in diameter between 27.79 µm in May and 57.87 µm in July (Laruelle *et al.*, 1994). The mean diameter of oocytes in clams from the south coast of Ireland was 43.2 µm (Xie and Burnell, 1994). Ripe oocytes of *T. philippinarum* in Southampton Water varied temporally from an average diameter of 35.9$\pm$0.5 µm in June 2008 to 43.3$\pm$0.2 µm in September 2008 (Cooke, 2009). The size of mature oocytes in the population from Poole Harbour was about 34.2 µm (Grisley, 2003). There is also variation in the fecundity of *T. philippinarum* for instance; the average fecundity of *T. philippinarum* observed by Utting *et al.* (1996) was 1.54 million eggs per female which is in the lower part of the range reported by Park and Choi (2004) (0.90-11.79 million eggs with a mean of 4.15 million). The relationship between fecundity and shell length was seen when the number of eggs in a clam of 20 mm in shell length was found to be 4.32 $x10^5$ eggs increasing to $1.3x10^6$ eggs in a 40 mm shell length clam (Yap, 1977).

1.5.3 Larval development

Tapes philippinarum synchronously broadcast spawn gametes (egg and sperm) into the water, where fertilization occurs (Jones *et al.*, 1993). The stage of larval development and their duration are shown in Table 2. The duration in each developmental stage varies depending on the environmental conditions such as temperature and diet (Emmett *et al.*, 1991). After the fusion of egg and sperm, a zygote is produced which develops into a trochophore larva within 24 hours (Jones *et al.*, 1993). The trochophore, the first free swimming stage of the clam, is characterized by a band of cilia (prototroch) (Sastry, 1979). The larvae are filter feeders, grazing on live algae (Laing *et al.*, 1990). The trochophore larvae develop to straight hinge veliger or D-shaped veliger when large velum is formed for swimming and feeding and the first larval shell, thin and transparent with a straight hinge, is secreted by the shell gland. The stage at which the second shell is formed is considered as umbo stage

because of a pronounced umbo on the larval shell. The larva continues to develop to a pediveliger once the foot appears to explore the substratum (Gosling, 2003). Certain key environmental stimuli such as light, gravity and pressure influence the swimming behaviour of the larvae (Gosling, 2003). The early larval stages respond positively to light and are sensitive to pressure. Thus the younger larvae generally remain in the surface water. The later stage larvae are, conversely, positively geokenetic and insensitive to pressure (Ishida *et al*., 2005; Ishii *et al*., 2005). They, therefore, tend to sink to the sea bed to find an appropriate substratum for settlement. The larvae attach to a pebble or shell on the sea floor using a byssus during settlement. The newly settled veliger metamorphoses to a juvenile and becomes sedentary (Munari *et al*., 2009; Tezuka *et al*., 2012)

Table 2 Life stages and characteristics of *Tapes philippinarum* (Grisley, 2003; Hur *et al*., 2005; Jones *et al*., 1993; Laing and Utting 1994; Munari *et al*., 2009; Tezuka *et al*., 2012)

Stage	Size (length)	Age and Characteristics
Fertilised egg	60-70 µm	0-24 hours; non motile
Trochophore	75 µm	10-48 hours; ciliated and motile
Veliger		
D-shaped	88-116 µm	1-6 days; straight-hinged shell
Umbo	130-200 µm	7-14 days; umbo on shell
Pediveliger	200-220 µm	2-4 weeks; development of foot
Juvenile	>240 µm	1-3 years; sexually immature
Adult	>15 mm	up to 25 years; sexually immature

The larval period of several bivalve species is minimized when the culture temperature is increasing (Bayne, 1965; Loosanoff and Davis, 1963; Walne, 1965). The optimum temperature for larval development of *T. philippinarum* is 23-25°C (Helm and Bourne, 2004). Lovatelli (1985) recorded that the larvae of this bivalve species spent 14 days to develop from fertilization to the pediveliger at 25.9°C, but took 24 days at 21.5 and 15.8°C. Food is another significant factor manipulating larval development of *T. philippinarum*. Yan *et al*. (2009) revealed that starvation resulted in decreasing in growth, survival, metamorphosis rate and metamorphosis size of the umbo-veliger of of *T. philippinarum*. For example, the early and middle umbo-veliger of 100 and 140 µm length seemed to stop growing after 1.5 and 4 days of starvation. In addition, the delay in duration to metamorphosis of the 100, 140 and 190 µm larvae starved for 4, 16, 20 days, respectively were 9, 11, and 17 days, respectively (Yan *et al*., 2009).

1.6 Ecology

Tapes philippinarum is most commonly found in stable, loosely packed substrata; in particular sand, sandy-silt or muddy-gravel bottoms ranging from the intertidal zone to several metres below Chart Datum. They live just under the surface (3-5 cm) (Jensen *et al*., 2005; Nie, 1993; Toba *el al*., 1993). The density of juveniles (3-10 mm shell length) of *T. philippinarum* in the Venice Lagoon, Italy in the sandy bottom or substrata mainly covered by shell debris was 2-3 times higher than those observed on a muddy bottom (Pelizzato, 2011). The clam has been found in high abundance in eutrophic and sheltered environments, for example Starfish Bay; a protected sandy shore in Hong Kong; 258.6 ind.m^{-2} (Lee, 1996), tidal flats with low wave action where benthic diatoms flourished in Venice lagoon; 384 ind.m^{-2} (Breber, 2002) and Poole Harbour, a shallow estuary with a narrow entrance to the sea; 56 ind.m^{-2} (Humphreys and May, 2005). *T. philippinarum* is both euryhaline and eurythermal. It is able to survive in salinity ranges from 15-36; however the optimum salinity is between 20 and 30 (Bernard, 1983; Jensen *et al*., 2005). They can therefore occupy an estuarine area (Bernard, 1983). This species can tolerate water temperature ranging from 0-25°C, but grows best between 13-21°C (Bernard, 1983).

T. philippinarum is a suspension feeder and its natural diet is phytoplankton and organic detritus (Dang *et al*., 2009), however it is non selective feeder consuming a wide range of suspended food (Sorokin and Giovanardi, 1995). An observation on the trophic characteristics of *T. philippinarum* using ^{14}C-labelled food showed that the clam can consume and digest green algae (*Chlorella, Tetraselmis and Dunaliella*), Diatom (*Nitzschia*), Cyanobacteria (*Oscillatoria* and a mixture of *Coelopshaerim)*, Bacterioplankton and Microzooplankton (Rotifer *Brachionus* sp.) (Sorokin and Giovanardi, 1995). Nevertheless, the assimilation index was maximal in diatoms (0.92-1.14%) and green algae (0.68-1.02%), but minimal (0.27%) in Cyanobacteria particularly *Coelopshaerim* (Sorokin and Giovanardi, 1995). The predators of *T. philippinarum* are fish, starfish, moon snail e.g. *Euspira lewisii* (Anderson *et al*., 1982), *E. fortunei* (Tanabe, 2012), crabs such as *Thalamita crenata* (Yap, 1977), *Cancer productus* (Quayle and Bourne, 1972), *C. maenas* (Cigarria and Fernandez, 2000), and diving ducks i.e. *Melanilla deglani*, *M. perspincillata*, and *M. nigra* (Bourne, 1984). The crab *Thalamita crenata* was responsible for up to 57% of the mortality of *T. philippinarum* in Kaneohe Bay, Honolulu island (Yap, 1977). Cigarria and Fernandez (2000) showed that the green crab *C. maenas* ranging from 7-65 mm carapace length (antero-posterior axis) was able to eat *T. philippinarum* of 8 mm shell length. Only crabs larger than 55 mm can eat clams of 23.5 mm shell length (Cigarria and Fernandez, 2000).

Paillard (2004) reviewed the occurrence of Brown ring disease, an infection of *Vibrio tapestis* which frequently causes the mortality of *T. philippinarum*. A brown deposit (conchiolin) on the surface of the internal shell between the pallial line and the growing shell edge is a characteristic symptom of the disease. The clam beds in Brittany, France have suffered from the disease since 1987. It consequently has spread along the European Atlantic coast including Spain, Ireland, Italy, Norway, and England. *V. tapestis* is gram-negative, non-sporulating motile bacterium which is a pathogen to the venerupid bivalves in the genera *Ruditapes*, *Tapes*, and *Venerupis* (Paillard, 2004).

The mortality of *T. philippinarum* is occasionally recorded as a consequence of the infection of the protozoan parasite *Perkinsus* spp. along the Mediterranean and Atlantic coasts of Europe (Chagot *et al.*, 1987; Da Ros and Canzonier, 1985; Sagrista *et al.*, 1995) and Japanese and Korean Waters (Choi and Park, 2010). The prevalence of *Perkinsus olseni* infection (100%) was detected from the *T. philippinarum* examined during its mass mortality in Gomsoe Bay, the west coast of Korea, with the high intensity ranging 11,000-2,000,000 cells g^{-1} wet tissue (Park, 1999 in Choi and Park, 2010). In other marine molluscs, the genus *Perkinsus* is responsible for the mortality of oyster, scallop, clam and abalone (Choi and Park, 2010). *P. honshuensis* was recorded in *T. philippinarum* from Japan (Dungan and Reece, 2006). White nodules on the surface of the mantle of *T. philippinarum* from both Japan (Hamaguchi *et al.*, 1998) and Korea (Park and Choi, 2001) were evident in heavily infected clams as a result of tissue inflammation to the infection. Organs where *Perkinsus* trophozoites were observed were gill, digestive gland, mantle, and gonad connective tissues (Choi *et al.*, 2002; Park and Choi, 2001).

1.7 Growth

Growth in bivalves is typically described in terms of an increment in one or more dimensions of the shell. In clams, for example, length is the maximum distance between the anterior and posterior margin of the shell. Shell height, the maximum distance from the dorsal (hinge) and ventral shell margin, is usually used in scallop. The measurement of growth can be examined in two ways. One is absolute growth, the size of the whole organism related to age; the other is allometric growth representing the rate of growth of one size variable related to that of another variable (Gosling, 2003). Absolute growth can be measured by different methods including size-frequency distributions, annual growth rings and marking on shells. Size-frequency distribution is employed to measure growth of a population while the annual growth rings can be examined either from rings on the external shell, growth lines in the shell matrix or ligament cross sections. Such rings or lines are annually produced because

growth is much lower in winter (Gosling, 2003). The growth can also be determined in the form of marking shell of animals and maintaining them in situ or in cages for a known period of time prior to the measurement of an incremental shell dimension. There are a number of factors affecting growth, however trophic resource is considered as the most important factor. This is because sustained growth is impossible without food (Seed and Suchanek, 1992). Other factors such as temperature, salinity and water depth not only directly influence growth but also indirectly manipulate food supply (Gosling, 2003). Growth rates therefore vary due to both environmental and animal conditions. Geographic location determines bivalve growth because of the variation in environmental factors. For example, a variation in total length was seen in *T. philippinarum* at the same age from different areas (Table 3)

Table 3 Shell length of one-year old *Tapes philippinarum* from different areas (Emmett *et al*., 1991)

Study area	Shell length of one-year old clam (mm.)
Hokkaido, Japan	8
Inland Sea, Japan	11
Southern Japan	27
Strait of Georgia, BC	10-15
Hood Canal, Washington	24

Growth rate is also affected by season, mainly involving food availability and seawater temperature changes. Ren *et al*. (2008) reported a seasonal pattern in the growth of *Tapes philippinarum* in Jiaozhou Bay, China. The highest growth rate in both farmed and wild specimens was seen in summer between May and September. The clams grew slowly from October onwards and stopped growing in winter (November until the following January) with weight loss. The onset of growth resumed in next March or April. Furthermore, intrinsic factors such as age and reproduction regulate growth of bivalves (Gosling, 2003). Ponurovskii (2008) revealed that an annual average growth rate in shell length of *T. philippinarum* aged below 3 years old from Amursky Bay, Sea of Japan was 11.6$\pm$0.3 mm. The rates decreased as the age progressed and did not exceed 3 mm year^{-1} in 6-7 year-old animals. In the same bivalve species from Jiaozhou Bay, China, the clam at age 1 year old revealed a faster growth rate (83.8% in shell length and 123% in gross weight) than the older groups (age 2 years old; 12.0% and age 3 years old; 9.7% in shell length) (Ren *et al*., 2008).

1.8 Population structure

Population structure is used not only to quantify population growth but also to reveal the standing stock of the study species. It is usually presented in form of a size-frequency distribution. Two main patterns of population structure include unimodal and bimodal pattern (Gosling, 2003). Unimodal pattern is the size distribution characterised by a single size category. On the other hand, a large proportion of the population consisting of two discrete size classes is referred to as being a bimodal pattern. Nonetheless, polymodal distribution dominated by several size classes can be observed in some bivalve species such as *Crassostrea rhizophorae* in Venezuela (Montes-M *et al*., 2007) and *Limopsis tajimae* (Nakaoka and Ohta, 1998).

The size-frequency distributions of *Tapes philippinarum* in many locations are unimodal (Dang *et al*., 2010; Grisley, 2003; Ponurovsky, 2008). The population of *T. philippinarum* in Poole Harbour presented the unimodal pattern which was dominated by the 'middle' size classes (Grisley, 2003), clams under 20 mm and above 40 mm in shell length were absent. The author suggested that the juveniles ($\leq$20 mm) were excluded because of a limitation in sampling gear (a large mesh size), but fishing in the previous season was likely to remove the larger bivalves. The impact of the fishery on *T. philippinarum* population in Poole Harbour was confirmed by Humphrey *et al*. (2007), who found the small asymptotic size (40-50 mm shell length) and young age (5-6 years old) of the oldest clam in spite of records of the longevity of 13-15 years old and a maximum shell length of 60 mm. The population of *T. philippinarum* in Amursky Bay, Sea of Japan was numerically dominated with clams of shell length 35-45 mm (67.8%) (Ponurovskii, 2008). An unbalanced population structure was reported in the same bivalve species from Arcachon Bay (SW France) since 2003 (Dang *et al*., 2010). Dang *et al*. (2010) recorded low proportions of juveniles (<20 mm) and larger adults (>40 mm). The majority of the population were 28-40 mm in shell length. A lack of juveniles was attributed to low recruitment success. Sixty five to seventy percent of total adult mortality was affected by fishing (MLS 40 mm). These observations raised serious concerns about the sustainability of the population and the continued exploitation by fishing (Dang *et al*., 2010).

The effect of selective removal on a population with a bimodal size-frequency distribution were described by Sejr *et al*. (2002), who studied the bivalve *Hiatella arctica* in a high-Arctic Fjord, Young Sound, Northeast Greenland. A group of very small individuals (6-8 mm in shell length) and another group of individuals around the maximum shell length (30-40 mm) formed the majority of the population. An absence of intermediate sizes was attributed to predation. Fukuyama and Oliver's study (1985)

also found a bimodal size distribution of small and large populations in bivalve mollusks in Norton Sound, Bering Sea, Alaska. It is suggested that small individuals in the populations were not worth eating from an energetic viewpoint and large individuals had the capability to escape predators by leaping in large *Serripes groenlanticus*, rapid burrowing into the sediment in large *Yoldia hyperborea*, and living deep in the sediment (>15 cm) in large *Mya truncata* and *Macoma calcarea*. Therefore, the middle size animal was intensively predated.

1.9 Evaluation of health status in *Tapes philippinarum*

1.9.1 Using immunological parameters as indicators of health status

A number of laboratory experiments and field trials have attempted to explore how *Tapes philippinarum* responds to a new environment; however the information is limited (Brooks, 1994; Matozzo *et al*., 2003; Flye-Sainte-Marie *et al*., 2009; Paillard *et al*., 2004; Soudant *et al*., 2004). Immune response is one of the most common indicators used as a biomarker to assess how environmental conditions may be affecting mollusc health. It enables us to have a better understanding of not only the animal's distribution in nature, but also its limits of tolerance to natural changes (Leinio and Lehtonen, 2005; Matozzo *et al*., 2003; Soudant *et al*., 2004).

The immune system protects against diseases by identifying and killing foreign material. It needs to be very sensitive to a variety of stressors both inside and outside the organism. Not only extrinsic factors (such as temperature, pollutants, and pathogens) but also intrinsic factors (i.e. physiological condition) can affect the structure and function of immune system. The direct effect of such factors is the alteration in the concentration and efficiency of components including cytokines, cytokine receptors and cells related with immune response. Generation of a general stress response is considered to be an indirect effect (Mydlarz *et al*., 2006). Internal defence in bivalve molluscs is mainly based upon a non-adaptive, non-specific, and innate immune system (Janeway, 1994).The self defence of bivalves is accomplished by haemocyte circulation in the open circulatory system of animals. Haemocytes can kill foreign materials through cellular (whole cell) and humoral (cell product) defences. The primary cellular responses in bivalves are phagocytosis and haemocytic infiltration, whilst the humoral defence mechanism is shown by either the presence or induction of a multitude of haemolymph factors for instance lysosomal enzymes, agglutinins, lectins and antimicrobial peptides. These enzymes and oxygen metabolites are released in order to destroy invasive pathogens (Gosling, 2003; Hine, 1999; Matozzo

et al., 2003; Sindermann, 1990; Tiscar and Mosca, 2004). Both defence mechanisams are widely used as biomarkers for evaluating the stressors in *T. philippinarum* (Table 4).

In *T. philippinarum*, those parameters are able to reflect a number of environmental stresses such as pathogen infection; *Vibrio tapetis* (Allam *et al.*, 2000; Lopez-Cortes *et al.*, 1999; Paul-Pont *et al.*, 2010), *Vibrio* P1 (Oubella *et al.*, 1993), parasite challenging; *Perkinsus olseni* (da Silva *et al.*, 2008; Hegaret *et al.*, 2007), *Himasthla elongate* (Paul Pont *et al,* 2010), harmful algal exposure (Hegaret *et al.*, 2011; Hegaret *et al.*, 2007), fishing impact (Matozzo *et al.*, 2003), rearing site (Soudant *et al.*, 2004), polluted site (Da Ros and Nesto, 2005), and seasonal variation (Matozzo *et al.*, 2003; Soudant *et al.*, 2004).

Table 4 List of various cellular and humoral defences used as biomarkers for stress assessment in *Tapes philippinarum*

Type of Defences	Authors
Cellular	
Total haemocyte count	Allam *et al*., 2000, Brooks, 1994, da Silva *et al*., 2008, Ford and Paillard, 2007, Hegaret *et al*., 2007, Matozzo *et al*., 2003, Soudant *et al*., 2004
Haematocyte size	da Silva *et al*., 2008, Hegaret *et al*., 2007, Paul Pont *et al,* 2010, Soudant *et al*., 2004
Cell complexity	da Silva *et al*., 2008, Hegaret *et al*., 2007
Haemocyte viability	Allam *et al*., 2000, da Silva *et al*., 2008, Hegaret *et al*., 2007, Paul Pont *et al,* 2010, Soudant *et al*., 2004
Apoptosis; Percentage of apoptotic haemocytes	da Silva *et al*., 2008
Locomotion rate of haemocytes	Brook 1994, Lopez-Cortes *et al*., 1999
Adhesion of the haemocytes	Hegaret *et al*., 2007, Paul Pont *et al,* 2010
Phagocytosis	da Silva *et al*., 2008, Hegaret *et al*., 2007, Lopez-Cortes *et al*., 1999, Matozzo *et al*., 2003, Paul Pont *et al*., 2010
Humoral	
lysozyme content	Matozzo *et al*., 2003; Soudant *et al*., 2004
Leucine amino peptidase	Allam *et al*., 2000; Ford and Paillard, 2007;
Superoxide dismutase (SOD) activities	Matozzo *et al*., 2003
Protein content	Allam *et al*., 2000; da Silva *et al*., 2008; Soudant *et al*., 2004
Agglutination titer	da Silva *et al*., 2008
Gelectin	Kim *et al*., 2008
Lectin	Bulgakov *et al*., 2004
Haemocyte Reactive Oxygen (ROS) production	da Silva *et al*., 2008, Hegaret *et al*., 2007, Paul Pont *et al,* 2010
Soluble protein	Ford and Paillard, 2007
DOPA (3, 4 Dihydrozyphenylalanine)	Ford and Paillard, 2007
Digestive epithelial thickness And lysosomal membrane stability	Da Ros and Nesto, 2005

1.9.2 Haemocyte

Haemocytes, the cells that circulate in the whole body of animals within the haemolymph, play an important role in the immune system. They also function in wound and shell repair, digestion and transport of nutrients. Haemocyte's classification in bivalves is controversial, but two broad categories are recognised; granulocytes (containing many cytoplasmatic granules (10-20 μm diameter)) and agranulocytes (hyalinocytes containing few or no granules (4-6 μm diameter)). Granulocytes are the majority cell type in haemolymph with higher phagocytic activity (Hine *et al*., 1999).

Haemocytes of *Tapes philippinarum* have been described as 2-4 different cell types. According to Matozzo *et al*. (2008), the haemolymph of this species contained two classes of haemocytes; small cells (2-3 μm in diameter)-hyalinocyte and large cells (6-10 μm in diameter)-granulocyte. Brooks (1994) proposed a scheme to identify and classify haemocytes into three groups. Firstly, a hyalinocyte is an agranulocyte which is small, dense, and spherical in shape (2-4 μm in diameter) producing hydrogen peroxide when it contacts with foreign material by 'bursts' of respiratory activity. Secondly, a small granulocyte is a spherical cell containing numerous dense granules with a diameter of 8-10 μm. Lastly, a large granulocyte (or amoebocyte) is irregularly shaped and motile often containing a small number of granules with a diameter of 10-15 μm. The large granulocyte is capable of producing pseudopodia 2 to 3 times bigger than this size and engulfing foreign particles.

Four cell types in haemolymph of *T. philippinarum* were reported as (A) granulocytes (48.05 $\pm$ 1.43 %), (B) hyalinocytes (32.18 $\pm$ 0.99 %), (C) haemoblasts (18.97 $\pm$ 0.63 %), and (D) serous cells (0.80 $\pm$ 0.19 %) (Cima *et al*., 2000). Granulocytes have an oval and eccentric nucleus with a diameter of 3-16 μm. It also contains a number of characteristic cytoplasm granules, approximately 0.5 μm in diameter. Hyalinocyte, the second major population, is normally smaller than a granulocyte. They are approximately 4-12 μm in diameter and had a round nucleus containing no granules appreciable under the light microscope. Both granulocyte and hyalinocyte appeared as either spherical cells (round) or amoebocyte (spreading). Haemoblasts circulating in haemolymph were spherical or oval in shape with a 3-5 μm in diameter with a nucleus surrounded by a thin layer of cytoplasm. The last population was serous cell which was non motile, 7-15 μm in diameter, and spherical in their shape. The cytoplasm was packed with large yellowish-brown pigment globules around 2 μm in diameter.

Changes in environmental factors such as temperature can influence the haemolymph of bivalves. This is because bivalves are both poikilothermic osmo- and thermo-conformers (Cheng, 1981; Shumway, 1977). Haemocyte measurement has demonstrated susceptibility to temperature variations in European flat oyster *Ostrea edulis* (Fisher *et al*., 1987; Hawkins *et al*., 1993; Hawkins and Hutchinson, 1992; Hauton *et al*., 1998), *Crassostrea virginica* (Fisher *et al*., 1989), *C. gigas* (Gagnaire *et al*., 2006), the Taiwan abalone *Haliotis diversicolor supertexta* (Cheng *et al*., 2004), Venus clam *Chamelea gallina* (Monari *et al*., 2007), Zhikong scallop *Chlamys farreri* (Chen *et al*., 2007), and Mussel *Mytilus galloprovincialis* (Nesto *et al*., 2006).

In *Tapes philippinarum*, cellular parameters increase according to the temperature (Paillard *et al*., 2004; Soudant *et al*., 2004). Paillard *et al*. (2004) recorded that the total haemocyte count of clams incubated at 21°C in a laboratory experiment for 30 days were significantly higher (one way ANOVA, $p < 0.05$) than the specimens held at 8°C (all samples at day 0, 3, 14, 24 and 30) and 14°C (only samples at day 14 and 30). An elevated metabolism at a higher temperature in clams could lead to the higher concentration of circulating haemocytes due either to a higher production rate of haemocyte at 21°C or mobilisation of haemocytes from tissue into the haemolymph (Paillard *et al*., 2004). In agreement with the field study of Soudant *et al*. (2004), seasonal variations showed distinctive effects on some immunological factors of *T. philippinarum*. In the case of total haemocyte count, they were high in spring-summer ($1.35 \pm 0.11 \times 10^6 / 0.91 \pm 0.06 \times 10^6$ cells ml^{-1}) and low in autumn-winter ($0.27 \pm 0.18 \times 10^6 / 0.42 \pm 0.04 \times 10^6$ cells ml^{-1}).

These results were in contrast to the study of Brooks (1994) that no effects of temperature on haemocytic indices were observed. *T. philippinarum* were kept at five different temperatures (5, 10, 15, 20, and 25°C) and haemolymph was then sampled for counting their number and measuring the diameter of large granulocytes, small granulocytes, and hyalinocytes. The results indicated that *T. philippinarum* from each of the temperature regimes showed no significant differences in total haemocyte and large granulocyte number. The number of small granulocytes, however, rose significantly between 5°C and 10°C and 5°C and 20°C ($p < 0.05$). The hyalinocyte number revealed a significant decrease between 5 and 25°C ($p < 0.05$), but no significant differences were observed between the other combination of temperatures. There were, furthermore, no significant differences over the range of temperatures tested in hyalinocyte and large granulocyte initial diameter. The locomotion rate of large granulocytes gave no statistically significant differences between the temperatures ($p < 0.05$). It has been assumed from the observation that the

temperature had very little significant effect for all these haemocyte measurements at high salinity tested (33).

In spite of the effect of temperature, several studies have revealed that the physiological status of the clams could also have an influence on haemocyte number. Matozzo *et al.* (2003) emphasised that total haemocyte count of *T. philippinarum* from the Venice lagoon was maximal in January 2001 and minimal in September 2000 as well as July 2001. This was probably the result of changing physiological condition, in particular the reproductive cycle, seemingly altering haemocyte number. Those periods showing minimal total cell count (September 2000 and July 2001) were coincident with post spawning, the Venice lagoon clams spawn in June and August. Similar patterns were found in other bivalves for instance *M. galloprovincialis* from the Venice lagoon (Pipe *et al.,* 1995) and from Ria de Vigo, Spain (Santarem *et al.,* 1994), *C. gigas* in a laboratory experiment (Delaporte *et al.,* 2006) and *C. virginica* from Apalachicola Bay, Florida (Fisher *et al.,* 1996). Suresh and Mohandas (1990) proposed that haemocytes probably mobilise towards gonads in order to remove cell debris remaining after spawning.

1.9.3 Phagocytosis

Phagocytosis is the cellular defence mechanism to eliminate foreign substances and dead cells. Granulocytes play the major role by engulfing foreign particles such as bacteria, algae, protozoan parasites and cell debris through a number of stages including chemotaxis, recognition, adhesion, ingestion, destruction and elimination of the particles (Hine, 1999; Song *et al*., 2010; Tiscar and Mosca, 2004). Chemotaxis, the first step of phagocytosis, which is poorly understood in bivalves, is the phenomenon in which haemocytes direct their movement through chemoattractant substances secreted by the target (Fawcett and Tripp, 1994). Haemocytes of many bivalves exhibit chemotactic and chemokinetic reactions, the type of which depends upon the nature of molecules presented. In the mussel, *Mytilus edulis*, the cell movement is stimulated by lipopoly saccharide (LPS) from both *Serratia marcescens* and *Escherichia coli* (Schneeweiss and Renwrantz, 1993). After *Bonamia ostreae* infects the European flat oyster *Ostrea edulis*, the migration of haemocytes from circulatory system to connective tissues is observed (Cochennec-Laureau *et al*., 2003). Peptides or small proteins secreted by both Gram-positive and Gram-negative bacteria attract haemocytes of *Mercenaria mercenaria* (Fawcett and Tripp, 1994). When a haemocyte contacts and recognises the foreign particle, adhesion between them takes place at cell surface receptors. A haemocyte ingests or engulfs the target by extending projections called pseudopodia and surrounding it (Sindermann, 1990). The destruction happens

when the target is taken into the cell and enclosed in a vesicle called a primary phagosome (Song *et al.,* 2010). A phagolysosome is then formed by merging of phagosome and lysosome. The fate of phagocytised particles varies. Digestible substances are degraded by lysosomal enzymes, reactive oxygen species (ROS), nitric oxide (NO) and antimicrobial factors within the phagolysosome. This releases nutrients to the bivalve which are considered as a supplementary source of nourishment (Chu, 2000; Tiscar and Mosca, 2004). The bivalve stores indigestible materials within haemocytes or they are physically removed through migration of haemocytes out of the body. Some pathogens are able to survive inside the phagolysosome. They resist killing, remain alive or multiply and eventually kill the host (Gosling, 2003; Song *et al*., 2010).

The majority of studies of phagocytosis in bivalves relate to the responses to pathogens. Infection by pathogens causes a decrease in phagocytic activities in various bivalves e.g. Pacific oyster *Crassostrea gigas* (Labreuche *et al.,* 2006), Palourde *Ruditapes decussatus* (Ordas *et al.,* 2000) and Taiwan abalone *Haliotis diversicolor supertexta* (Cheng *et al.,* 2004). Furthermore there may be a decrease in phagocytic activity when bivalves encounter unfavourable conditions such as an algal bloom, pollutants and 'beyond threshold' temperature. These factors might lead to increased susceptibility to disease which later results in bivalve mortality in either the short or long term. Take the case of an algal bloom, some algae act as an immunostimulant whereas others are immunosuppressive. Phagocytic activity, ROS, cell adhesion and living haemocyte number in *Mercenaria mercenaria*, *Mytilus arenaria*, *C. virginica*, *C. gigas* and *T. philippinarum* were activated by *Prorocentrum minimum,* but *Heterosigma akashiwo*, *Alexandrium fundyense* and *A. minutum* appeared to suppress the immunity of those species (Hegaret *et al*., 2011). However, some algal species can lessen the burden of parasitic infection of *R. philippinarum* heavily infected with *Perkinsus olseni*. The immunities of the clam were raised after exposure to the high concentration of alga *Karenia selliformis* (da Silva *et al.,* 2008). This was due to the direct effect of toxin from algae to the parasite (da Silva *et al.,* 2008). Phagocytosis is also inhibited or reduced by pollutants for example PAHs in *Mytilus edulis* (Grundy *et al.,* 1996) and Copper in *C. virginica* (Cheng, 1989).

Temperature, an important environment parameter, affects bivalve phagocytosis. Fisher and Tamplin (1988) revealed a positive correlation between temperature and rate of locomotion of *C. virginica* haemocytes. An increment of haemocyte locomotion at elevated temperature induced a number of haemocytes attacking to foreign particles which increased phagocytosis. The same pattern was observed in *M. edulis* (Parry and Pipe 2004). Percentage of phagocytosed haemocytes of *M. galloprovincialis* at 20°C

and 30°C were greater than at 10°C (Carballal *et al.,* 1997). It was in agreement with the results from *Mercenaria mercenaria* and *C. virginica* that phagocytosis were suppressed by low temperature (Alvarez *et al*., 1989; Chu and La Peyre, 1993; Fisher and Tamplin, 1988; Foley and Cheng, 1975; Tripp, 1992). A significantly positive correlation (G-test, $p < 0.001$) between phagocytosis of *Chamelea gallina* and temperature was reported by Ballarin *et al.* (2003). Temperature challenges at 10°C represented a stressful condition for the Floridian green mussel *Perna viridis* resulting in low phagocytic activity in comparison to those at 20°C and 30°C (Donaghy and Volety, 2011). So, generally, phagocytosis activity positively correlates with temperature, however this not always the case. Munari *et al.* (2007) considered functional responses of the haemocytes from *Chamelia gallina*. The phagocytic inhibition in animals kept at 30°C for 7 days were greater than those at 20°C and 25°C, it would seem that temperatures above or below a certain threshold result in haemocyte stress so that they are less active (Chu and La Peyre, 1993; Fisher *et al*., 1989; Fisher and Tamplin, 1988). Chen *et al.* (2007) noted that low phagocytic haemocytes and acid phosphatase production (ACP) were observed in *Chlamys farreri* transferred from 17°C to 23°C and 28°C for a period of 72 hours. However, no obvious negative effect was found in the scallops moved to 10°C (Chen *et al.,* 2007). Decreases of phagocytic index with increases of temperature were also reported in *C. virginica* (Hegaret *et al.,* 2003) and *Mactra veneriformis* (Yu *et al.,* 2009). *H. diversicolor supertexta* when transferred from 28°C to 32°C, showed a higher susceptibility (lower phagocytosis and phenoloxidase activity) to *Vibrio parahaemolyticus* than at 20°C, 24 °C and 28°C (Cheng *et al.,* 2004). According to Perrigault *et al.* (2011), the optimal temperature of phagocytic activity in *Mercenaria mercenaria* was revealed when a higher activity was seen at 21°C than at 13°C and 27°C.

Seasonal variation of phagocytosis in bivalves seems to have received limited attention. Ballarin *et al*. (2003) found that phagocytic activity of *Chamelea gallina* was higher in summer than winter. Nevertheless, there was no evidence of seasonality in phagocytosis of *T. philippinarum* (Flye-Sainte-Marie *et al*., 2009; Matozzo *et al*., 2003). Matozzo *et al*. (2003) reported that the phagocytic index of this species from the Venice Lagoon varied greatly throughout the year (September 2000-July 2001) and suggested that several factors (environmental parameters and physiological status of clam) may contribute to this variation, even if reasons are uncertain. Flye-Sainte-Marie *et al*. (2009) showed a similar result that a significant variation in phagocytosis of *T. philippinarum* from Gulf of Morbihan (France) was seen during the study period (July 2004- September 2005). However the measured environmental parameters (temperature, salinity and trophic resources) did not seem to influence phagocytosis.

Although, no observation of reproductive cycle impact on phagocytosis of *T. philippinarum* has been documented so far, a decline of phagocytosis during gonad development in particular spawning and post spawning period was observed in other bivalves such as *Crassostrea gigas* (Li *et al*., 2009a), *Placopecten magellanicus* (Pichaud *et al*., 2009), and *Mytilus* spp. (Lemaire , 2006). However, the phagocytosis of *Crassostrea gigas* examined during pre- and post-spawning showed no difference (Li *et al*., 2009b).

1.9.4 Digestive Tubule

The hepatopancreas or digestive gland is a molluscan organ producing digestive enzymes and absorbing digested food. It also accumulates toxins and actively participates in their detoxification and elimination (Marigomez *et al*., 2002). It has been used to detect early signs of health impairment. Digestive cells, particularly abundant in lysosomes, are a major component of the hepatopancreas and act as the main interface between the organism and the environment. These cells are highly susceptible to environmental damage (Nicholson, 2001) and changes under certain physiological and pathological conditions (Bitensky *et al.,* 1973). The various stresses such as low salinity, poor nutrition, parasitic infection and pollutants could change cell type composition of digestive epithelium such as volume density of basophilic cells and the structure of digestive gland i.e. reduction the epithelial thickness of digestive tubule and diverticular, as well as lysosomal membrane stability (Cajaraville *et al*., 1993; Couch, 1984; Da Ros *et al*., 2000; Garmendia *et al*., 2011; Lee *et al*., 2001; Lowe *et al*., 1981; Marigomez *et al*., 2002; Thompson *et al*., 1978; Usheva *et al*., 2006; Weinstein, 1997). To illustrate this, digestive epithelium cells are destabilised by overloaded toxic compounds through an enhancement of lysosomal enzymes in the cytoplasm and cellular autolysis leading to pathological alterations in the height of digestive cells (Bayne *et al*., 1988; Lowe *et al*., 1981; Marigomez *et al*., 1990). The degeneration firstly takes place in the individual cell or groups of cells. Such changes possibly bring about disfunction of organs or whole animal physiological processes. The cellular responses are therefore the rapid and sensitive indicators with which to diagnose the impact of environmental factors.

Normal digestive gland has large, finely vacuolated cells in the absorptive digestive gland epithelium, narrow to medium digestive tubular lumen, and high digestive epithelial thickness. Meanwhile, less finely vacuolated cells, enlarged lumen and digestive tubular cells smaller in height are categorised as digestive gland atrophy (Bricelj *et al.,* 2004). The digestive epithelial thickness has been used as a biomarker to evaluate the health status or impact of environment changes in several bivalves. The

tubular structure of digestive gland in mussel *Mytilus californianus* is changed by elevated temperature (Thompson *et al.,* 1978). Couch (1984) reported a decrease in epithelial thickness of almost 100% in the oyster *Crassostrea virginica* exposed to base-neutral, organic pollutant chemicals. Weinstein (1997) recorded that *C. virginica* in a fluoranthene-contaminated site revealed low mean epithelial thickness. In addition, a thinner mean thickness was reported in *C. virginica* with many helminth parasites than those with few or no parasites (Weinstein, 1997). Although no histopathological abnormality was detected at the cellular level in the hard clam *Mercinaria mercinaria* and mussel *Mytilus edulis* fed with toxic picoplankton *Aureococcus anophageffereus*, the height of their digestive epithelium was reduced in a manner similar to those in starvation (Bricelj *et al.,* 2004). Mussel *Mytilus galloprovincialis* in the polluted area in the Venice lagoon, Italy revealed a reduction in mean epithelial thickness (MET) and tubular dimension (Daros *et al.,* 2000). Marigomez *et al.* (2006) examined the digestive alveoli morphology of the mussel *Mytilus galloprovincialis* including mean luminal radius/mean epithelial thickness (MLR/MET). It was found that the MLR/MET of the mussel in a clean site at the Bay of Biscay, Spain was 0.7-1.2 μm/μm, whilst in the Prestige oil spill (POS) area, it was valued at >1.4 μm/μm (Marigomez *et al.*, 2006). Exposure to the POS also altered the cell type composition in digestive epithelium and structure of digestive tubule in the mussel (Marigomez *et al.*, 2006). Garmendia *et al.* (2011) also reported that the MLR/MET of *Mytilus galloprovincialis* before POS was between 0.7 μm/μm during spring-summer and 1.2 μm/μm in winter; nevertheless, it was raised to over 1.6 μm/μm after POS exposure. Daros and Nesto (2005) reported that mean epithelium thickness of *Tapes philippinarum* in the industrial area at the Venice lagoon was significantly lower than the control site. Conversely, *Mytilus galloprovincialis* in the industrial site expressed thicker epithelium than those in the clean area. The authors explained that the very high content of particulate organic matter in the industrial area might enhance the growth rate of the mussel. The negative effect of pollution was thus suppressed. According to Usheva *et al.* (2006), abnormally large light vacuoles were observed in the digestive cells of Gray's mussel *Crenomytilus grayanus* in polluted water at the southern entrance cape of Sivachya Bay, Japan. However there were no changes in cell structure of the epithelium.

1.10 Spring mortality in *Tapes philippinarum*

There was mortality (>50% of the total population in some years) of *T. philippinarum* during spring (Feb-May) in Poole Harbour (Wordsworth, pers. comm.). Grisley (2003) proposed that the mortality was possibly caused by the synergistic effects of water/mud temperatures (in Poole Harbour *T. philippinarum* is mostly intertidal), poor nutritional condition and reproductive stress. Grisley (2003) has integrated the measures of physiological and reproductive condition of *T. philippinarum* with environmental variables including temperature and food supply (Fig. 5). Fig. 5 shows that the percentage of total lipid of *T. philippinarum* in Ower Bay in winter and spring (approximately 5-6% of dry weight; DW) were lower than those in other seasons (about 7-8% of DW) (Grisley, 2003). This was due to the low levels of chl_a concentration in winter (below 1µg l^{-1}). The percentage of total lipid revealed the low energy reserve of the clams in winter and spring. In general, when spring is coming, temperature rising enhances metabolic rate and initiates sexual development which both requires a large amount of energy. An illustration of this is the study of Delgado and Camacho (2003), the gonadal growth accounted for 90% and 98% of the total increase in flesh dry weight (DW) of *T. decussatus* fed with the diet ration of 0.96% and 0.10% of DW, respectively. Both food rations gave the clams the positive energy balance. Fig. 5 also indicated that, in May 2001, more than 50% of ripe oocytes were seen in the clams from Ower Bay, Poole Harbour when day degree was increasing. However, chl_a concentration representing the food availability had remained low. The food availability in spring 2001 delayed until a peak of Chl_a was seen in June. Based on this fact, it can be said that the mortality of *T. philippinarum* in spring possibly occurred because of the negative energy balance. This was because the clams had low energy reserve and low energy input and the energy were demanded for increasing of metabolic rate and the onset of gametogenesis in spring.

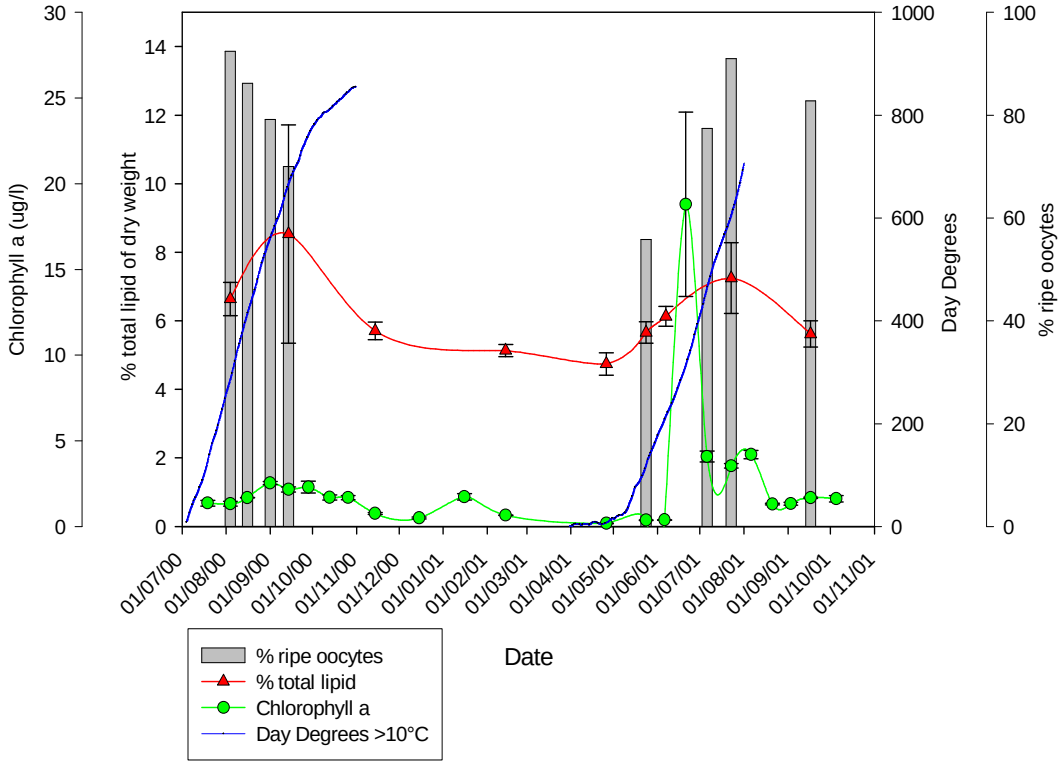

Figure 5 Food availability (chlorophyll a) in relation to percentage total lipid with respect to percentage ripe oocytes and increasing day degrees in Ower Bay, Poole Harbour for naturalised *Tapes philippinarum* (Mean±SD) (Grisley 2003).

Some studies stressed that the temperature rise in spring results in an elevated growth of disease pathogen and parasites (Baross and Liston, 1968; Baross and Liston, 1970; Flye-Sainte-Marie *et al.*, 2007; Mackin, 1962). Animals weakened by low winter energy input are more susceptible to a possible fatal infection in spring than other seasons. The *T. philippinarum* population along the French Atlantic Coast suffered from high mortality in late winter (February) which was attributed to unfavourable environmental conditions, for instance low food supply, cold temperature and low salinity (Bower, 1992). The association with Brown Ring Disease (BRD), a bacterial disease induced by the pathogen *Vibrio tapetis* when clams exhibit low condition index and biochemical reserves stress, was discovered later (Flye-Sainte-Marie *et al.*, 2007). The highest infection intensity (monthly infection prevalence 86%) of the protozoan *Perkinsus* in *T. philippinarum* from the east coast of Jeju, Korea was observed in spring (March 2002) (Ngo and Choi, 2004). Similarly, in other bivalves, summer mortality in *Crassostrea gigas* occurred when seawater temperature was rising (Beattie *et al.*, 1980; Lipp *et al.*, 1976). A direct correlation was observed between temperature and *Vibrio* spp. count in *C. gigas* from Puget Sound, Washington, USA (Baross and Liston, 1968; Baross and

Liston, 1970). Parasitism and mortality caused by *Perkinsus marinus* in *C. gigas* was attributed to high temperature (Mackin, 1962)

1.11 Bioenergetics in *Tapes philippinarum*

Bioenergetics is the quantification of energy flows through living systems (production and utilisation of energy). It can be characterized either by investigation on a single factor, such as feeding, assimilation or respiration, or by a holistic approach such as Scope for Growth (SFG) (Bayne *et al.,* 1988). Because SFG measures the overall physiological status, it is capable of reflecting deterioration in the environment before effects reveal themselves in the specific population or the community. Apart from such a prediction, environmental and pollution stress, growth, reproductive fitness and disease susceptibility can be indicated by net energy production in a form of SFG (Bayne *et al.,* 1988). Maximum positive values of SFG are found in optimum environmental conditions. A decrease to negative values is seen when severe stress occurs and affects the utilisation of stored energy. If SFG <0, it means that energy or matter is mobilized from energy stores in order to sustain basal maintenance. The first reserve target is gonad and then somatic tissue resulting in weight loss of animals. If SFG>0, it shows that energy or matter obtained is allocated for shell, soma and gonad. (Bayne and Newell, 1983; Bayne *et al*., 1988; Smaal and Widdows, 1994).

1.11.1 Effect of temperature

Physiological performances and SFG of bivalves are influenced by various intrinsic (such as temperature, salinity, trophic resource and pollutants) and extrinsic factors (such as size, age and gametogenesis) (Bayne *et al*., 1988). In general, SFG and physiological indices directly relates to temperature because mechanical activities are suppressed under low temperature conditions. Clearance rate, respiration rate, excretion rate and SFG increase with temperature. But no correlation between absorption efficiency and temperature is seen (Bayne *et al.,* 1988). According to Goulletquer *et al.* (1989), filtration and respiration rate of *T. philippinarum* expressed a non-linear relationship with temperature, with the maximal levels at 15°C and 20°C, respectively.

Brooks (1994) reported that the filtration, oxygen consumption and excretion rate as well as SFG of *T. philippinarum* conditioned at temperature of 5, 10, 15, 20, and 25°C showed a direct relationship with temperature up to an optimum temperature. Although there was no difference in the filtration rate between 5°C and 10°C, the rate

significantly increased in the clams at 15°C and 20°C (one way ANOVA, $p < 0.05$). A significant difference in the oxygen consumption was seen between the low temperatures (5°C and 10°C) and the higher temperatures (15°C, 20°C and 25°C) (one way ANOVA, $p < 0.05$). The differences in the excretion rate in all temperature comparisons were significant (one way ANOVA, $p < 0.05$) except between 10°C and 15°C and between 20°C and 25°C. The SFG between the low temperatures (5°C and 10°C) was significantly different from the higher temperatures (15°C, 20°C and 25°C) (one way ANOVA, $p < 0.05$). Furthermore, the SFG was maximal at 15 to 20°C. There was no significant influence of temperature on absorption efficiency. However, an increase of temperature above the optimum caused a decrease in those parameters. Significant decreases in the filtration rate, excretion rate and SFG were recorded at 25°C, a decline in the oxygen consumption was observed at 20°C without significant difference (one way ANOVA, $p > 0.05$). Brooks (1994) also explained that the differences of these parameters between 10°C and 15°C might lend further support to the possibility of discrete winter and summer physiologies.

A similar pattern was observed by Han *et al.* (2008) in small (151±12 mg DW) and large (353±16 mg DW) *T. philippinarum* conditioned at 5, 10, 15, 20, and 25°C. The filtration rate in the clams of both sizes significantly increased with temperature (one way ANOVA, $p < 0.001$). The filtration rate of the small and large clams were from 1.22±0.19 at 5°C to 5.06±0.25 l g^{-1} DW h^{-1} at 25°C and from 0.85±0.01 at 5°C to 3.58±0.21 l g^{-1} DW h^{-1} at 25°C, respectively. The significant increases in oxygen consumption as the temperatures rose were also seen in both small and large clams (one way ANOVA, $p < 0.001$) with the values ranging from 0.16±0.03 at 5°C to 0.73±0.07 ml O^2 g^{-1} h^{-1} at 25°C in the small clams and from 0.10±0.01 at 5°C to 0.57±0.05 l ml O^2 g^{-1} h^{-1} at 25°C in the large clams. Similarly, SFG increased with increasing temperatures 22.69 at 5°C to 79.40 cal g^{-1} DW h^{-1} at 20°C in the small clams and from 17.86 at 5°C to 64.50 cal g^{-1}DW h^{-1} at 20°C in the large clams. At 25°C, the SFG, nonetheless, decreased to 71.70 and 54.31 cal g^{-1} DW h^{-1} in the small and large clams, respectively. This was due to a reduction in assimilation efficiency and an increment in metabolic costs. No significant relationship was seen between assimilation efficiency in clams with temperature.

Laing and Child (1996) showed that the food consumption and respiration rate in juveniles of fed *T. philippinarum* were directly correlated to temperature. The food consumption increased with increasing temperatures from 0.19 µg algal food mg DW^{-1} day^{-1} at 3°C to 0.69 and 2.00 µg algal food mg $DW^{-1}day^{-1}$ at 6 and 9°C, respectively. This reaction was similar to that seen by the respiration rate which was lowest at 3°C with the value of 0.28 µg O_2 mg $DW^{-1}day^{-1}$ and higher at 6°C with the value of 0.42 µg O_2 mg

$DW^{-1}day^{-1}$. The highest respiration rate was 0.64 µg O_2 mg $DW^{-1}day^{-1}$ at 9°C (one way ANOVA, p =0.042). The ingestion rates of *T. philippinarum* kept at different temperatures included group L (temperature of 14 and 18°C) and H (18 and 22°C) were examined by Fernandez-Reiriz *et al.* (2007). The food ration of group L was lower than those in that of group H in order to minimize the variation in energy balances at the different temperatures. The ingestion rate of group H was 2.4 times higher than group L despite no intra-group difference being seen.

1.11.2 Effect of food availability

In marine ecosystems, the availability of food whether over short or long periods conditions the physiology of the animals (Albensosa *et al*., 2007) and influences the accumulation and use of energy reserves in the animals (Gabbott, 1976). Bayne *et al.* (1988) pointed out that SFG is commonly negative at low food concentrations, increasing as the food increases and becomes positive when the food concentration rises above the maintenance ration. However, very high food concentration reduces the SFG because of a lower clearance rate and assimilation efficiency. Beiras *et al*. (1994) indicated that the clearance rate of *Venerupis pullistra* seed was inhibited at the high food ration but the ingestion rate depended on food ration. A slight decline of assimilation efficiency was seen at the high food concentration. A routine level of the metabolic rate of the clam was noticed at medium and high food rations, but low food concentrations lowered the metabolic rate. Similarly, Sgro *et al.* (2005) recorded that the clearance rate and absorption efficiency of *T. philippinarum* and mussel *Musculista senhousia* increased when food concentration increased. Nevertheless, at increasing food concentration, the clearance rate of *M. senhousia* continuously declined and the absorption efficiency of both species remained stable. Laing and Child (1996) revealed that the respiration rates of unfed juvenile *T. philippinarum* and *T. decussatus* maintained at 9°C were lower than those in fed group; the rate being 56% and 48% of the rate for fed clams, respectively.

The metabolic rate declines to minimum values under nutritive restriction so that basal metabolism can be sustained when the clams encounters a prolonged absence of food (Bayne and Newell, 1983; Bayne, 1973; Gabbott and Bayne, 1973). Albensosa *et al*. (2007) reported that the respiration rate of starved *T. decussatus* and *Vernerupis pullistra* decreased 20% and 40-50% of initial rate, respectively after 15 days and remained basal for the rest of the experiment (84 days). Both bivalve species utilised their energy stores to maintain vital functions as seen in the reduction in the total energy content 27-30% and 45% of initial energy content in *T. decussatus* and *Vernerupis pullistra*, respectively after 84 days starvation.

1.11.3 Synergistic effect of temperature and food availability

A number of studies have described the single effect of either temperature or food supply on physiology and SFG of bivalves (Bayne *et al.*, 1988; Brooks, 1994; Han, *et al.*, 2008; Laing and Child, 1996; Sgro *et al.*, 2005), but literature describing the combined effect of temperature and food availability is limited. A concurrent impact of temperature and food supply on energy reserve and survival of the clam was explored by Laing and Child (1996). In laboratory experiments, small fed and unfed juveniles of *T. philippinarum* and *T. decussatus* responded differently when conditioned to temperatures of 3, 6 and 9°C. All juveniles at 9°C survived for 11 weeks though the unfed clams lost their biochemical reserves, weight and condition whilst fed populations accumulated biochemical reserves, weight and condition. The organic content of unfed *T. philippinarum* and *T. decussatus* at the end of the experiment (week 11) declined to 18.5% and 14.5% of the initial organic content, respectively. At 6°C, weight and condition decreases were seen in all *T. decussatus* and unfed *T. philippinarum*. The mortality of both fed and unfed *T. decussatus* appeared at week 11 (24 and 46%, respectively). Meanwhile, all fed *T. philippinarum* survived with slight positive increments of growth and condition. A severe impact was revealed in the clams conditioned to 3°C. The mortality of starved juveniles in both species was observed from week 3 onwards (up to 10% in week 3) and a marked increase was recorded after 7 weeks (>50% of all *T. decussatus* and unfed *T. philippinarum*). At week 7, fed *T. philippinarum* showed low mortality (18%). All *T. decussatus* and unfed *T. philippinarum* died by week 11, but 58% of fed *T. philippinarum* had survived. The survivors utilised reserves and weight and condition were lost in all juveniles.

Based on this observation, it can be said that the combined effect of temperature and food supply induces either a positive or negative energy balance which influences the survival of animals. Energy balance seems to be positive in high temperatures if food is present. This is because of a great energy input in spite of high energy expenditures caused by an increased metabolism. Distinct growth and energy storage are therefore noticeable. High temperature and poor food availability conversely leads to an increased energy cost to maintain metabolic rate and a low energy intake gained from food consumption. Either zero or negative energy balance is the consequence. At low temperature, a positive energy balance may be achieved if food is available, but an absence of food can cause a severely negative energy balance. Nevertheless, the survival of the animal in any situations depends on its energy reserves.

In any field-based study, the combined effect of temperature and food availability on the animals is more complex as a consequence of the variation of those factors. For

examples, metabolic rates in bivalves are temperature-dependent so it is expected that energy expenditure will be reduced in typical, winter seawater temperature of 2-8°C compared to summer temperatures which may exceed 20°C on the South coast of the UK. However, winter temperatures are not always the same and 'warm' and 'cold' winters could have different impacts on the energy reserves of the animals. Zarnoch and Schreibman (2008) recorded a 'warm' winter in 2001-2002 in Jamaica Bay, New York when the seawater temperature remained between 6-10°C until March except for a decrease below 5°C for 2-4 weeks. In contrast, the seawater temperatures in a 'cold' winters in 2002-2003 were below 5°C for at least 14 consecutive weeks (Zarnoch and Schreibman, 2008). The temperature of the mud at Ower Bay, Poole Harbour declined below 5°C for 28 days between December 2002 and March 2003; the lowest temperature recorded was 1.7°C during January 2003 for 5 days (Jensen, unpublished data). This is assumed to be 'cold' winter. A 'warm' winter was noticed in 2004-2005 when the same logger showed that temperature went below 5°C for less than 24 hours between 27th and 29th December 2004, dropping to a minimum of 3.8 on 27th December (Jensen, unpublished data).

Zarnoch and Schreibman (2008) revealed high survival (no mortality) in the juvenile of *Mercenaria mercenaria* from Jamaica Bay, New York in spring 2002 following a mild winter 2001 (high temperature + poor food = positive energy balance). Condition index (CI), shell length (SL), and tissue dry weight (DW) were not changed throughout winter, but increased in spring. Carbohydrate and lipid content increased in late winter (January-February) and spring (April). Average growth rates between November and June were 1.1 mm week^{-1} in SL and 14.8 mg week^{-1} in DW. A 'cold' winter conditions in 2004-2005 prior to spring (but with a prevalence of phytoplankton) revealed low mortality (low temperature + high food = positive energy balance). No differences in CI, SL and DW were recorded during winter, but increases in these parameters were observed in May and June. Protein and carbohydrate content fluctuated between November and December, but increased in spring (March-May), coinciding with spring phytoplankton bloom (peak up to 64 µg l^{-1}). Average growth rates were between 1.02 and 6.02 mm week^{-1} in SL and 6.75 and 8.98 mg week^{-1} in DW. Conversely, the majority of deaths (0.99% per day) was seen in spring after a 'cold' winter (2002-2003) which was coincident with low food supply; 0.9-1.6 µg l^{-1} from March to June (low temperature + poor food = negative energy balance). There were decreases in CI, SL and DW during winter without significant difference (ANOVA, $P > 0.05$) and increases in these parameters in late spring without significant difference (ANOVA, $P > 0.05$). Carbohydrate content significantly (Mann-Whitney, $p = 0.02$) declined in March and April (up to 63% of an initial value recorded in November). Average growth rates in shell length and tissue dry weight were 0.43 mm week^{-1} in SL and 2.1 mg week^{-1} in DW,

respectively. They also proposed another possible cause of apparent 'spring mortality' in juvenile *M. mercenaria* that a large amount of energy is allocated for growth regardless of negative energy balance. In fact, it is necessary for the small clams to grow to a larger size in order to avoid predation (Zarnoch and Schreibman, 2008).

1.11.4 Effect of reproductive development

Reproductive development has an important intrinsic influence on the physiology of bivalves. The energy requirement during reproductive cycle is usually high (Bruce, 1926). Bayne *et al.* (1988) recorded that when the mantle tissue contained mature gametes, the respiration rate of *Mytilus edulis* increased 33% of the rate at other times of the year. Furthermore, the mussel increased its clearance rate in order to compensate for the metabolic loss and maintained the SFG value. The seasonality in oxygen consumption of cockles was significantly associated with gametogenic index (Newell and Bayne, 1980). Smaal *et al.* (1997) reported that the maximum respiration rate of *Cerastoderma edule* was observed in May 1986 and 1987 which were coincident with the end of the reproductive period, but no correlation between the clearance rate and reproductive development was seen. The respiration rates of *Spisula subtruncata* in active gonad states were higher than the rates of those in non-active gonad states (Rueda and Smaal, 2004). Sgro *et al*. (2005) indicated that the SFG of mussel *Musculista senhousia* was high in July because the maturity of gonad demanded energetic support for the gonad development in summer. In addition, the low SFG of *T. philippinarum* was observed in July because the clams had completely released their gametes in June (Sgro *et al*., 2005).

Mann (1979) recorded the combined effect of temperature and reproductive development in adult *T. philippinarum* conditioned at 12, 15, 18 and 21°C for a period of 19 weeks. Ripen gonads were seen at all temperatures, but spawning occurred in the clams maintained at higher temperatures (15, 18 and 21°C). At 21°C, high mortality was evident at week 11 and 13 (40% and 28%, respectively) which coincided with spawning and a decline in dry meat weight. It is believed that energy demand to allow metabolic rate increase at high temperature and histolysis and histogenesis of tissues after spawning was very high. A large amount of energy is required to degrade the residual gametes in gonad at post-spawning and to form the new tissues. The clams eventually died due to the stress of extremely negative energy balance as the consequence (Mann, 1979).

Delgado and Camacho (2003) revealed the relationship between energy budget and gonad development in *Ruditapes decussatus*. Gonads could develop even under at

negative energy balance (daily food ration of 0.025% algal dry weight per clam live weight. The lowest food ration (0.025%) resulted in weight loss in the clams (20% of their DW) at day 70 of the experiment period. However, there was a direct relationship between food availability and the extent of gonadal development. The gonad of the clam having a positive energy balance (fed with 0.24%, 0.48% and 0.96% food ration) became mature with the gonad occupation index (GOI representing the degree of maturity) of 30-40% in females and 60-75% males by between day 12 and day 45. In contrast, the gonad of the clams having poor food (0.05% ration) and a negative energy balance showed a similar GOI at day 70. It is in agreement with the observation of Urrutia *et al*. (1999) that *Ruditapes decussatus* from Urdaibai Estuary, Spain put less effort into reproduction, so promoting survival by delaying their sexual maturity under pressure of a negative energy budget.

1.12 Southampton Water

Southampton Water, located on the south coast of England, is a partially mixed to well-mixed coastal plain estuary wide (Dyer, 1982; Dyer, 1997; Sylaios and Boxall, 1998) that is approximately 10 km long and 1.9-2.5 km wide. The maximum depth is 12-15 m in the central channel and 1-8 m deep elsewhere (Soulsby *et al*., 1985). The estuary is characterized by a double high tide. It is essentially marine in character, with slight or no variation in salinity near the mouth (~30) (Dyer, 1997). The head of the estuary shows salinity stratification, where salinity values between 11 and 32 can be found (Hirst, 1999; Muxagata *et al*., 2004). A seasonality in temperature was reported with the lowest in winter (<7°C) and the highest in summer (>17°C) (Howard *et al*., 1995). Due to the unique topography and tides, Southampton Water has been used as a port which is one of the most important and busiest in the UK. It is also world-renowned for various recreational water sports i.e. yachting. From an ecological aspect, a great number of water birds such as waders and wild fowl are supported by this estuary (Kershaw and Acornley, 2009).

In addition, Southampton Water has been the focus of shellfisheries including native oyster (*Ostrea edulis*) (now in decline throughout the Solent), American hardshelled clam (*Mercenaria mercenaria*) (peaked in the late 1970s and now no longer a commercial species) and Manila clam (*Tapes philippinarum*) (Jensen, pers. comm.) (active). The native oyster beds are along the eastern shore of Southampton Water, the entrance to the River Hamble and the Solent. Six local fishing boats are given licenses and the harvest is done by dredging between November and February (Kershaw and Acornley, 2009). The public oyster beds between Weston and Netley (the eastern shore of Southampton Water) produce only a poor quality catch which means they have less

importance for the commercial fishery. The minimum landing size for *Ostrea edulis* is 70 mm in shell length (Vanstaen and Palmer, 2009).

According to Mitchell (1974), *M. mercenaria* was introduced to Southampton Water in the 1930s. The fishery harvest peaked during the late 1970s and early 1980s, however overfishing coupled with poor recruitment greatly reduced the clam stock. The present distribution of *M. mercenaria* in Southampton Water is mainly along the western side. No closed season exists for the *M. mercenaria* in Southampton Water and the Solent, but the fishery is temporarily closed by SIFCA for population management purposes (Kershaw and Acornley, 2009). There is a prohibition to landing *M. mercenaria* less than 63 mm in total length (SIFCA, pers. comm.).

T. philippinarum appeared in Southampton Water and the Solent system in about 2005, possibly transferred by commercial fishing interests from Poole Harbour, where it became established and has been intensively fished. It is distributed from Dock Head to the BP Terminal at Hamble-le-Rice and it is currently mainly harvested at Deans Lake south of Hythe (Kershaw and Acornley, 2009). *T. philippinarum* are certainly found throughout the Itchen estuary (John Humphreys, pers. comm.). There are two existing regulations controlling exploitation in the area which are the EU minimum landing size regulation (35 mm shell length) and the district byelaw prohibiting boats from over 12 m fishing within the SIFCA district (SIFCA, pers. comm.).

Chapter 2 Materials and Methods

2.1 Study site: Bird Pile, Southampton

The study site (Bird Pile area), around a navigation marker, is situated on the western side of Southampton Water (coordinate 50°51'.72 N 001° 22'.33 W) (Fig. 6).

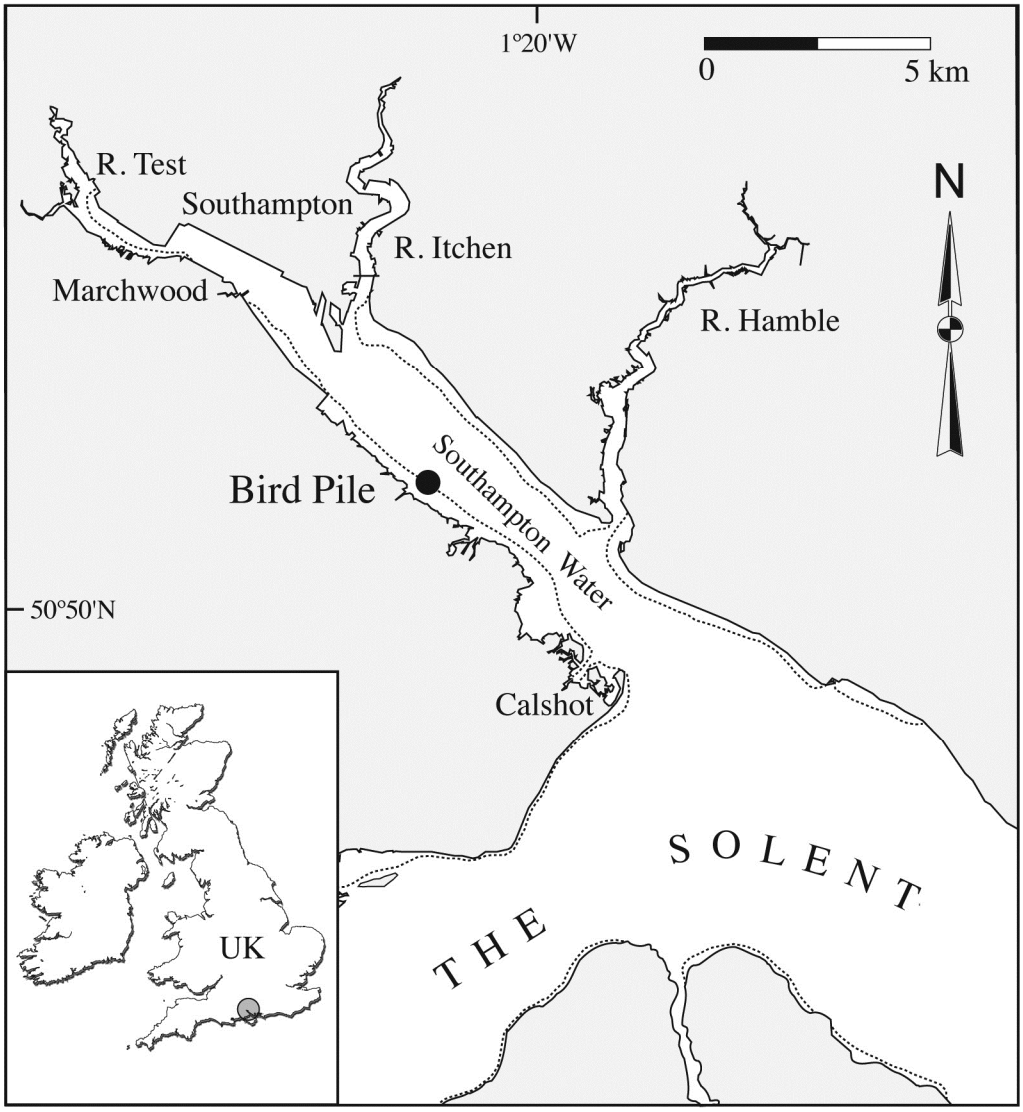

Figure 6 The location of the *Tapes philippinarum* sampling site (Bird Pile) in Southampton Water

2.2 Environmental parameters

The environmental parameters were examined on a monthly basis between February 2009 and January 2010. The ambient temperature and salinity were recorded at 1 metre above the sea bottom using a T-S Probe. Three replicates of seawater were sampled at the same depth using Niskin bottle for Chlorophyll_a (Chl_a) analysis by fluorometry (Parsons *et al.*, 1984). One hundred ml of seawater samples were immediately filtered onto 25 mm glass fibre filter (GF/F). Next the filters were folded over and wrapped in aluminum foil, with algal material inside, placed in a sealable plastic bag, labelled and stored at -80°C to prevent photodecomposition of the pigments until analysed. The Chl_a extraction can be done right away by sonication in 5 ml 90% acetone for 30 seconds followed by a 15 min extraction period. For frozen filters, total Chl_a was extracted by placing the filters in 5 ml 90% acetone, covered with Parafilm to reduce evaporation and left in a refrigerator overnight. The filters were then centrifuged at 3000 rpm for 10 min. The pigment extract was measured the absorbance using a 10-AU Fluorometer.

2.3 Population structure

Tapes philippinarum (approximately 100 individuals) were collected monthly by dredging using a commercially clam dredge (70x136x23 cm; mesh size 2x2 cm) from the area of Bird Pile, Southampton Water between December 2008 and May 2010. Total shell length to the nearest 0.1 mm of all specimens was measured using vernier callipers prior to categorising them into 9 different classes (5mm interval). The population structure histograms were then created based on the number of animals in each size class.

2.4 Growth rate

Eighty-five *T. philippinarum* (35.17$\pm$2.98 mm shell length) were collected in March 2009 from Bird Pile, Southampton Water using the same method described in section 2.3. Each individual was labeled and maintained inside a net bag suspending in the water column off the NOCS pontoon for annual growth monitoring between March 2009 and April 2010. The growth was determined monthly by biological measurements. Total length, height, and width of shell of the clams were measured using a vernier caliper with a precision to the nearest 0.1 mm. An electronic scale was

used to weigh the total live wet weight to the nearest 0.01 g. The monthly growth rate was then calculated as follows.

$$\text{Monthly cumulative growth rate} = (G_t - G_0 / G_0 \times 100\%)$$

Where G_t is the average growth (shell length, height, width or total weight) at month t.
G_0 is the average growth (shell length, height, width or total weight) at the initial

2.5 Gonad histology and oocyte measurement

2.5.1 Gonad examination

Tapes philippinarum was collected monthly from three sampling sites including Bird Pile, Southampton Water, Poole Harbour and Portsmouth Harbour to assess the stage of gonad development. Sampling periods were:

Bird Pile, Southampton Water: December 2008 - June 2010 (14 months)
Poole Harbour: March 2009 – July 2010 (11 months)
Portsmouth Harbour: May 2010 (1 month)

Each month, at least 10 individuals per size range were collected from Southampton Water using the same method described in section 2.3 in order to investigate the reproductive condition and the relationship between shell length and oocyte diameter. Size range (5 mm interval) was categorized by shell length of the clam. To compare the reproductive cycle with the specimens from Southampton Water, least 6 individuals of *T. philippinarum* were monthly sampled from Poole Harbour (43.7$\pm$8.3 mm shell length) and Portsmouth Harbour. All specimens were preserved in 4% formaldehyde. The soft tissue of each clam was then removed from the shell using a scalpel, then weighed (wet flesh weight), and longitudinally cut through the mid region of the body to provide transverse section containing some gonad, digestive gland, and muscular foot tissue. The tissue was placed into Bouin's fixative and left at least 24 hours. The specimens were subsequently dehydrated by immersing in a series of increasing concentrations of ethyl alcohol solutions (50%, 70%, 96% and dehydrated alcohol) overnight for each concentration. To clarify the tissues, they were then immersed in xylene and xylene with a few flakes of wax overnight each respectively.

The melted paraffin wax was poured out into the tissue, dabbed dry and placed in the oven at 60°C for 24 hours allowing wax to infiltrate to the whole tissue. The tissue was quickly transferred from melted wax with forceps and placed sectioned face down into

a square mould containing fresh melted wax. A backing cassette was then placed on top of the mould followed by the label. The wax was left to set before the block was turned out of the mould. Wax blocks were sectioned between 7-8 µm thickness using Cambridge microtome, floated out in a water bath (set at 45-47˚C), and positioned on a labelled glass slide. All slides were left to dry on a heated platform before staining. Staining was carried out by passing the slides through the following solutions and times: de-waxed in xylene (few seconds), dipped in a graded series of ethyl alcohol (96%, 70%, 50% - few seconds each), stained with haematoxylin (15 minutes), rinsed in tap water (few seconds), counterstained with 1% eosin (2-3 minutes), dehydrated in 96% ethyl alcohol (2-3 minutes) and clarified in xylene (2-3 minutes). The slides were left to dry, mounted with DPX and a cover slip applied.

The histology slides were microscopically observed to determine the gender and reproductive stage. The Chi-square test was used to analyse the sex ratio of *T. philippinarum* from Southampton Water and Poole Harbour against the null hypothesis of a 1:1 ratio. The stage of oogenesis (female: 7 stages) and spermatogenesis (male: 7 stages) in *T. philippinarum* was categorised using a modified version of the maturity scale described by Grisley (2003) and Xie and Burnell (1994) (Table 5 and Fig. 7-10)

Table 5 Description of reproductive stages for *Tapes philippinarum*

Female	
Inactive	Gonad predominantly composed of connective tissue. Sex is difficult to be distinguishable
Early active	Small acini are loosely scattered in the visceral mass of the molluscs. In this stage, oogonia and previtellogenic oocytes are round with a maximum diameter of 25 μm, and are attached to the acini walls. Ovarian acini are filled with follicle cells.
Developing	Acini walls are well defined and the interfollicular connective tissue has decreased. Oocytes enter the vitellogenic phase.
Late active	Oocytes continue to grow and attain a polyhedral shape ranging from 25-60 μm in diameter. Some oocytes appear free in the lumen and others remain attached to the acini walls.
Mature	This stage marks the end of the vitellogenic process. The nucleus is round and occupies a large area in the middle of the oocyte. Post-vitellogenic oocytes measure a maximum of 70 μm along the major axis, and maintain their polyhedral shape because of oocyte crowding. Interfollicular and inter-oocyte spaces are minimal. Oocytes appear free in the lumen of the acinus.
Spawning	Acini appear full of mature post-vitellogenic oocytes with a broken germinal vesicle.
Spent	The acini are collapsed as a result of oocyte evacuation. A few unspent oocytes with the germinal vesicle intact remain in the acini in the process of degradation. Haemocytes proliferate during this stage, contributing to gonad repair.
Male	
Inactive	Gonad predominantly composed of connective tissue. Sex is difficult to be distinguishable.
Early active	The germinal epithelium produces spherical spermatogonia with a size of 3 μm in diameter after a centripetal evolution from the internal wall to the lumen. Spermatogonia are located along the internal wall of the acini in bands of several cells. Some spermatocytes are present in the lumen.
Developing	Acini begin to show stratification and all developmental stages are present including spermatogonia, abundant spermatocytes in the following layer, spermatids, and scarce spermatozoa located towards the acinus lumen. Cell diameter decreases from 3 to 0.5 μm.
Late active	Spermatogenesis takes place in the whole area of the acini. The spermatogonia layer becomes thinner. The numbers of spermatozoa increase and their tails are directed towards the lumen.
Mature	Acini appear full of spermatozoa with their tails towards the acini lumen. Merging of some acini is evident.
Spawning	Acini become elongated and the boundaries between acini are not easily distinguished. Evacuating ducts are evident from which a great number of spermatozoa is spent.
Spent	Numerous empty spaces towards acini lumen are evident because of the release of spermatozoa during spawning. If the spawning is not complete, some acini are empty but others remain full of ripe sperm. The empty acini show a great number of haemocytes. No signs of active spermatogenesis are observed in any regions of the gonad.
Indifferent	Gonad is not distinguishable or sex is undifferentiated.

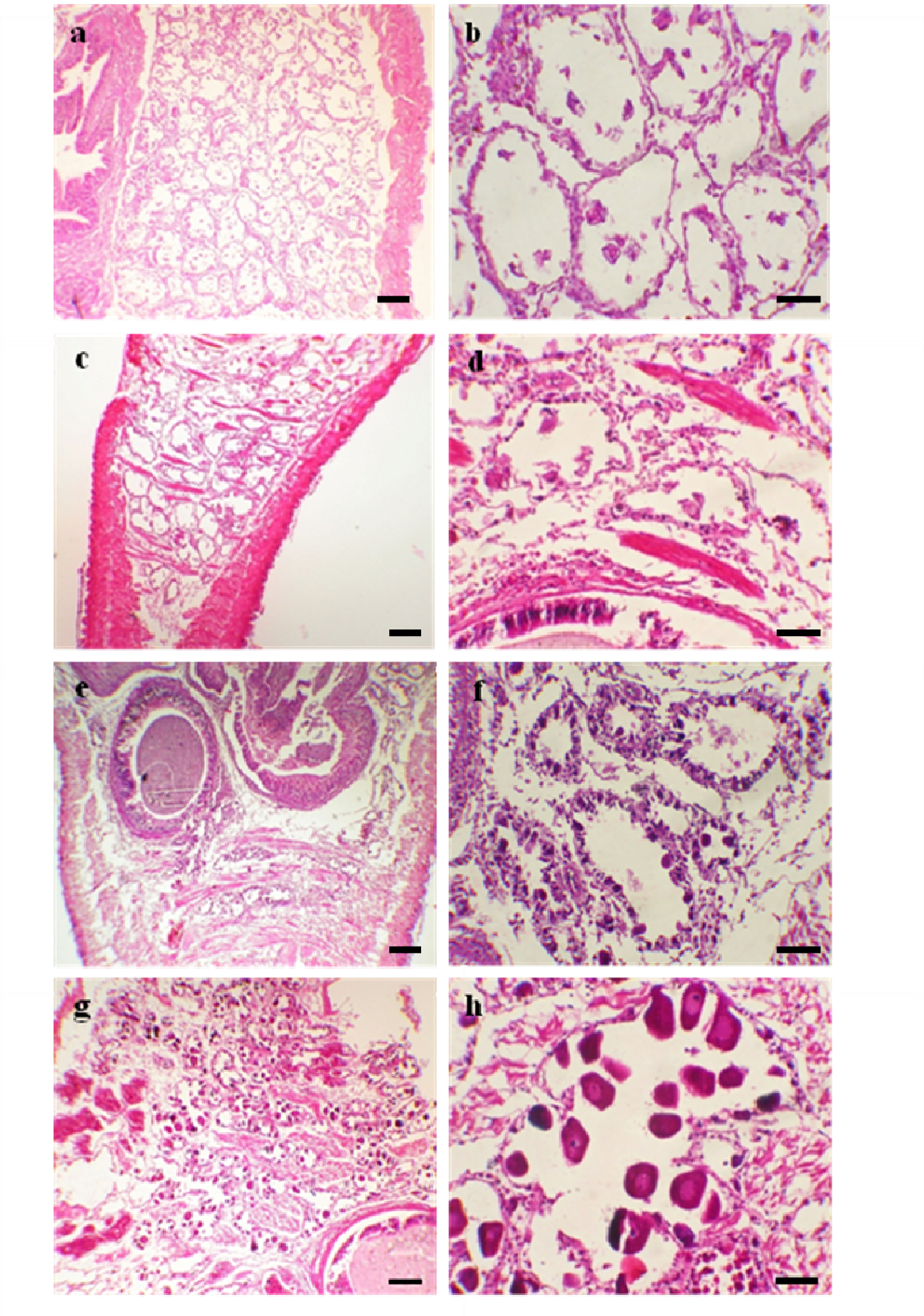

Figure 7 Female reproductive stages in *Tapes philippinarum* (Inactive-Developing), a-b: Inactive stage, c-d: Early active stage, e-f: Developing stage, g-h: Late active stage. Scale bar in a, c, e, g: 200 μm; in b, d, f, h: 50 μm.

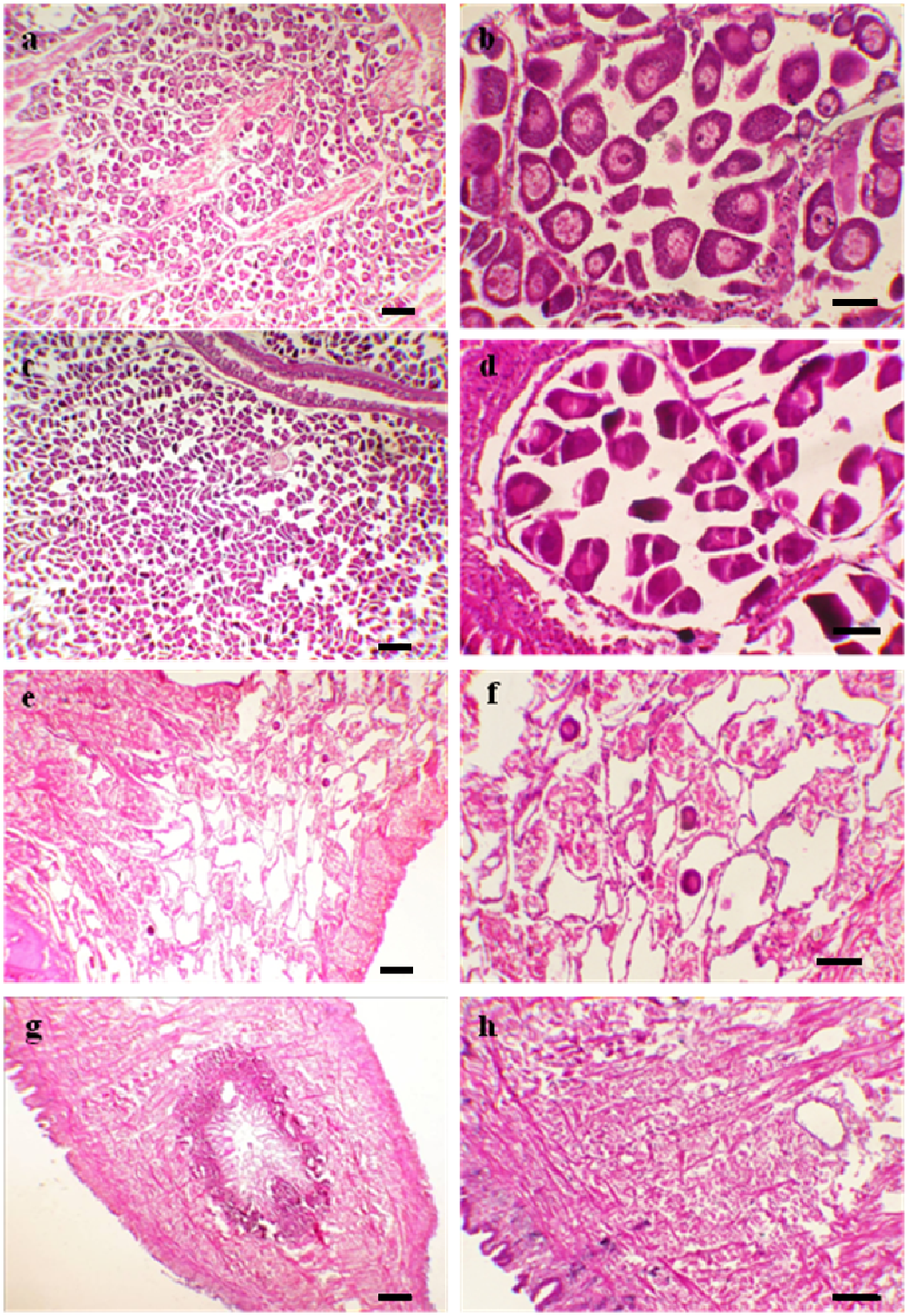

Figure 8 Female reproductive stages (Mature-Spent; a-f) and indifferent stage (g-h) in *Tapes philippinarum*. a-b: Mature stage, c-d: Spawning stage, e-f: Spent stage. Scale bar in a, c, e, g: 200 μm; in b, d: 50 μm; f, h: 100 μm.

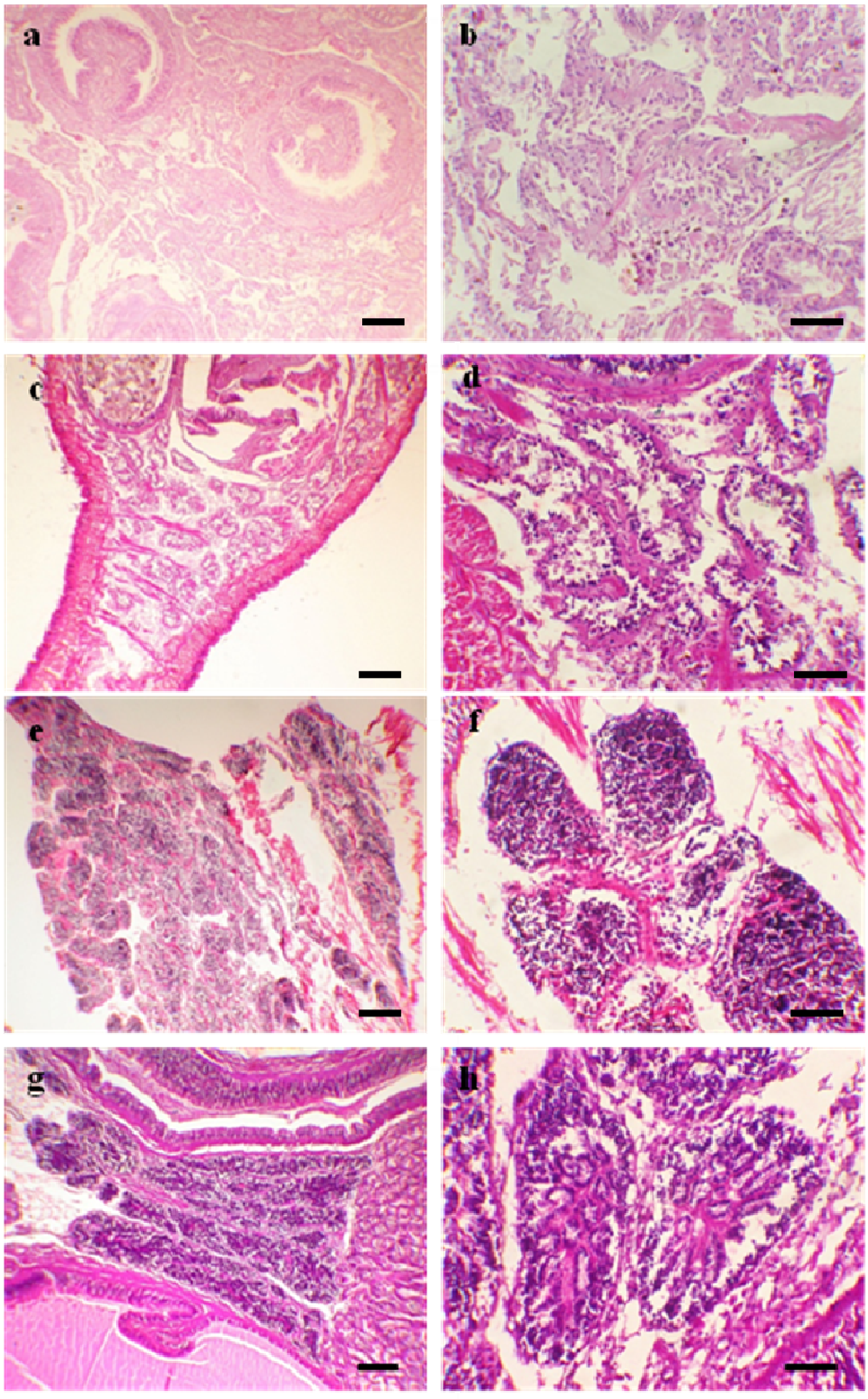

Figure 9 Male reproductive stages in *Tapes philippinarum* (Inactive-Developing), a-b: Inactive stage, c-d: Early active stage, e-f: Developing stage, g-h: Late active stage. Scale bar in a, c, e, g: 200 μm; in b, d, f, h: 50 μm.

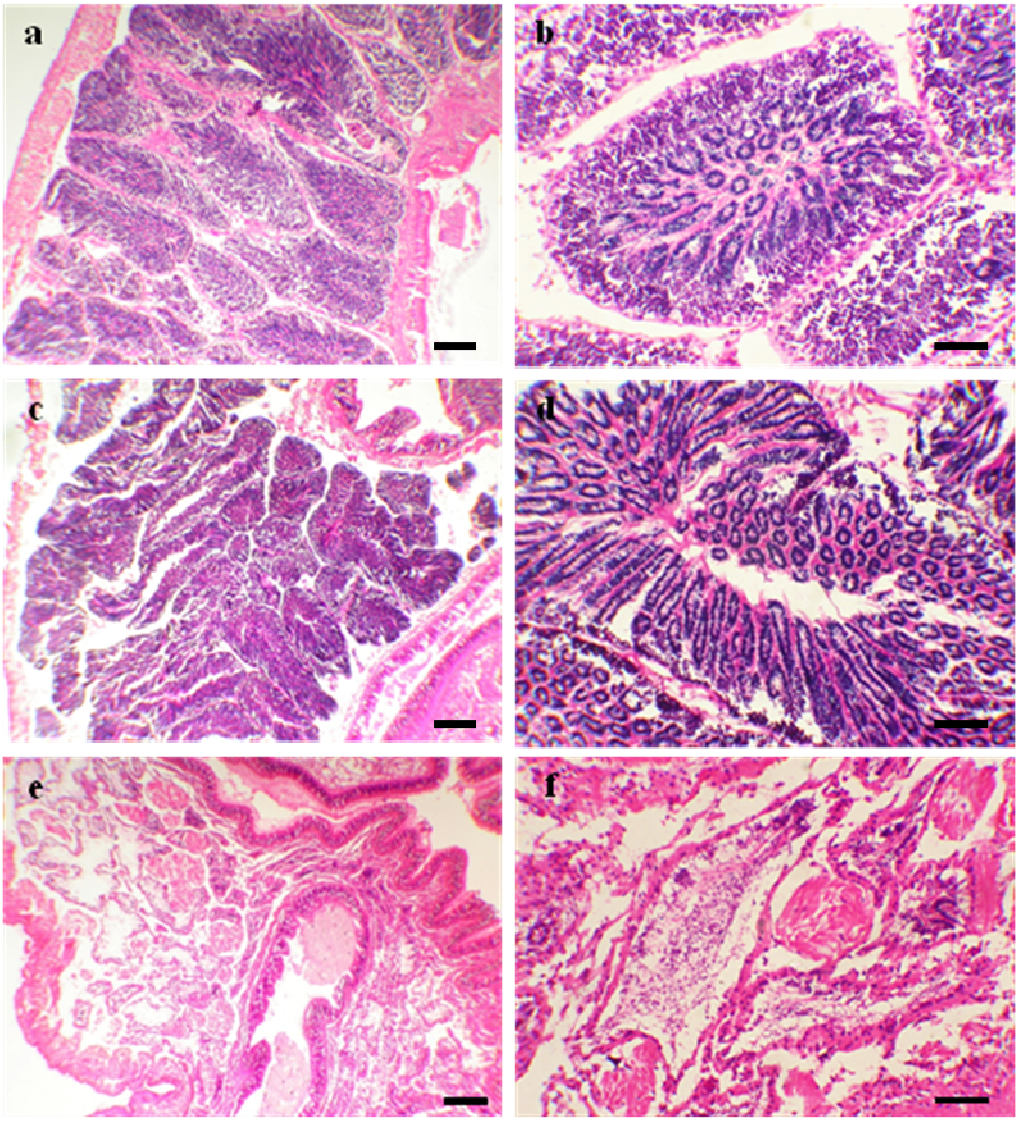

Figure 10 Male reproductive stages in *Tapes philippinarum* (Mature-Spent), a-b: Mature stage, c-d: Spawning stage, e-f: Spent stage. Scale bar in a, c, e: 200 μm; in b, d, f: 50 μm.

Maturity index (MI) was calculated by grouping both male and female gonads into different stages. The MI will reveal quantitative results and is defined as:

$$MI = \Sigma \text{ (\% of each sexual state} \times \text{maturity factor)}/100$$

and was calculated with arbitrary maturity factors: 0 = Inactive; 1 = Early Active; 2 = Developing; 3 = Late Active; 4 = Mature; 5 = Spawning; 6 = Spent (Etchian *et al.*, 2004).

2.5.2 Oocyte measurement

The oocyte diameter of *T. philippinarum* was examined in all females having plenty mature oocytes (over 100 oocytes per individual). The specimens from Southampton Water were assessed during spawning season from May to August 2009 and May to June 2010. The oocyte measurement from Poole Harbour samples was conducted from June to September 2009. The image analysis technique was applied to measure the maximum diameter of the oocytes. The prepared slides were examined under light microscope and images of oocytes were taken at x10, x20 and x40 magnifications. Images of a 1 mm graticule were also taken at magnifications of x10, x20 and x40 for calibration purposes. Over 100 mature oocytes chosen in random per female were measured the size using the Jandel Scientific SigmaScan Pro4 software package. Only those oocytes exhibiting clearly visible nucleoli were selected for a measurement to ensure that the value obtained was closest to the actual oocyte diameter. The silhouettes of particular oocytes were traced using a mouse pen and drawing tablet. The ferret diameter measurement from each oocyte (i.e. the estimated spherical diameter of oocyte) was calibrated to the correct magnification and transferred onto a SigmaScan spreadsheet. Multiple regression analysis was applied to determine the effect of shell length, of month and of year on the oocyte diameter of *T. philippinarum* from Southampton Water. The software Minitab 16 was used for data analysis and significance was set at $p<0.05$ in all cases.

2.6 Immunological parameters

The health status of *Tapes philippinarum* was examined and quantified in the form of immunological parameters including haemocyte numbers, phagocytosis and digestive epithelial thickness. Eight replicates of live clams (41.8$\pm$5.7 mm shell length) were collected from Bird Pile, Southampton Water using the same method described in section 2.3 between June 2009 and September 2010 for haemocyte count and phagocytosis measurement.

2.6.1 Haemocytes

Haemolymph was withdrawn from clam posterior adductor muscle sinus using a Pasteur pipette. The number of total haemocytes, granulocytes, and hyalinocytes (agranulocytes) were counted using a Neubauer haemocytometer under a light microscope.

2.6.2 Phagocytosis

Cellular defence abilities of *T. philippinarum* were quantified by phagocytic activity and capacity using yeast cells (*Saccharomyces cerevisiae*) as foreign material. A known volume of haemolymph was diluted with an equal volume of 0.45 µm filtered sea water. A 20 µl of yeast suspension (0.025 g of yeast in 50 ml filtered sea water) was dropped on a microscope slide and an equal volume of haemolymph was then added. The slide was incubated in a culture chamber at the ambient temperature for 1 hour. The number of granulocytes containing ingested yeast particles and the number of yeast cells engulfed by each granulocyte per slide were counted under a light microscope. Phagocytic activity and capacity can be calculated using the following equations.

$$\text{Phagocytic Activity} = \frac{\text{Total number of granulocytes containing ingested yeast cells}}{\text{Total number of granulocytes per treatment}}$$

$$\text{Phagocytic Capacity} = \frac{\text{Total number of yeast cells engulfed by granulocytes}}{\text{Total number of granulocytes containing ingested yeast cells}}.$$

2.6.3 Digestive tubule

To investigate the digestive gland, animals (37.5±7.5 mm shell length) were sampled at the same sampling site using the same method described in section 2.3 between June 2009 and June 2010. Specimens were dissected through the entire middle part of their digestive gland and fixed in Bouin's fixative. The same standard histological technique as used in the reproduction study was used to prepare the histological slides. The digestive gland tubules of ten individuals (50 tubules/organism) from each sample collection were individually examined and quantified using the image analysis technique (Jandel Scientific Sigma Scan Pro 4 software package). The thickness of the epithelium of each tubule was determined by subtracting the lumen diameter from the alveoli radius and dividing the remainder by 2 (2 cells on either side of the alveoli). The lumen radius of each digestive tubule was measured. Mean luminal radius (MLR) and mean digestive epithelial thickness (MET) were measured and the MLR /MET ratio was calculated as an integrative measure of changes in the tubular morphology.

2.6.4 Statistical analyses

One-way analysis of variance (ANOVA) was used to determine the seasonal changes in haemocyte number, phagocytosis and digestive tubule structure. Comparison of mean

was subsequently carried out by post hoc Turkey test. The software Minitab 16 was applied for data analysis and significance was set at $p<0.05$ in all cases.

2.7 Bivalve larvae abundance

Bivalve larvae from Bird Pile, Southampton Water were monthly sampled using a conventional cod end plankton net of 120 µm mesh size between April 2009 and January 2010. Three replicates were towed approximately one metre above sea bottom for 5 min each. The volume of each sampling was shown in Appendix 1. The samples were preserved in 4% formalin seawater buffered with borax. Next the larvae were identified using morphological characteristics for examples general shapes and prominence of the umbones described in taxonomic keys (Chanley and Andrews, 1971; Loosanoff *et al.*, 1966)

2.8 Spring mortality laboratory experiment

Grisley (2003) proposed that the mortality of *Tapes philippinarum* during spring (February-May) in Poole Harbour possibly related to the nutritive stress as a result of the gonad started to develop prior to the onset of the spring phytoplankton bloom (see more detail in chapter 1 section 1.10). Hence, this laboratory experiment attempts to scrutinize how *T. philippinarum* allocates their energy budget under the pressure of low food availability in winter at different temperatures during three months. Physiological responses including scope for growth and reproductive development, were examined at different temperatures (5°C and 10°C) for 12 weeks. The temperature of 5°C represents a 'warm' winter temperature. 10°C is the minimum temperature initiating gametogenesis in *T. philippinarum* (Millican and Williams, 1985).

2.8.1 Collection and maintenance of animals

Mature *T. philippinarum* (35.5±5.4 mm shell length) were collected from Southampton Water using the same method described in section 2.3 in October 2011, the post spawning period. All animals were cleaned and their lengths and total wet weight were measured. They were divided into different groups of 12 individuals, labeled and then maintained in aquarium tanks without substrate under controlled temperature conditions. Animals were acclimated at an initial seawater temperature of 12°C (temperature at the sampling site) and salinity of 33. The temperature was gradually decreased at a rate of 1-2°C day^{-1} to obtain experimental temperatures (5 and 10°C).

These temperatures were constantly held (± 1.5°C) for the entire period of experiment (12 weeks) from the day of attaining the conditioned temperatures.

Seawater in the tanks was continuously aerated and changed. The animals were fed with a mixed phytoplankton culture of *Isochrysis galbana, Phaeodactylum tricornutum, Chaetoceros ceratosporum, Tetraselmis suecica* and *Pavlova lutheri.* A mixed algal diet was applied so as to more closely resemble a natural seston (Bayne *et al.*, 1987; Doering and Oviatt, 1986). No preferential uptake of algal species was assumed as it is known that a number of bivalve species showed no size selection of particles up to 120 µm (Foster-Smith, 1975; Yonge, 1926). The daily food ration was 0.0025% algal dry weight per clam live weight which led to a zero energy balance and also matched the Chl_a concentration during winter in Southampton Water.

For each group, clearance rate, food absorption efficiency and respiration rate were measured following the procedures reported by Bayne *et al.* (1988). At the end of week 1, 4, 8 and 12, twelve animals were selected and the physiological responses and total weight were measured for each temperature. Cumulative growth rate was calculated using the following equation.

$$\text{Cumulative growth rate (\%)} = (W_t - W_0 / W_0 \times 100\%)$$

where W_t is the total weight of clams at week t.

W_0 is total weight of clams at the beginning of the study.

After the physiological measurement, the animals were sacrificed to quantify the degree of gonad development. In February 2012, twelve *T. philippinarum* were also dredged from Southampton Water in order to compare their physiological responses with the animals from the laboratory experiment. The specimens from Southampton Water had been experiencing a 'warm/mild' winter 2011-2012. The investigations of clearance rate, absorption efficiency and respiration rate were conducted at 5°C which was similar to the seawater temperature when sampled. The animals were then sacrificed for gonad analysis.

2.8.2 Clearance rate

Clearance rate (CR), the volume of water cleared of suspended particles per hour, was determined. Twelve animals were placed in a separated chamber containing 400 ml of filtered seawater positioned on magnetic stirrer base plates to keep the water homogeneous and oxygenated. A chamber without clams was employed as a control. After the clams were left in the chambers for a period of 20 min to 4 hours allowing them to open and resume pumping, algae cells (*Phaeodactylum tricornutum*) were

added to each container giving an initial concentration of 25,000 cells ml^{-1} in the water. Twenty ml aliquots were sampled from each chamber after 1 hour. Next, the samples were preserved with 2% lugol fixative and kept in the dark. The decline in cell concentration was monitored using sedimentation chamber. The algae were concentrated by leaving to settle at least 24 hours in the chamber before counting. The cell density was then counted under a microscope. The individual CR was standardized to an equivalent of 1 g dry weight of animal using the conversion factor of *T. philippinarum* from Southampton Water. The CR was then calculated using the following equation (Coughlan, 1969).

$$CR\ (l\ h^{-1}) = V \times (\ln C_1 - \ln C_2)/t$$

where C_1 is the initial algal concentration

C_2 is the final algal concentration

V is the volume of seawater in the chamber

t is the time between C_1 and C_2

2.8.3 Food absorption efficiency

Absorption Efficiency (AE) was measured by comparing the proportion of organic matter in the food algal cells and the clam faeces (Conover, 1966). Each bivalve was placed in a separate beaker containing 500 ml of filtered seawater such that faeces produced by each individual clam could be identified. The algae (*Chaetoceros ceratosporum*) were dosed into each container at the density of 25,000 cells ml^{-1}. Any faeces egested by each individual were collected after 24 hours using a Pasteur pipette and deposited on pre-ashed (450°C for 4 hours) and pre-weighed GF/C filters. A control without an animal was also filtered onto pre-ashed and pre-weighed GF/C. The filters of algal food and faecal samples were washed out with 3x10 ml of deionised water in order to remove the salt content. The filters were dried at 70-80°C overnight prior to weighing and subsequent ashing in a muffle furnace at 450°C for 4 hours. The ashes were reweighed to obtain ash-free dry weights. Finally, AE was calculated by the following equation (Conover, 1966).

$$AE\ (\%) = (F-E) / [(1-E) \times F] \times 100$$

where F is the ratio ash-free dry weight / dry weight of food

E is the ratio ash-free dry weight / dry weight of faeces

2.8.4 Respiration rate

Respiration rate (RR) was measured by placing individual animals in a closed chamber containing air-saturated filtered seawater which was thoroughly mixed using a magnetic stirrer bar beneath a perforated glass plate supporting the clam. The chamber was sealed without air and the decline in O_2 concentration was measured using YSI ProODO optical dissolved oxygen instrument at 40 min intervals for an experimental period of 120 min. Respiration time was determined from the time when their valves opened in each animal. A closed chamber without a clam was used as a control. The overall RR was calculated as the mean of the three consecutive measurements (0-40, 40-80 and 80-120 min). Each individual RR was standardized to an equivalent of 1 g dry weight of the animal using the conversion factor of *T. philippinarum* from Southampton Water. The O_2 consumption was calculated using the following equation.

$$RR\ (mg\ O_2\ l^{-1}\ h^{-1}) = ([O_2]t_0 - ([O_2]t_1) \times (Vchamber - Vanimal) / (t_1 - t_0)$$

where $[O_2]$ is the oxygen concentration

t_0 is the initial time

t_1 is the final time

V is the volume of chamber and animal

2.8.5 Scope for Growth

Each physiological rate was then converted to energy equivalent ($J\ h^{-1}\ g^{-1}$) in order to calculate energy budget and scope for growth (SFG). SFG represents the difference between the energy absorbed from the food (food consumption x absorption efficiency) and energy loss via metabolic energy expenditure (O_2 consumption). In order to calculate SFG, total energy consumed, total absorbed energy and energy respired are required. Equation for calculating those parameters are below (Smaal and Widdows, 1994):

$$P\ (J\ h^{-1}) = A - R$$

where P is scope for growth ($J\ h^{-1}$)

A is total absorbed energy

R is energy respired

Total absorbed energy

$$A = C\ (J\ l^{-1}) \times AE$$

where A is total absorbed energy

C is total nergy consumed

AE is food absorption efficiency

Total energy consumed

$$C = CR\ (l\ h^{-1}) \times \text{particle concentration} \times \text{energy content of algae}\ (J\ l^{-1})$$

where C is total enery consumed

CR is clearance rate

NB Algal cell numbers were converted from cells l^{-1} to mg l^{-1} and then to J l^{-1}. The energy of *Chaetoceros ceratosporum* is assumed to be 14.247 J mg^{-1} dry weight (Gonzalez *et al*., 2002; Velasco, 2007).

Energy respired

$$R = V_{O2}\ (mg\ O_2\ l^{-1}\ h^{-1}) \times 13.98$$

Where R is energy respired

V_{O2} is oxygen consumption (O_2 was 13.98 J mg O_2^{-1})

2.8.6 Statistical analyses

The effect of temperature, time and their possible interaction on CR, AE, RR, SFG, and cumulative growth rate were determined using two-way ANOVA. Percentage of cumulative growth rate was transformed by angular transformation (arcsine square root percentage) prior to analysis using two-way ANOVA. One-way ANOVA was performed to determine the difference of CR, AE, RR and SFG among two different temperatures and wild population at week 12. The post hoc Turkey test was applied for mean comparison. The data analysis was conducted using the software Minitab 16 and significance was set at $p<0.05$ in all cases.

Chapter 3 Results

3.1 Environmental parameters

Sea water temperatures recorded from February 2009 to January 2010 at Bird Pile, Southampton Water varied between 4.7 and 19.7°C (Fig. 11). The seasonal fluctuation showed the highest temperature in summer (August 2009) and the lowest value in winter (February 2009). Moreover the variation of salinity from February 2009 to January 2010 was between 29.64 and 35.20 (Fig. 11). The salinities were high and relatively constant throughout the entire sampling period. In addition to temperature and salinity, Chlorophyll a concentrations examined from April 2009 to January 2010 varied between 0.32±0.06 (January 2010) and 7.96±0.76 µg l^{-1} (April 2009) (Fig. 11). A spring peak of Chl_a was seen in April 2009 followed by intermediate summer and low winter values.

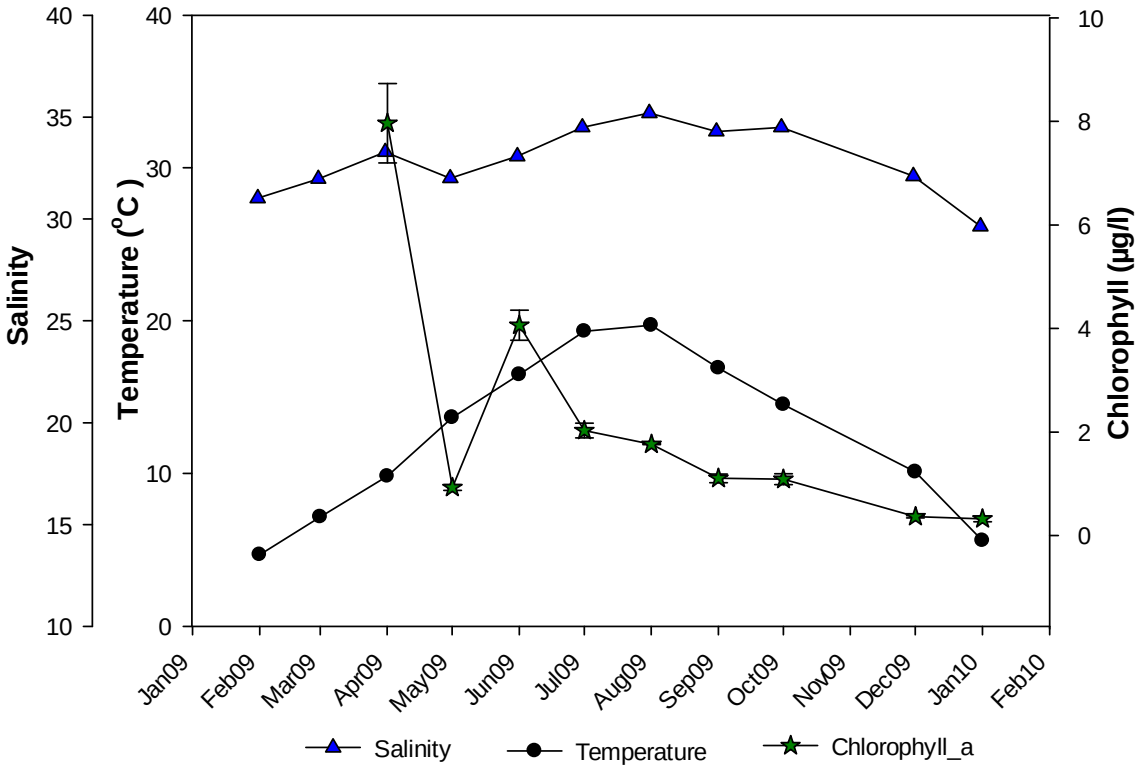

Figure 11 Seasonal variation of seawater temperature, salinity and Chlorophyll_a concentrations at Bird Pile, Southampton Water from February 2009 to January 2010 (Mean Chl_a±SD, n=3 at each point).

3.2 Population structure

The population structure of *Tapes philippinarum* in Southampton Water was examined over a 13 month period between December 2008 and May 2010. A total of 1156 individuals were collected and then categorized into 9 different size classes (5 mm intervals) (Fig. 12). The shell length of animals ranged between 10.3 and 50.1 mm with an average shell length of 31.47$\pm$8.56 mm. The size classes 30-35 and 35-40 mm were numerically dominant 25.09% and 27.61% of the samples, respectively. Only 11.67% of the samples were larger than 40 mm. An increase in proportion of small clams (<25 mm) was evident in August, October and December 2009.

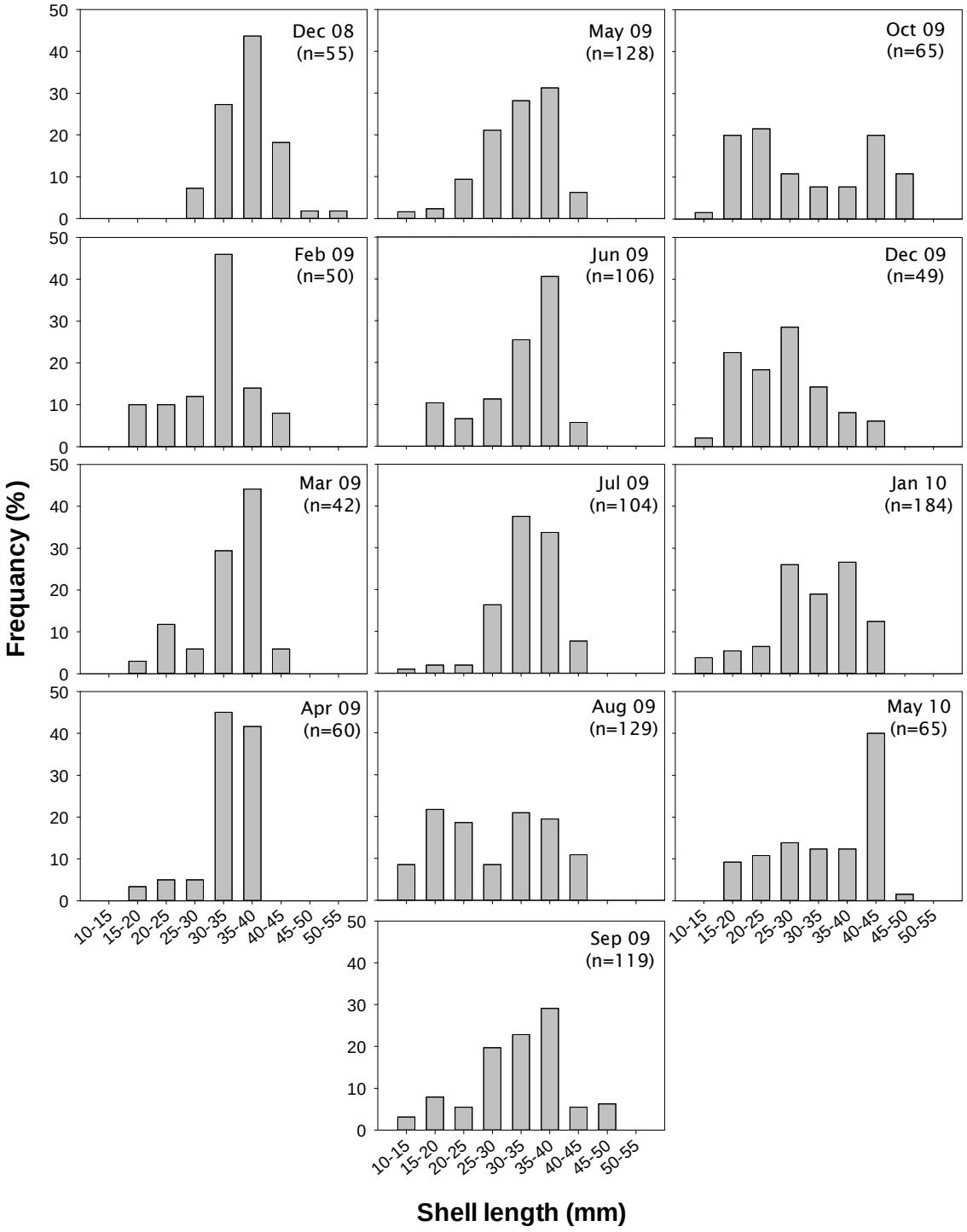

Figure 12 Monthly size-frequency distributions of *Tapes philippinarum* between December 2008 and May 2010 at Bird Pile, Southampton Water (n varies each month)

3.3 Growth rate

Eighty-five adult *Tapes philippinarum* collected from Bird Pile, Southampton were maintained in natural condition (under the NOCS pontoon) for 13 months between March 2009 and April 2010. The initial shell length, height and width and total wet weight were 35.17±2.98 mm, 26.77±2.43 mm, 17.68±1.85 mm and 11.00±3.10 g, respectively. Total length, height and width of shell as well as total live wet weight were measured monthly to track the growth rate of each individual. A similarity of growth patterns in all factors was observed showing that the clams grew slowly between March and June 2009 (Fig. 13). Rapid growth then occurred until September 2009, followed by a cessation in shell growth until February 2010. A little decline in gross weight was noticeable in January 2010. A minor increase of all parameters was seen again from February 2010 onwards. Growth rates in summer (June-August 2009) accounted for more than 70% of annual growth (total length; 88.9%, height; 72.1%, width; 80.5% and total weight; 75.4%). The final cumulative growth rates of length, height, width, and total weight in the present study were 2.93±1.23 mm (8.34% of the initial shell length), 2.80±1.25 mm (10.46% of the initial shell height), 2.73±0.95 mm (15.43% of the initial shell width), and 4.43±1.64 g (40.30% of the initial total weight), respectively.

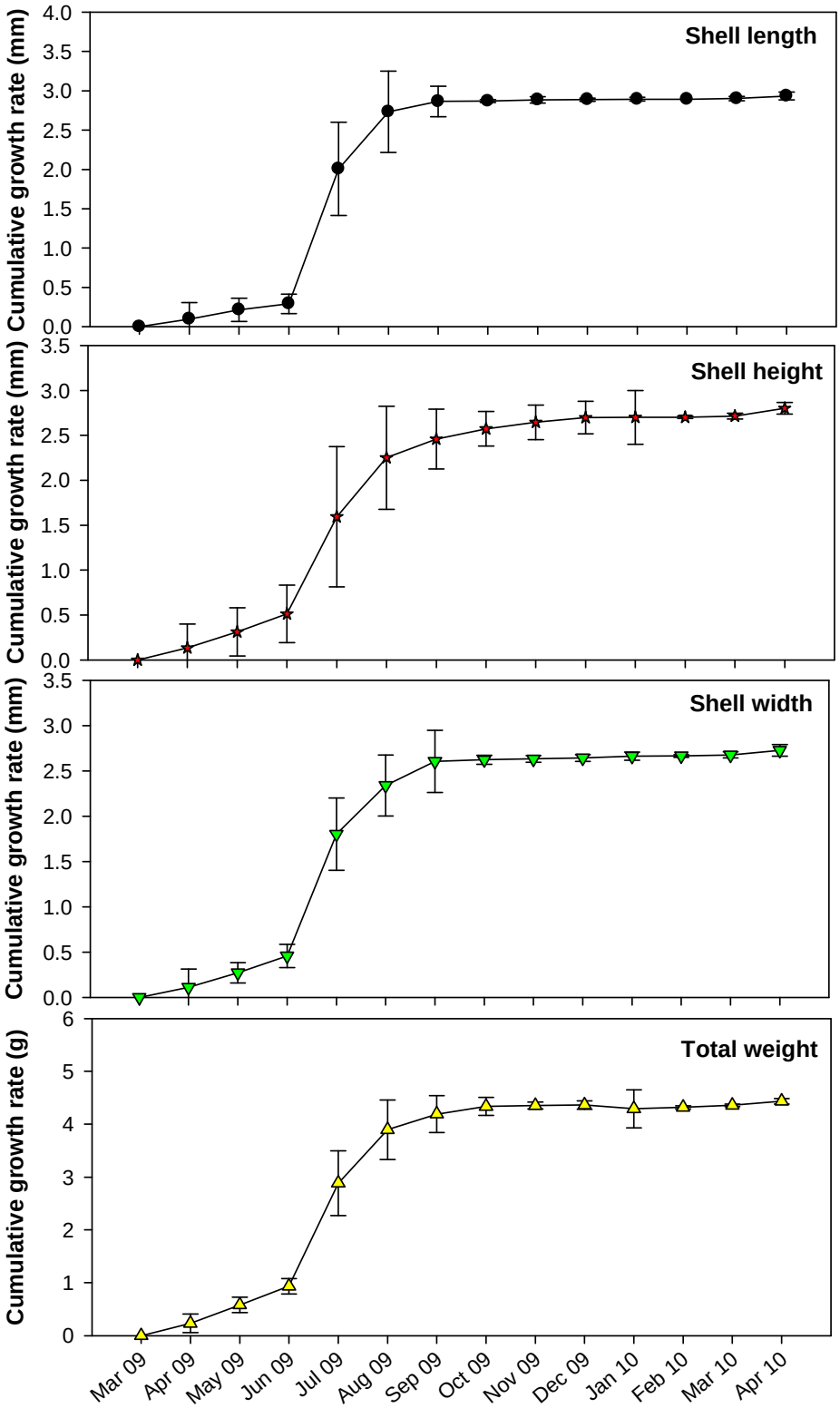

Figure 13 Monthly cumulative growth rates of shell length, height, width and total weight of *Tapes philippinarum* in Southampton Water from March 2009 to April 2010 (Mean±SD, n=85 each month).

3.4 Gametogenesis and maturity index

3.4.1 *Tapes philippinarum* from Bird Pile, Southampton Water

A quantitative study of the gonads of *Tapes philippinarum* ranging from 10.3 to 50.1 mm shell length was histologically examined over 14 months between December 2008 and June 2010. A total of 275 animals were histologically processed. Of these, 134 (48.73%) were males and 107 (38.91%) were females, whereas the remaining 34 (12.36%) were sexually undifferentiated. The sex ratio of male to female was 1:0.8 which showed no significant difference from a 1:1 ratio (χ^2=3.02; df=1; p=0.08). Additionally, all animals found were gonochoric and no cases of hermaphroditism were detected among the examined clams.

Stacked percentage frequency histograms (Fig. 14) and maturity index (MI) (Fig. 15) show that gonad development of both males and females was synchronised. Males reached each phase earlier than females. Sexual activity was quiescent between December and February in both 2009 and 2010, the inactive stage accounting for 87.50-100% of individuals (MI=0). The onset of gametogenesis started from February 2009 in Males (MI=0.25) and March 2009 in females (MI=0.57). Late active stage (23.08%), sexual maturing (7.69%) and spawning (7.69%) in males were first noticeable in May 2009 (MI=2.38). The majority of male clams from June to August 2009 were in spawning phase (46.15 – 90.00%) and some were recorded until September 2009 (MI=3.77-5.62). Similarly, females were initially ripe in May 2009 (18.18%; MI=2.45) and spawned from June 2009 (62.50%; MI=4.19) to September 2009. Over 75% of mature and spawning females were reported between June and August 2009, respectively (MI=3.89-4.19). The majority of males (61.53-88.89%) and females (87.90-91.67%) consequently stoped breeding and started reabsorbing their residual gonad tissue between September and October 2009 (MI=5.33-5.88).

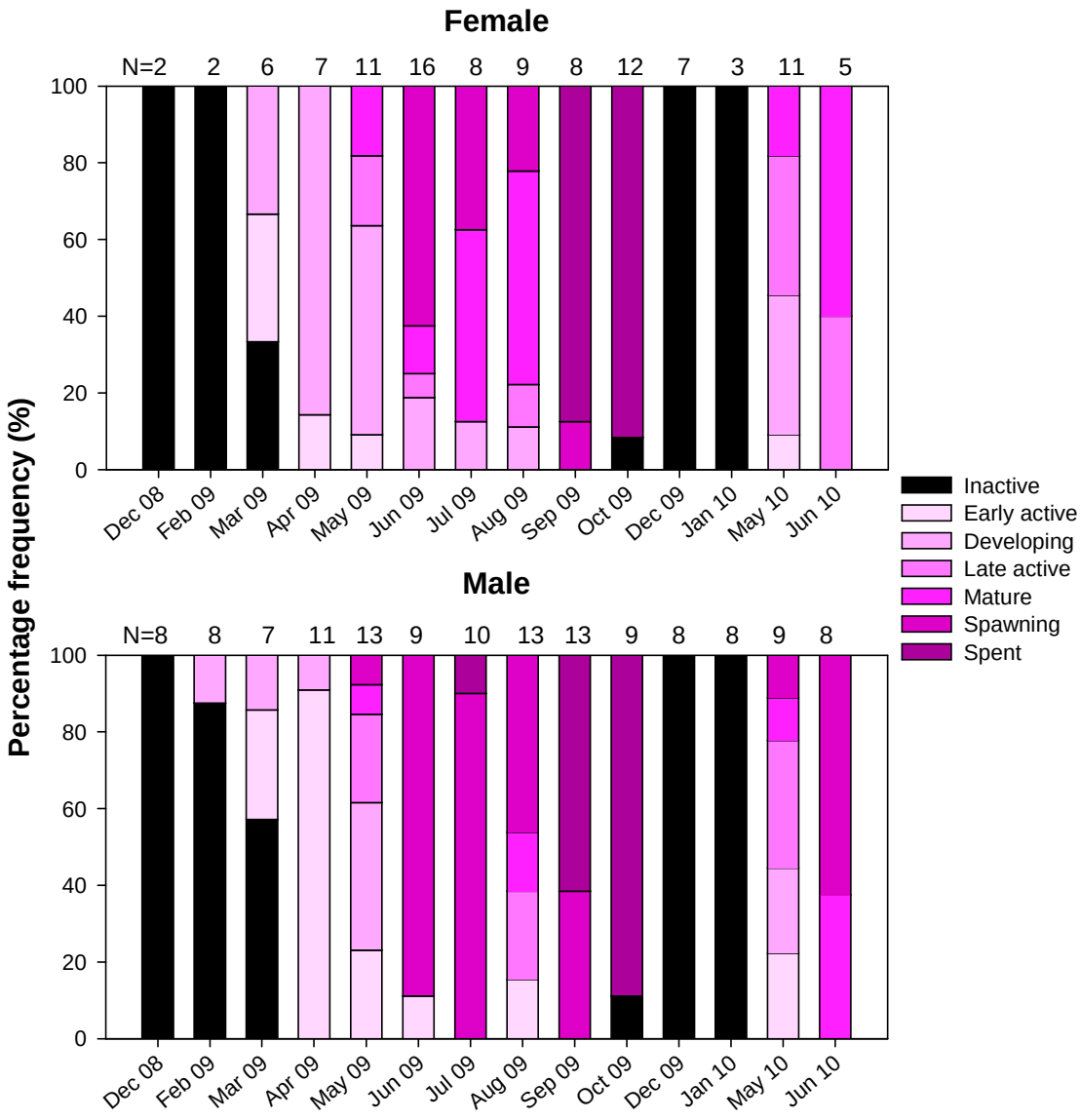

Figure 14 Stacked percentage frequency histograms of *Tapes philippinarum*'s reproductive stages from Bird Pile, Southampton Water between December 2008 and June 2010 (n varies each month)

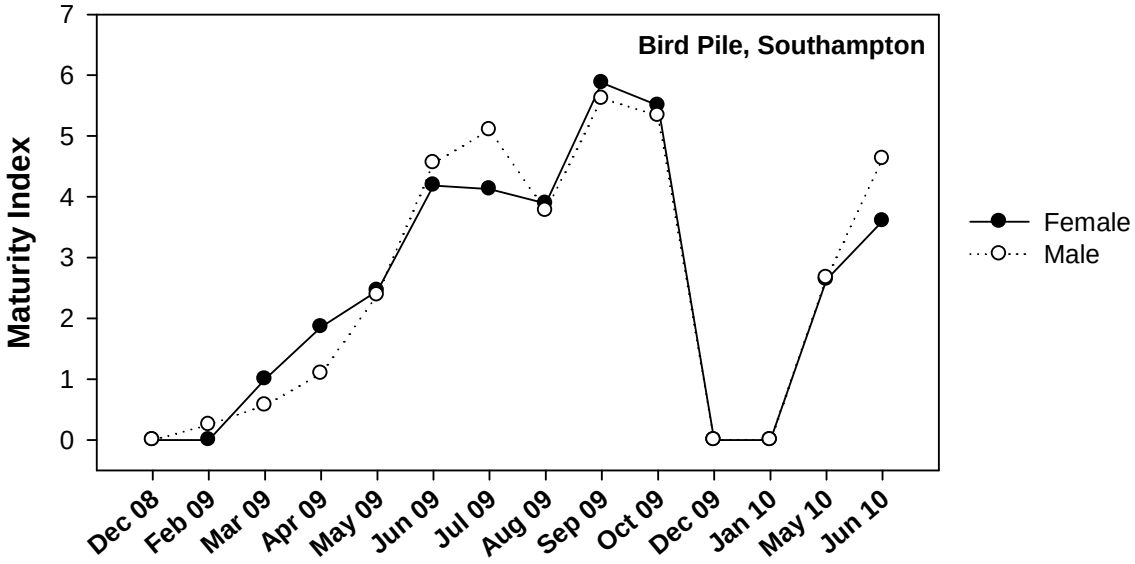

Figure 15 Maturity index of *Tapes philippinarum* from Bird Pile, Southampton Water between December 2008 and June 2010

3.4.2 *Tapes philippinarum* from Poole Harbour and Portsmouth Harbour

Histological observation in *Tapes philippinarum* ranging between 21.8 and 58.8 mm total length from Poole Harbour were examined over 11 months (March 2009 to July 2010). Of the 68 clams examined, 35 were male and 33 were female. The overall male/female sex ratio was 1:0.94 which was not significantly different from a 1:1 ratio (χ^2=0.06; df=1; p=0.81). The five *T. philippinarum* from Portsmouth in May 2010 that were histologically examined had shell lengths between 34.5 and 50.2 mm. There were 2 males and 3 females. All specimens of both study sites were gonochoric and no hermaphrodite clam was found.

Fig. 16 reveals the percentage of clams in various stages in reproduction at Poole Harbour whereas Fig. 17 quantitatively illustrates the gametogenesis as maturity index (MI). The patterns of gonad development in both sexes were synchronised. Gametogenesis in females began in March 2009 (MI=2) at developing stage (50%) whilst all males were in resting phase (MI=0). The onset of gonadal activity in males possibly started in April 2009 because by May 2009 the majority of their gonads had developed to 'late active' (MI=2.75; 75%). The first ripe males and females appeared in June 2009 (MI=4.0; 80% and 3.8; 100% respectively). Mature individuals were present in samples from June to September 2009 and June to July 2010 (MI=3.00-5.67). Spawning in both sexes had occurred from July 2009 to September 2009. Partially spent individuals were also recorded during the spawning period of July - September. From

November 2009 through to January 2010, most of the gonads were either being reabsorbed or were inactive (MI=0). Although the gametogenesis in the clams from Poole seems to be normal, gonad abnormality was obvious in that gametes were not entirely released in both 2009 and 2010, this being especially notably in 2009. Indeed, spawning in Poole Harbour probably occurred in a small scale throughout 2009. From May to September 2009, a large proportion of *T. philippinarum* in both sexes (50.00-83.33%) were seen to be reabsorbing gametes. This phenomenon during advanced gametogenesis is called “atresia” (Fig. 18 and 19).

Tapes philippinarum obtained from Portsmouth in May 2010 were in an advanced reproductive stage with maturity indices of 4.33 in females and 4.50 in males. 66.67% of females and 50% of males were spawning whereas the remaining were in late active and mature stages.

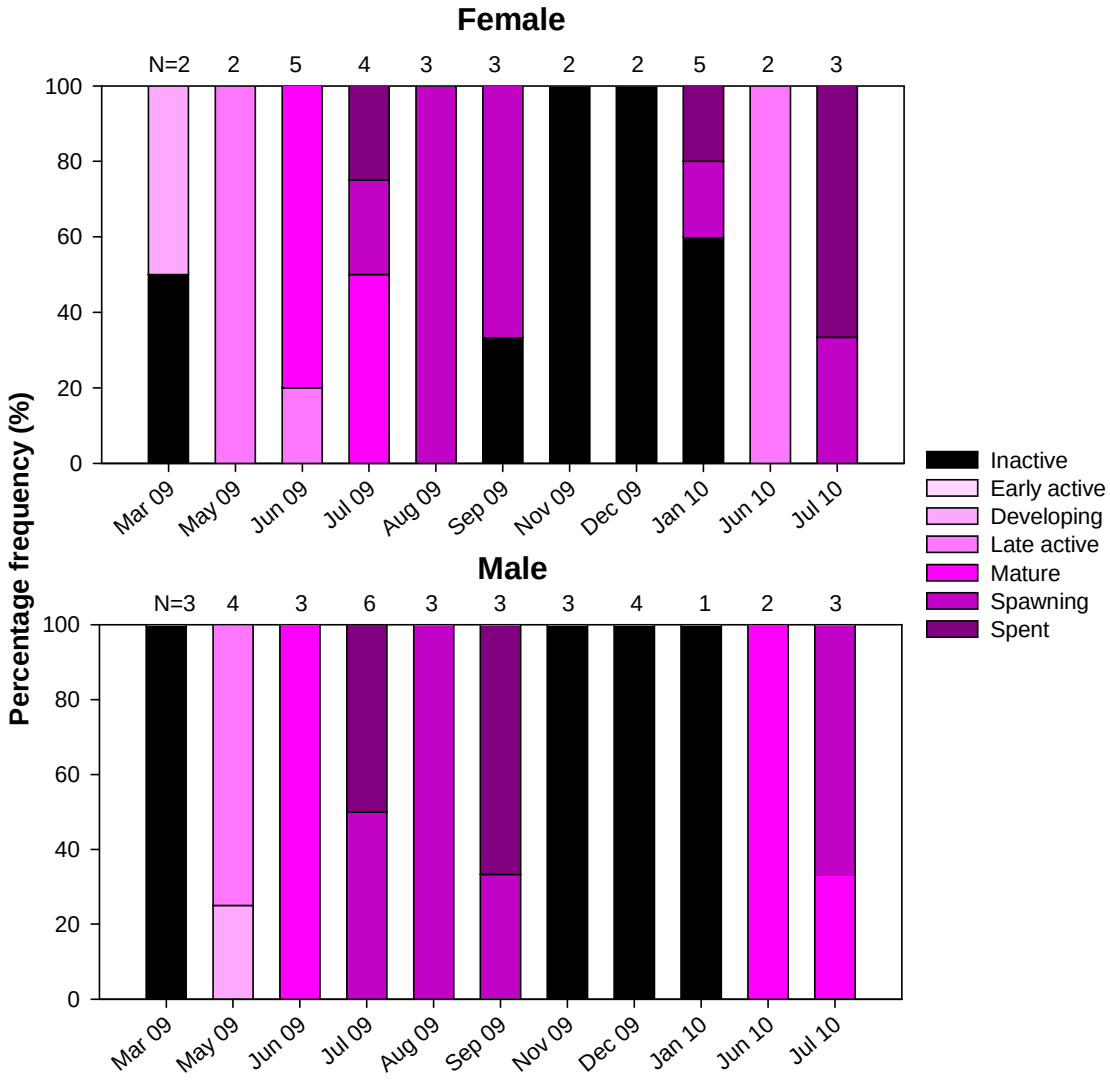

Figure 16 Stacked percentage frequency histograms of *Tapes philippinarum*'s reproductive stages from Poole Harbour between March 2009 and July 2010 (n varies each month)

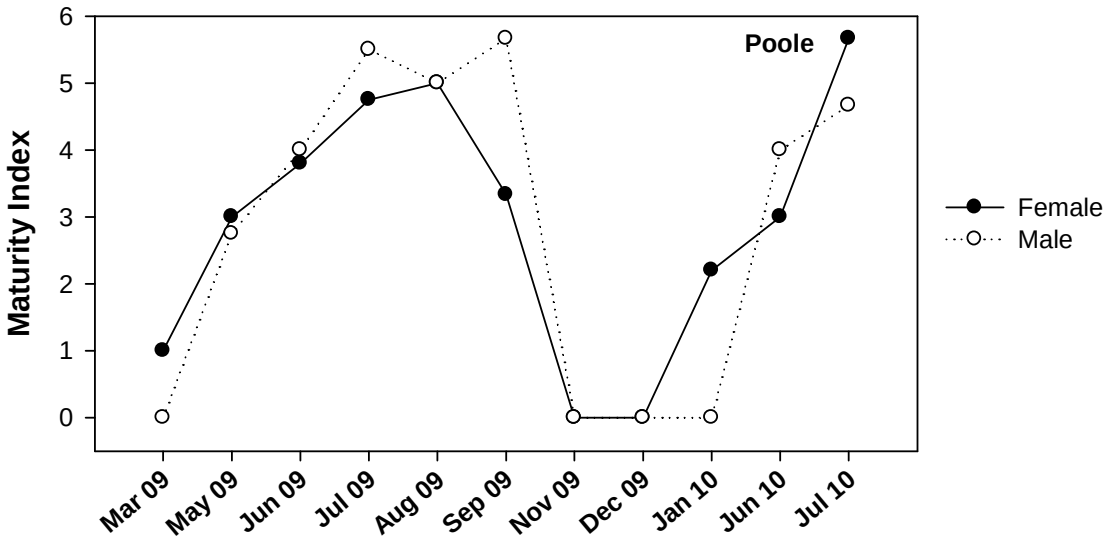

Figure 17 Maturity index of *Tapes philippinarum* from Poole Harbour between March 2009 and July 2010

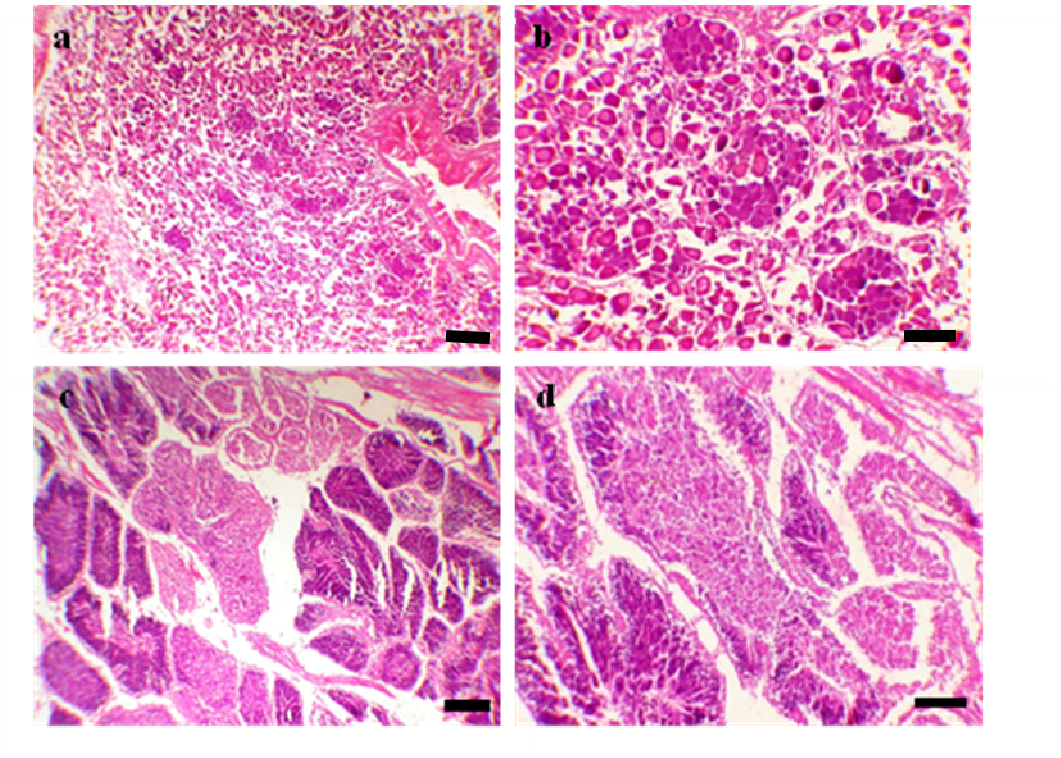

Figure 18 Prevalence of atresia in *Tapes philippinarum* from Poole Harbour, a-b: gonads in female, c-d: gonads in male. Scale bar in a, c: 200 μm; in b, d: 100 μm.

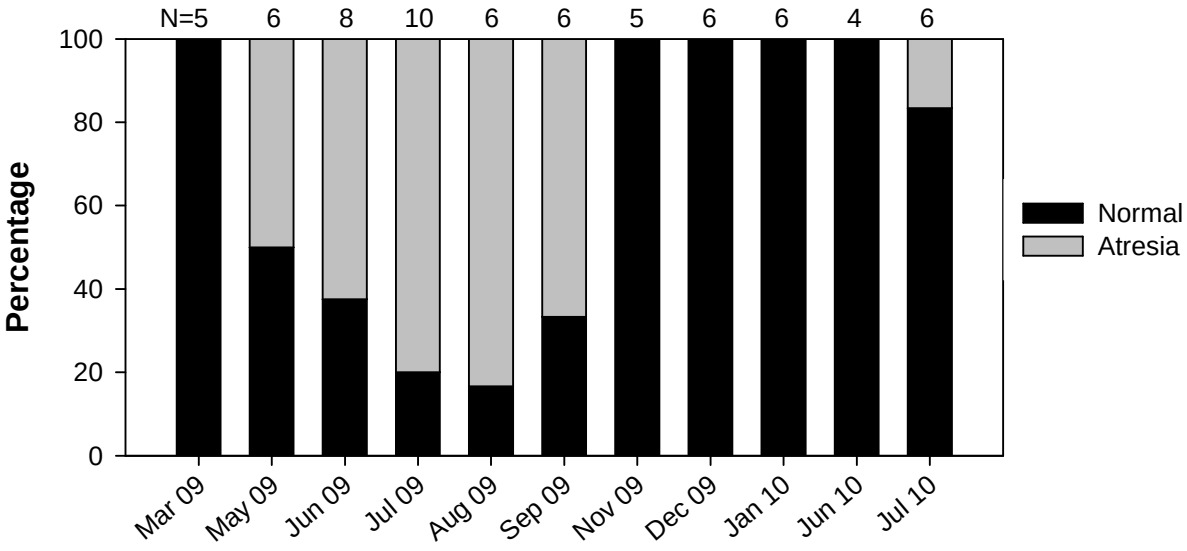

Figure 19 Monthly percentages of normal and atresia individuals in *Tapes philippinarum* from Poole Harbour between March 2009 and July 2010 (n varies each month)

3.5 Size at sexual maturity of *Tapes philippinarum* from Bird Pile, Southampton Water

Tapes philippinarum collected in this study ranged from 10.3–50.1 mm in shell length. The smallest sexually mature animals were 17.9 (male) and 20.2 mm (female) in shell length. The smallest individual female found to be sexually mature contained ripe oocyte with an averaged diameter of 34.3±10.7 μm. The gonad of the smallest ripe male was thought to have reached spawning stage because evacuating ducts (from which spermatozoa are released) were obvious, however some sperms remained.

The lowest size at maturity in *T. philippinarum* during the spawning season (June to August 2009 and June 2010) occurred in individuals within the 15-20 mm and 20-25 mm size range. However, not all animals in these size classes reached maturity. Despite being sampled during the breeding season, most of the clams less than 25 mm in shell length were categorised as immature. Fig. 20 reveals that none of the clams with total length less than 15 mm became mature. Small clams (15-20 mm and 20-25 mm in length) showed a low percentage of maturity (22.22% and 28.57%, respectively). In contrast, 87.50% of individuals between 25 and 30 mm were mature. Furthermore, all individuals larger than 30 mm (100%) were found fully mature and spawned. In fact, all *T. philippinarum* above 29.7 mm shell length were mature during breeding season.

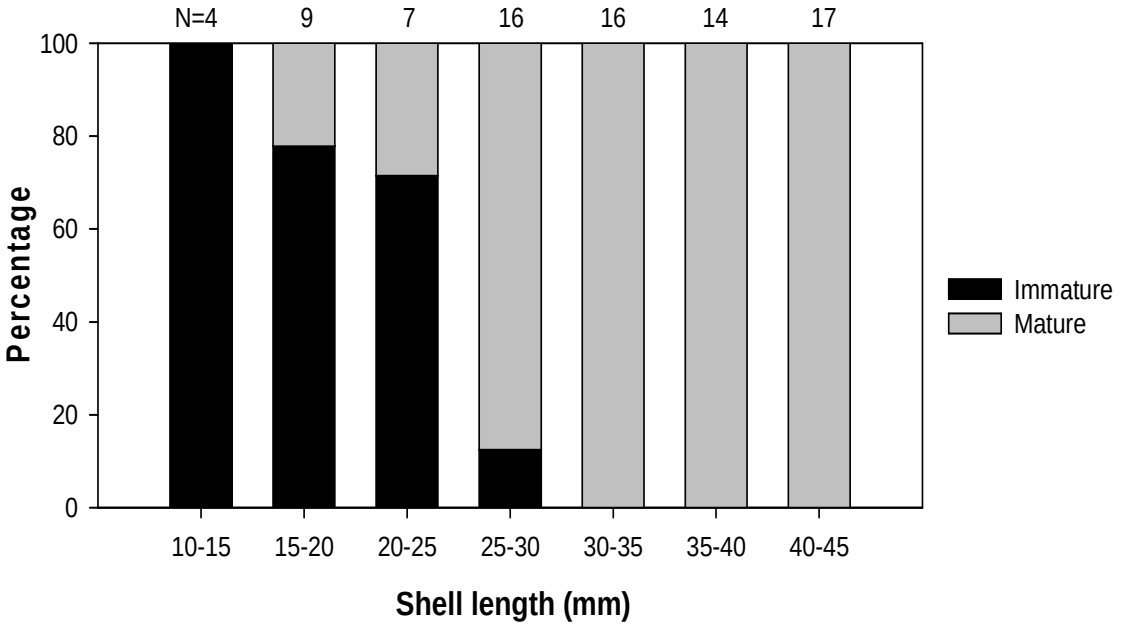

Figure 20 Percentage of immature and mature individual in each size class of *Tapes philippinarum* from Bird Pile, Southampton during spawning season. (n varies for each size class)

3.6 Oocyte size

3.6.1 *Tapes philippinarum* from Bird Pile, Southampton Water

Oocyte diameter measurement was conducted in ripe females of *Tapes philippinarum* with the size ranging from 20.2 mm to 43.1 mm having sufficient mature oocytes. The examination was possible only from May to August 2009 and May to June 2010 because oocytes had developed and reached ripe phase. Fig. 21-23 showed monthly size frequency distribution of mean oocyte diameter of *T. philippinarum*. Multiple regression analysis indicated a significant positive relationship of oocyte diameter with shell length of the clam (df=1, F=8.5636, r^2=0.63, p<0.01) and month (df=1, F=8.5636, r^2=0.63, p<0.001), but no significant relationship between year was seen.

The multiple regression equation is:

Oocyte diameter = 17.7393 + 0.34645 shell length + 5.12842 month + 3.06465 year

The oocyte diameter had a significant linear relationship with the shell length of the clam (Multiple regression, df=1, F=8.5636, r^2=0.63, p<0.01). For example, in June 2009 (Fig. 21), the average oocyte diameter of *T. philippinarum* was smallest in the 20-25 mm shell length range (33.42±11.45 µm) and increased to 36.38+3.81 µm in the 25-30 µm shell length range. Larger oocytes were seen in the 30-35 mm and 35-40 mm shell length range; average diameter of 42.49±2.39 and 43.12±6.22 µm, respectively. The 40-45 mm shell length range had the largest oocyte size of 48.02±1.29 µm. It is interesting that none of the clam below 29.8 mm shell length produced oocytes larger than 44 µm despite the range of average egg diameter in all individuals was between 31.13±6.31 and 55.74±6.20 µm. Fig. 24 visualises these data to show this relationship.

The oocyte diameter significantly increased over time during the spawning season (May-August) (Multiple regression, df=1, F=38.9251, r^2=0.63, p<0.001). For instance, within the 40-45 mm shell length range (Fig. 21-22), the average oocyte diameter (39.84±8.35 µm) was minimal in May 2009 and grew to 48.02±1.29 µm in June 2009. The sizes then increased to 51.66±5.04 and 52.09±1.50 µm in July and August 2009, respectively.

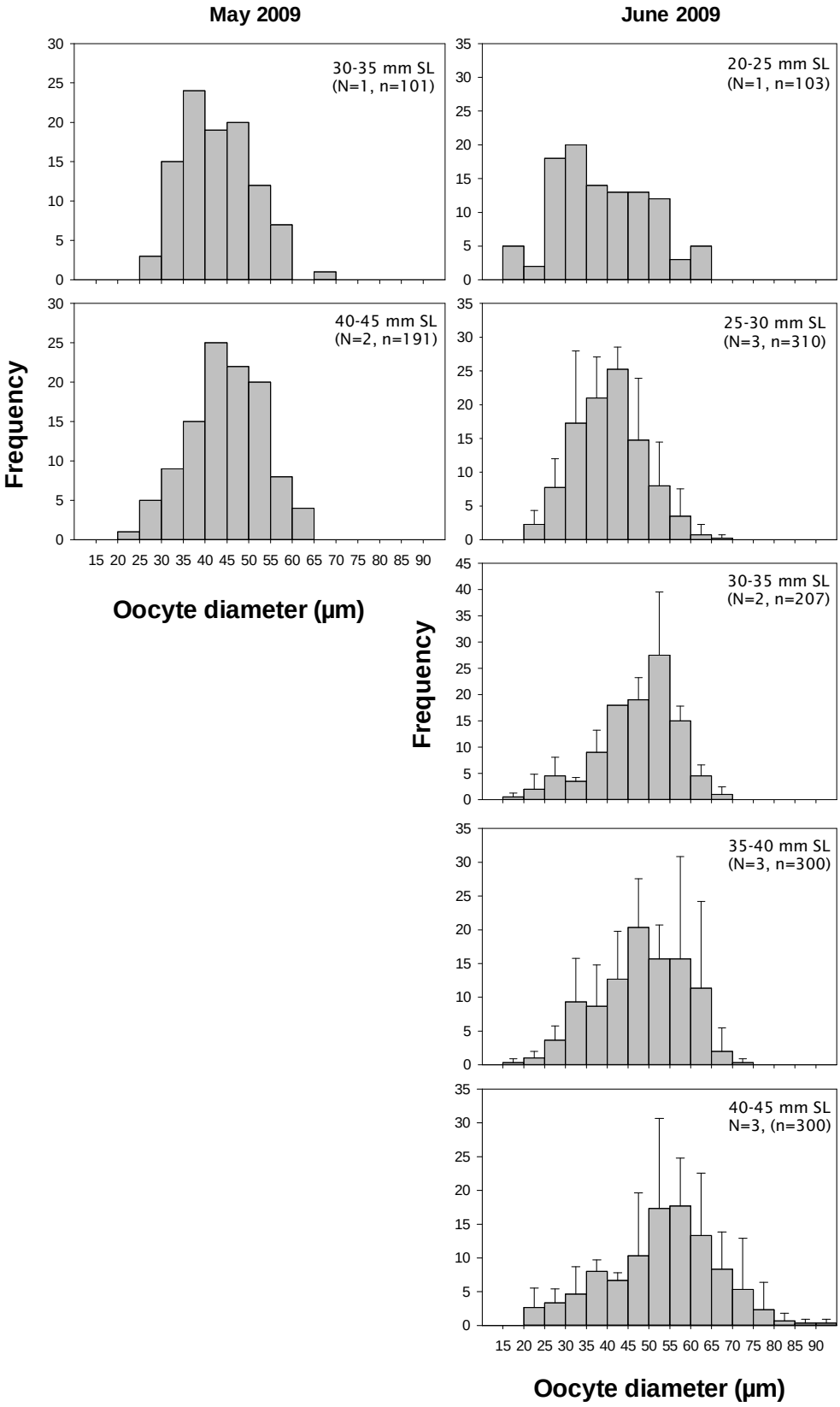

Figure 21 Oocyte diameter frequency histograms of *Tapes philippinarum* from Southampton Water in May and June 2009. (Mean±SD, N=number of females, n=number of oocytes, both vary with size class)

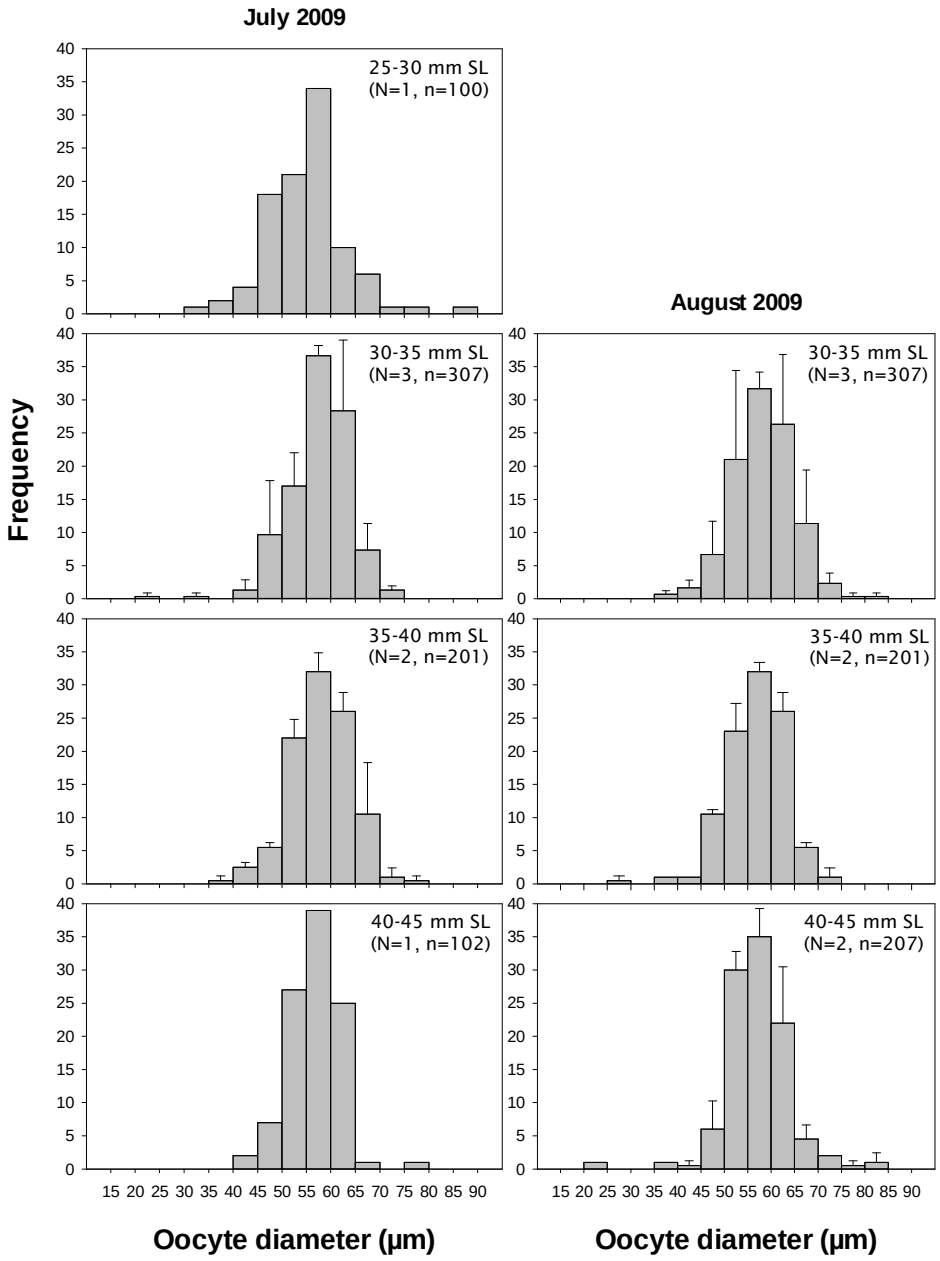

Figure 22 Oocyte diameter frequency histograms of *Tapes philippinarum* from Southampton Water in July and August 2009. (Mean±SD, N=number of females, n=number of oocytes, both vary with size class)

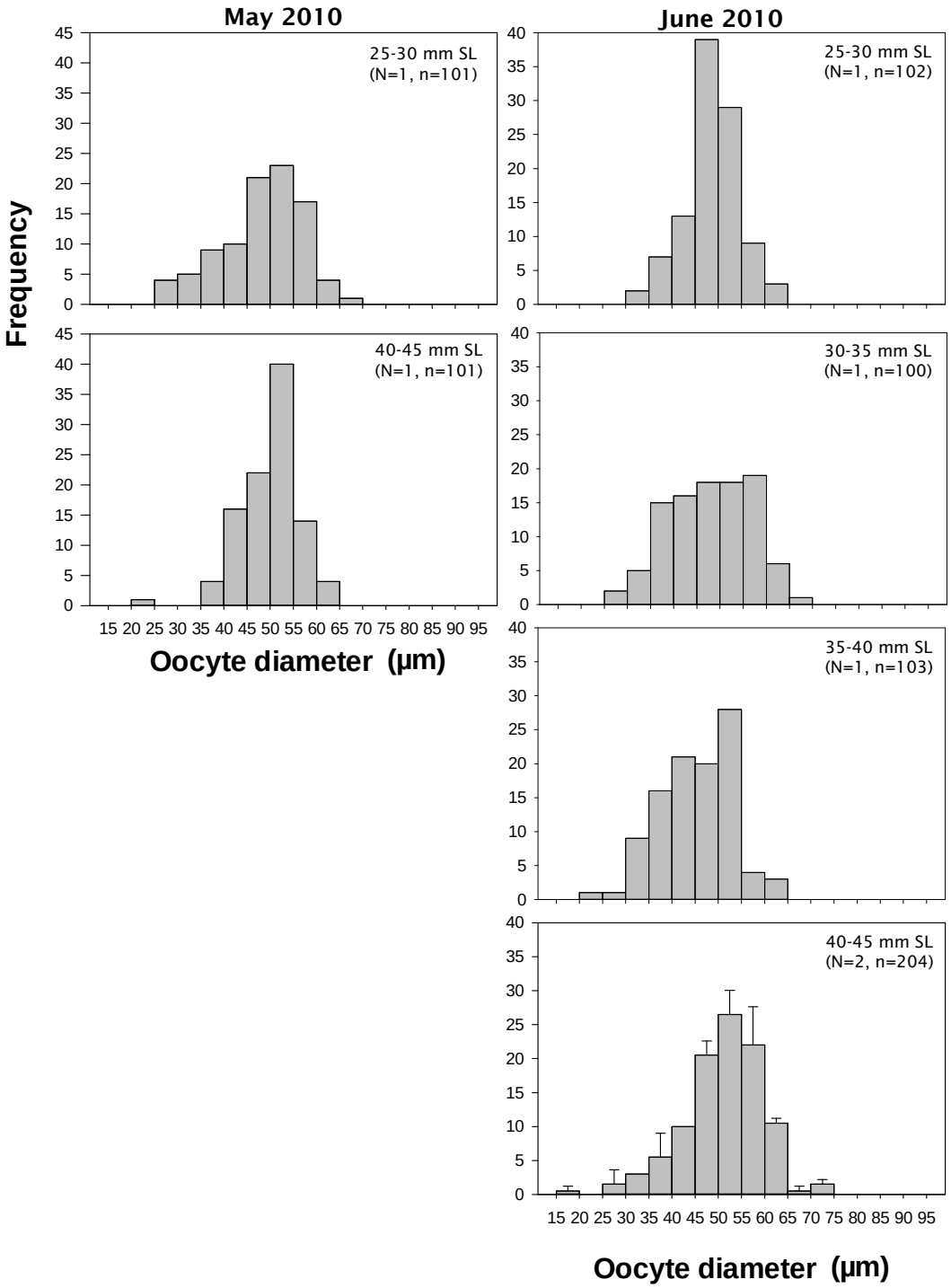

Figure 23 Oocyte diameter frequency histograms of *Tapes philippinarum* from Southampton Water in May and June 2010. (Mean±SD, N=number of females, n=number of oocytes, both vary with size class)

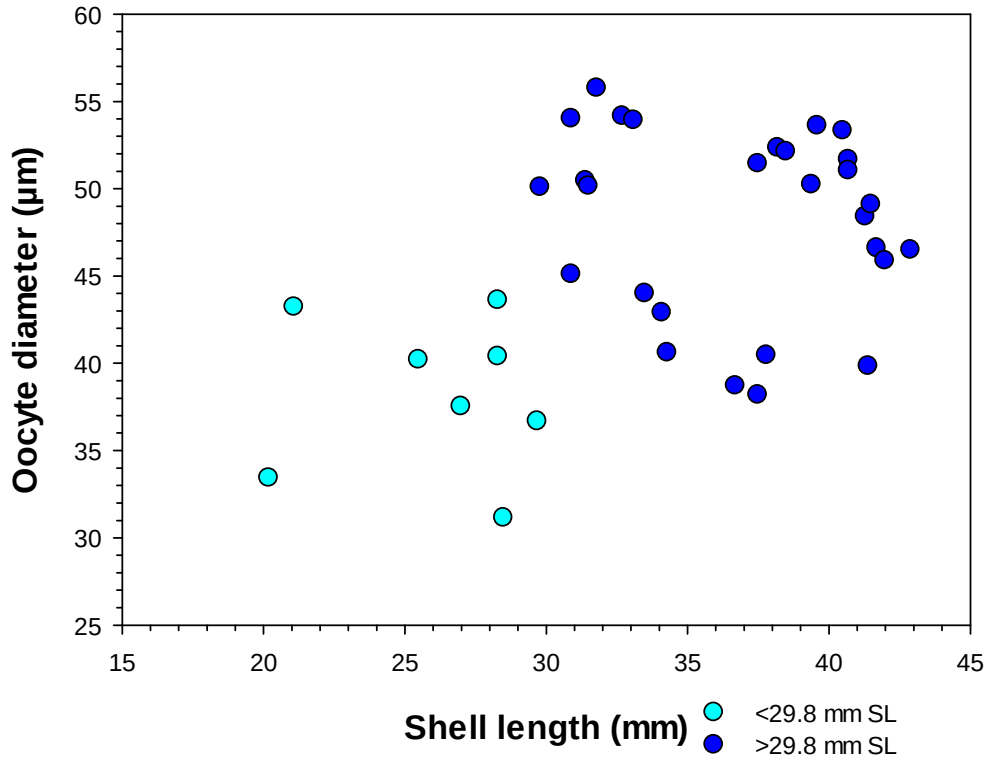

Figure 24 Average oocyte diameter in relation to shell length of *Tapes philippinarum* from Southampton Water (n=35)

3.6.2 *Tapes philippinarum* from Poole Harbour and Portsmouth Harbour

T. philippinarum from Poole Harbour provided mature oocytes for measurement between June and September 2009 (Fig. 25). Monthly variation in oocyte diameter was obvious in the *T. philippinarum* from Poole Harbour. Mature follicles were smallest between June and July 2009 with the average diameter of 41.17±2.76 and 40.45±3.31 µm, respectively. Significantly largest oocytes were observed in September with the average diameter of 49.77±0.47 µm (One-way ANOVA, df=3, F=7.98, r^2=0.63, p<0.01).

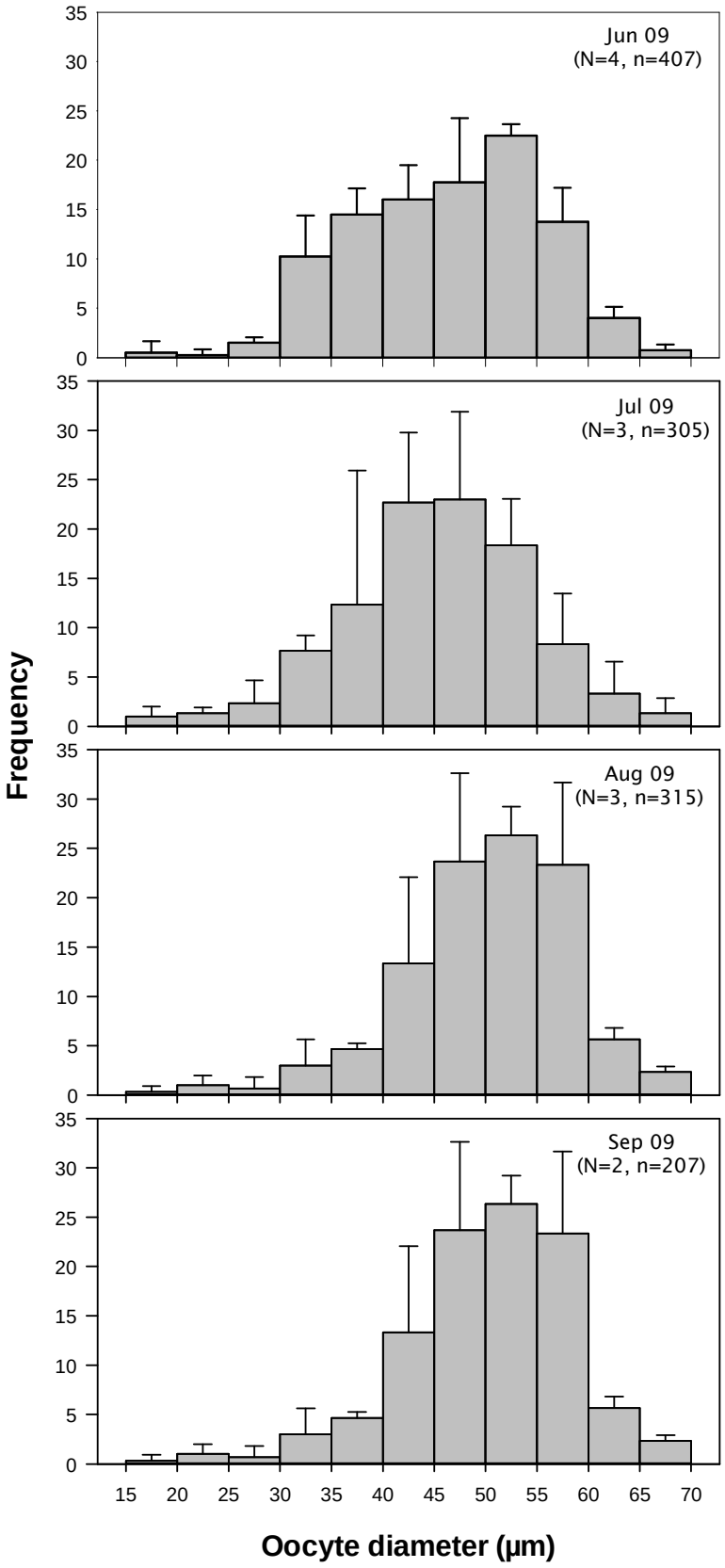

Figure 25 Oocyte diameter frequency histograms of *Tapes philippinarum* from Poole Harbour between June and September 2009. (Mean±SD, N=number of females, n=number of oocytes, both vary with each month)

3.7 Immunological parameters

3.7.1 Haemocytes

Eight *Tapes philippinarum* (41.8±5.7 mm shell length) were monthly collected for haemocyte count from June 2009 to September 2010. Fig. 26 presents the changes of total haemocyte count (THC), granulocytes and hyalinocytes (agranulocytes) during the eleven months observed. Haemocyte numbers seemed to show a seasonal variation. In 2009, peaks of THC and hyalinocytes were observed in summer (July) with concentrations 21.13±17.04 x 10^6 and 19.15±17.56 x 10^6 cells ml^{-1} respectively. They then gradually decreased to the lowest values (5.10±3.00 x 10^6 and 3.76±2.52 x 10^6 cells ml^{-1}) in winter (December). Granulocytes also showed a similar trend; the maximum concentration was seen in summer (August; 3.31±2.05 x 10^6 cells ml^{-1}) and the minimum was observed in winter (December; 1.34±0.65 x 10^6 cells ml^{-1}). There were significant differences between the highest and lowest concentrations in THC, granulocytes and hyalinocytes (One-way ANOVA; THC, df=11, F=6.42, p<0.001, granulocytes, df=11, F=3.67, p<0.001, hyalinocytes, df=11, F=6.20, p<0.001). The results in 2010 confirmed the observation in 2009 that THC and hyalinocytes were greatest in summer (June; 23.90±6.88 x 10^6 cells ml^{-1} and 18.35±6.69 x 10^6 cells ml^{-1} respectively). However, they decreased to the lowest in September (6.36±2.68 x 10^6 cells ml^{-1} and 3.31±2.02 x 10^6 cells ml^{-1}, respectively). The maximum and minimum values of both parameters were significantly different (One-way ANOVA; THC, df=11, F=6.42, p<0.001, hyalinocytes, df=11, F=6.20, p<0.001). Similarly, an increase of granulocytes was found in June (5.55±3.74 x 10^6 cells ml^{-1}) and next dropped to the minimum in August (2.83±1.67 x 10^6 cells ml^{-1}) with no statistical differences.

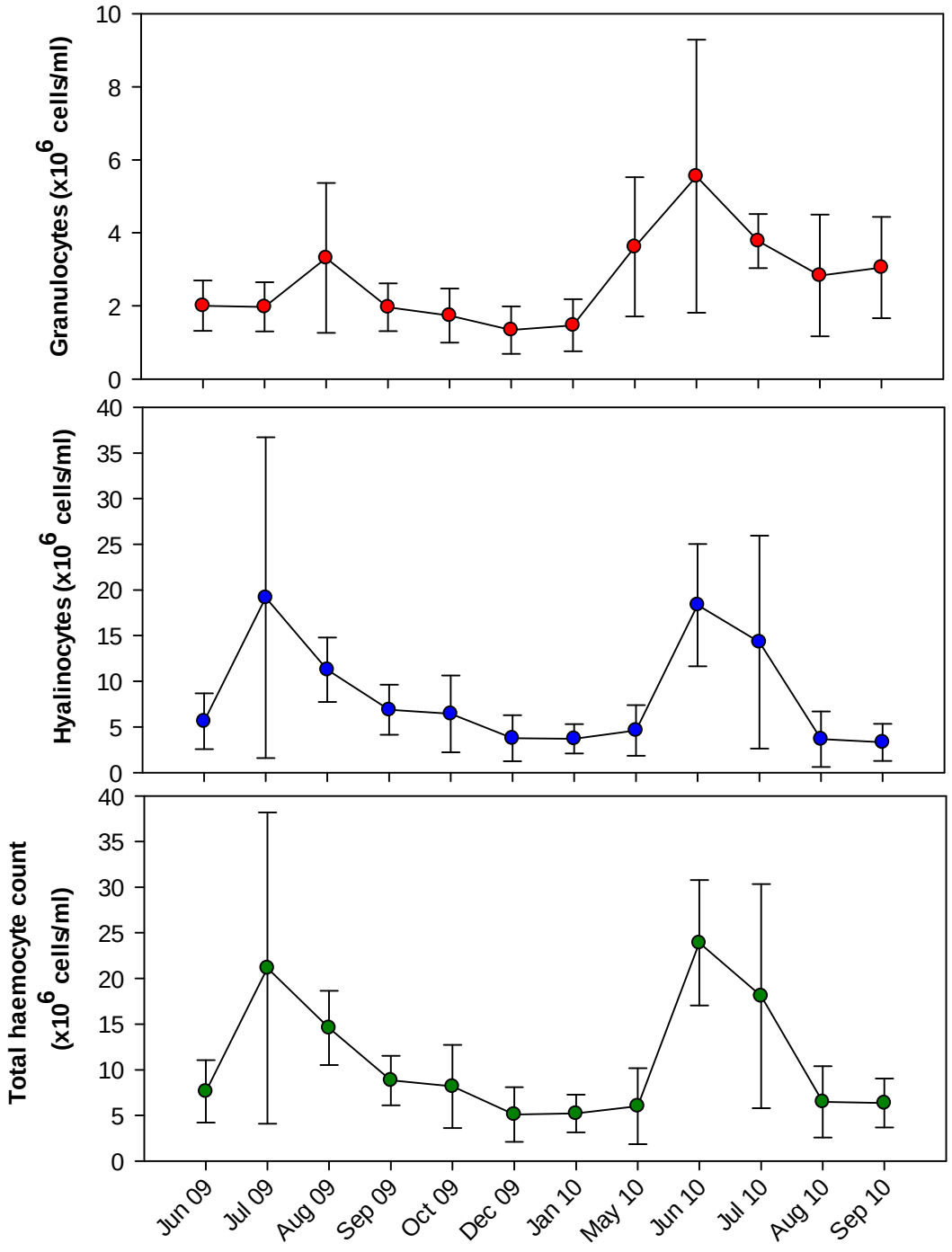

Figure 26 Total haemocyte count, Granulocyte numbers and Hyalinocyte numbers in *Tapes philippinarum* from Bird Pile, Southampton Water between June 2009 and September 2010. (Mean±SD, n = 8 at each point)

The relationship between haemocytes and reproductive stages was shown in Fig. 27. All types of haemocytes revealed the similar trend that the numbers were low during early stages of gametogenesis including early active and developing phases. Granulocyte concentration then peaked in the late active reproductive stage. A significant reduction in granulocyte number was observed in spawning and spent stages (One-way ANOVA; df=5, F=7.53, p<0.001). THC and hyalinocytes increased from the late active phase onwards until the end of spawning phase when they began to decline (One-way ANOVA; THC, df=5, F=5.54, p<0.001, hyalinocytes, df=5, F=4.20, p<0.01). The increments of haemocytes in first three stages of gonad development (early active, developing and late active) were not significantly different, probably as a result of a small sample size.

Granulocyte concentration appeared to be weakly positively correlated with that of hyalinocytes. Pearson correlation between haemocytes and seawater temperature indicated that the strength of association between THC, granulocytes and hyalinocytes and temperature are weakly positive (Table 6).

Table 6 Estimative of Pearson's product-moment correlation coefficients among total haemocyte count, granulocyte numbers and hyalinocyte numbers

Variables		r	*P*-Value
Temperature	Total haemocyte count	0.433	< 0.01
	Granulocytes	0.333	< 0.01
	Hyalinocytes	0.397	< 0.01
Granulocytes	Hyalinocytes	0.309	< 0.01

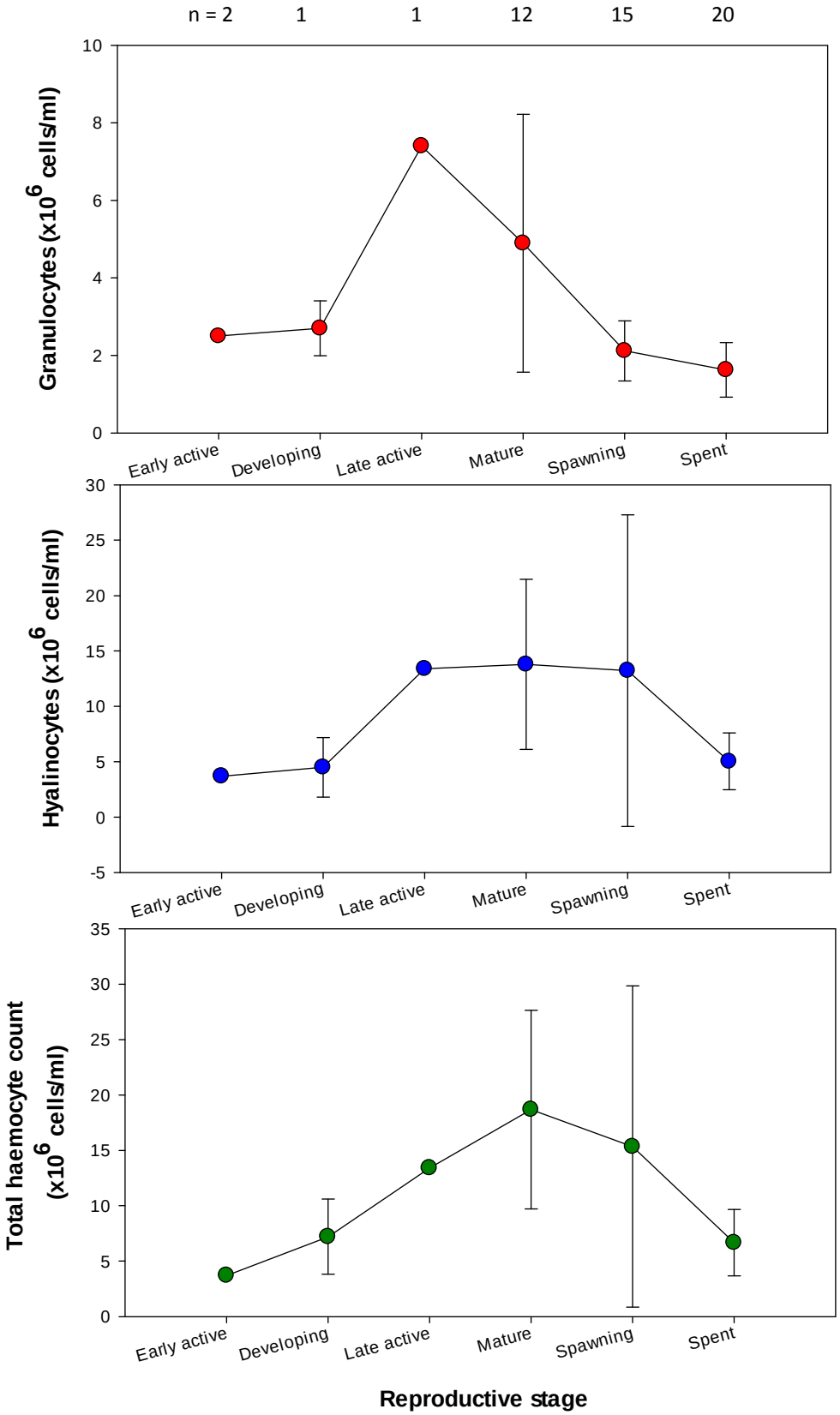

Figure 27 Total haemocyte count, Granulocyte numbers and Hyalinocyte numbers in *Tapes philippinarum* from Bird Pile, Southampton Water in each stage of gonad development. (Mean±SD, n varies at each point)

3.7.2 Phagocytosis

Phagocytic activity is an indicator revealing the self-defense competence of the clam through the proportion of active granulocytes. Phagocytic capacity reflects the carrying capacity of the granulocytes to contain foreign substances. The phagocytic activity values throughout the twelve months examined (June 2009–September 2010) ranged from 0.03±0.02 – 0.34±0.08 (Fig. 28). Although no clear-cut pattern was shown, phagocytic activities both in 2009 and 2010 declined during the same period (August and September) without significant differences. In the same way, phagocytic capacity fluctuated between 0.92±0.08 and 1.75±0.72 during the period of study, the highest activity was seen in summer of both years (June 2009; 0.34±0.08 and July & August 2010; 0.08±0.05).

The linkage between phagocytosis and the reproductive cycle is shown in Fig. 29. Phagocytic activities were low during the early stages of gonad development (early active, developing and late active) and then increased to a peak during the spawning phase. The value consequently decreased after gamete release. However, no statistically significant temporal differences in phagocytic activity were recorded. Phagocytic capacities seemed to be constant in all stages of gametogenesis except for a sudden decrease in the late active gonad. No significant differences in phagocytic capacities were observed in consequence of the small sample size in the first three stages. The Pearson correlation between phagocytic activity and capacity is -0.072 which showed no association. Moreover, phagocytosis revealed no correlations with haemocytes and seawater temperatures (Table 7).

Table 7 Estimative of Pearson's product-moment correlation coefficients among phagocytosis and other variables evaluated

Variables		r	*P*-Value
Phagocytic activity	Total haemocyte count	-0.136	0.217
	Granulocytes	-0.090	0.417
	Hyalinocytes	-0.128	0.245
Phagocytic capacity	Total haemocyte count	0.116	0.294
	Granulocytes	-0.113	0.307
	Hyalinocytes	0.152	0.168
Temperature	Phagocytic activity	0.197	0.071
	Phagocytic capacity	-0.063	0.566
Phagocytic activity	Phagocytic capacity	-0.072	0.514

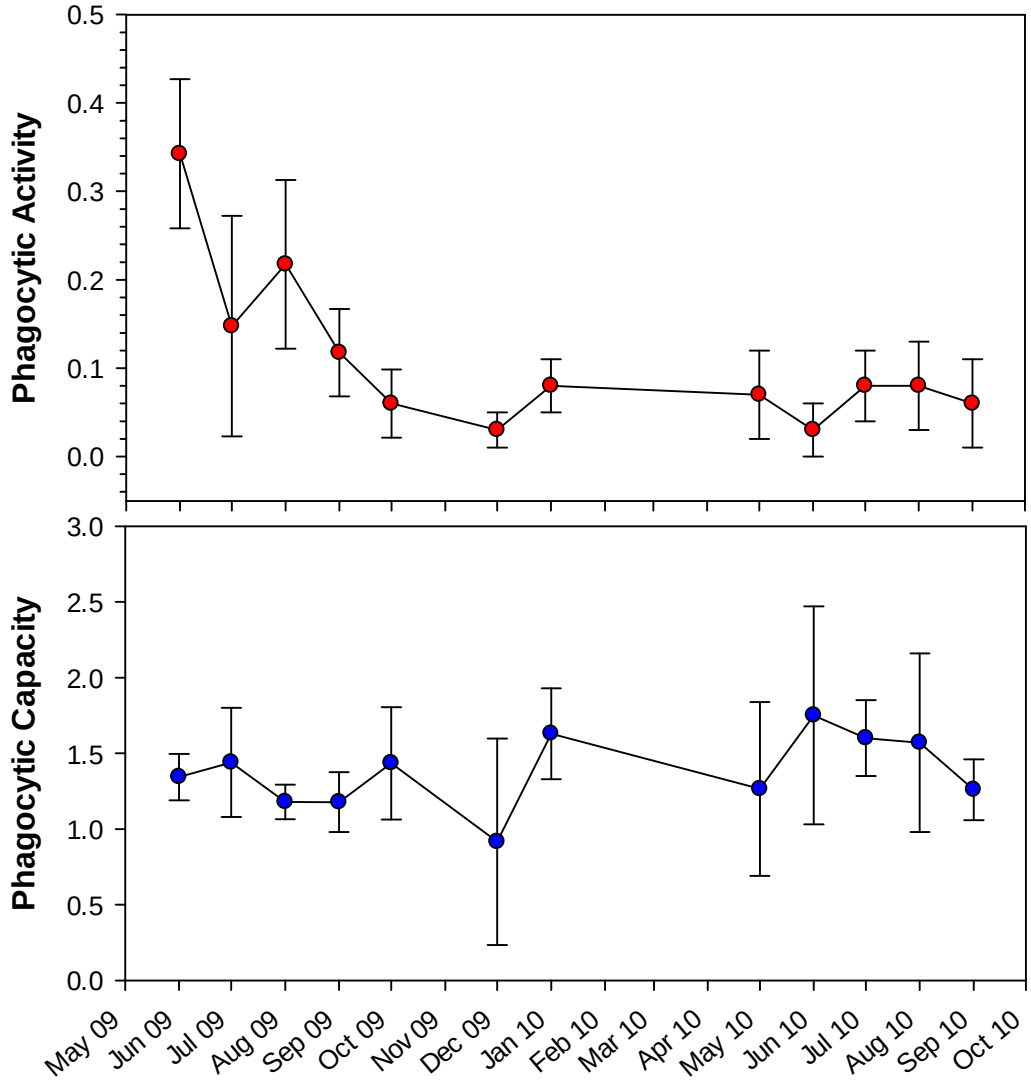

Figure 28 Phagocytic activity and capacity in *Tapes philippinarum* from Bird Pile, Southampton Water between June 2009 and September 2010 (Mean±SD, n = 8 at each point)

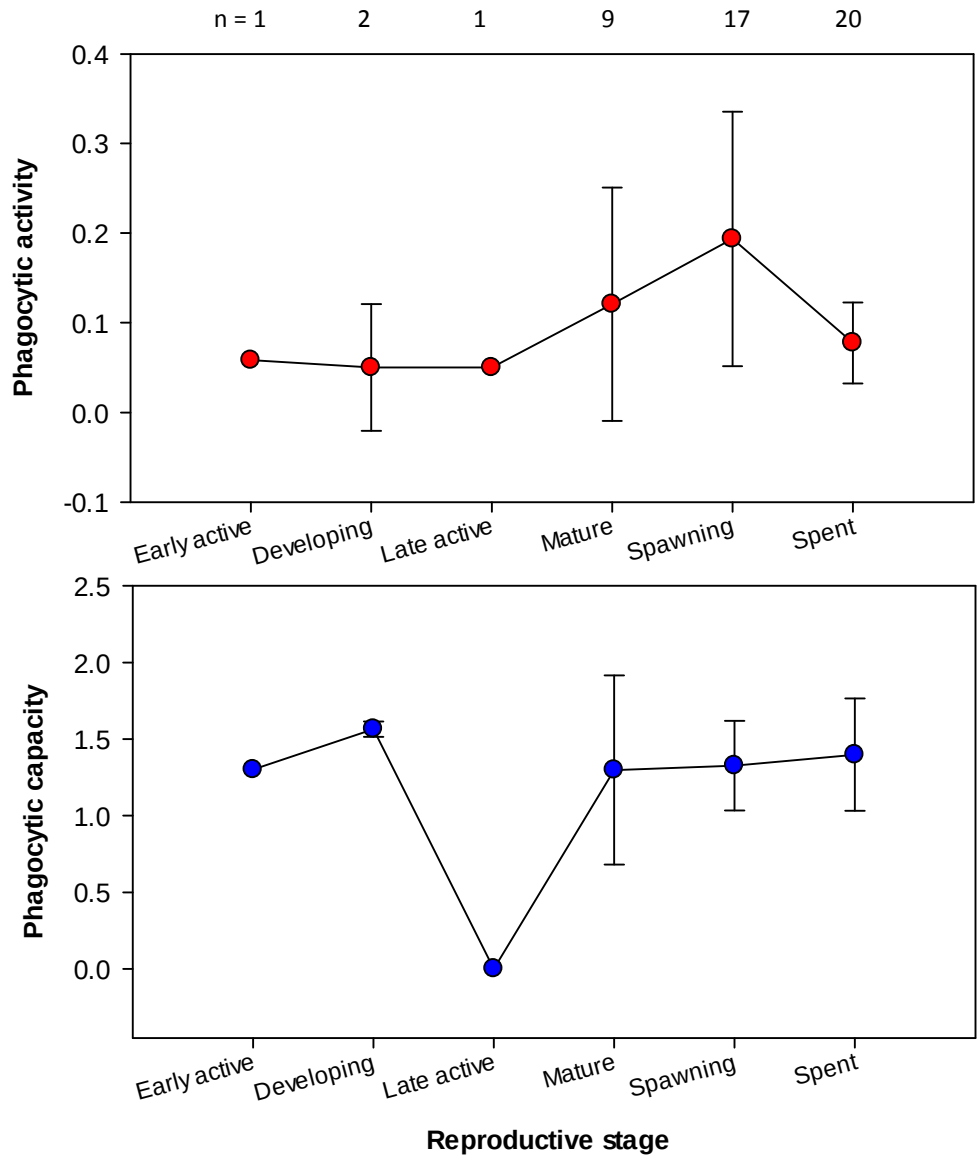

Figure 29 Phagocytic activity and capacity in *Tapes philippinarum* from Bird Pile, Southampton Water in each stage of gonad development (Mean±SD, n = 8 at each point)

3.7.3 Digestive tubule

The structure of digestive tubules from *Tapes philippinarum* was determined between June 2009 and June 2010 (9 months). Seasonal variation in average epithelial thickness (Fig. 30) was demonstrated when the value peaked in summer (June 2009; 19.39±2.47 µm), and then gradually decreased to a minimum in winter (December 2009; 13.36±1.98 µm). The epithelial thickness again increased and reached a maximum in the next summer (June 2010; 18.85±1.36 µm). The epithelial thicknesses in summer (Jun-Jul 2009 and Jul 2010) were significantly greater than those in winter (December 2009 – January 2010) (One-way Anova; df=8, F=12.61, p <0.001).

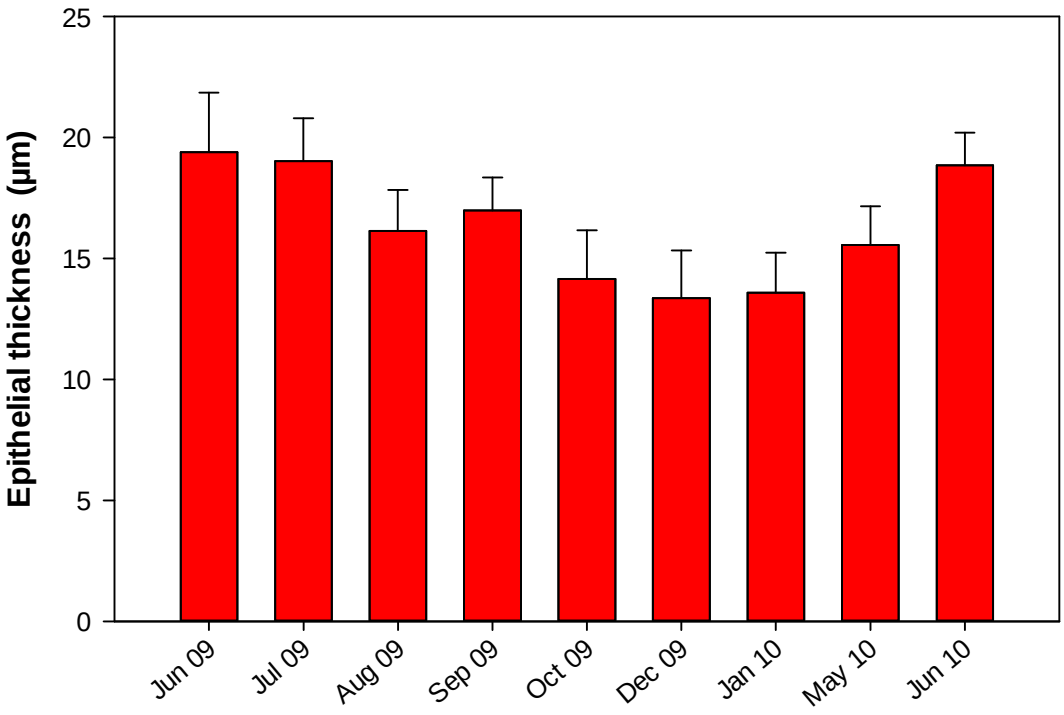

Figure 30 Average digestive epithelial thickness of *Tapes philippinarum* from Bird Pile, Southampton Water between June 2009 and June 2010. (Mean±SD, n=10 each month)

The average luminal radius remained relatively constant (24.32+3.96 µm - 29.40+3.21 µm) and showed no seasonal pattern throughout the year (Fig. 31). Only the luminal diameter in July 2009 (24.32±3.96 µm) was significantly different from that in June 2010 (33.72±3.61 µm) (One-way Anova, df=8, F=2.33, p<0.05).

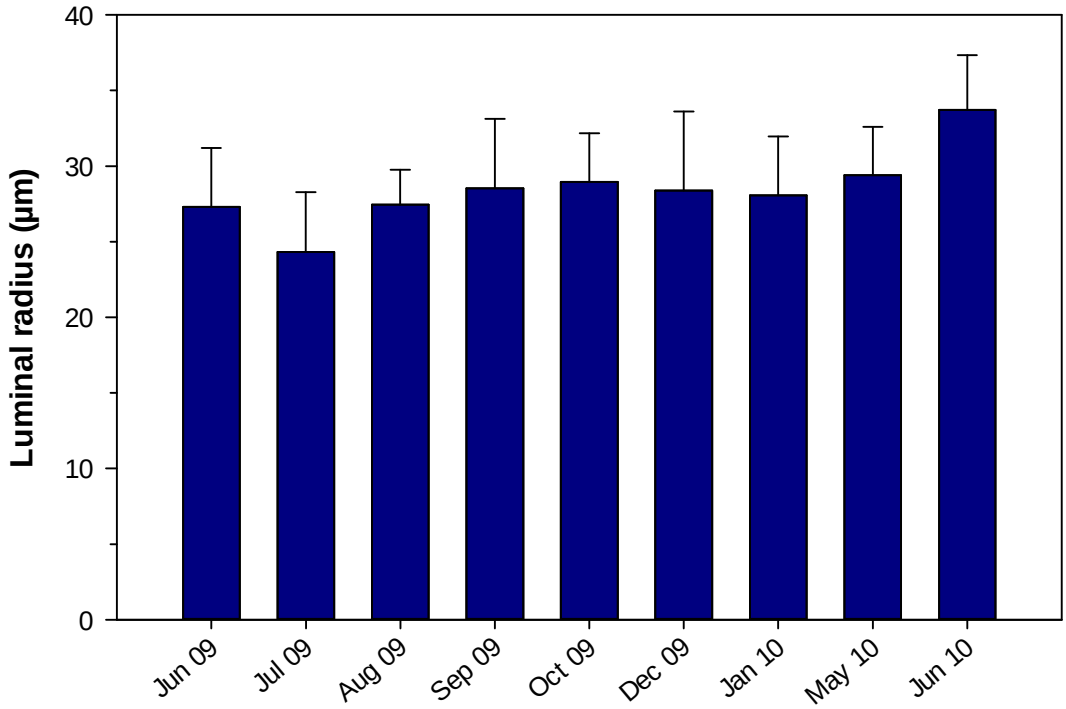

Figure 31 Average luminal radius in digestive tubule of *Tapes philippinarum* from Bird Pile, Southampton Water between June 2009 and June 2010. (Mean±SD, n=10 each month)

Mean luminal radius/Mean epithelial thickness (MLR/MET) ratios in the clam were between 1.30±0.28 and 2.19±0.66 µm/µm (Fig. 32). A temporal variation was reflected by a significantly high ratio in autumn-winter (October 2009 – January 2010; 2.07±0.26 – 2.19±0.66 µm/µm) compared to summer in 2009 (June-July 2009; 1.43±0.26 and 1.30±0.28 µm/µm) (One-way ANOVA, df=8, F=5.59, p<0.001).

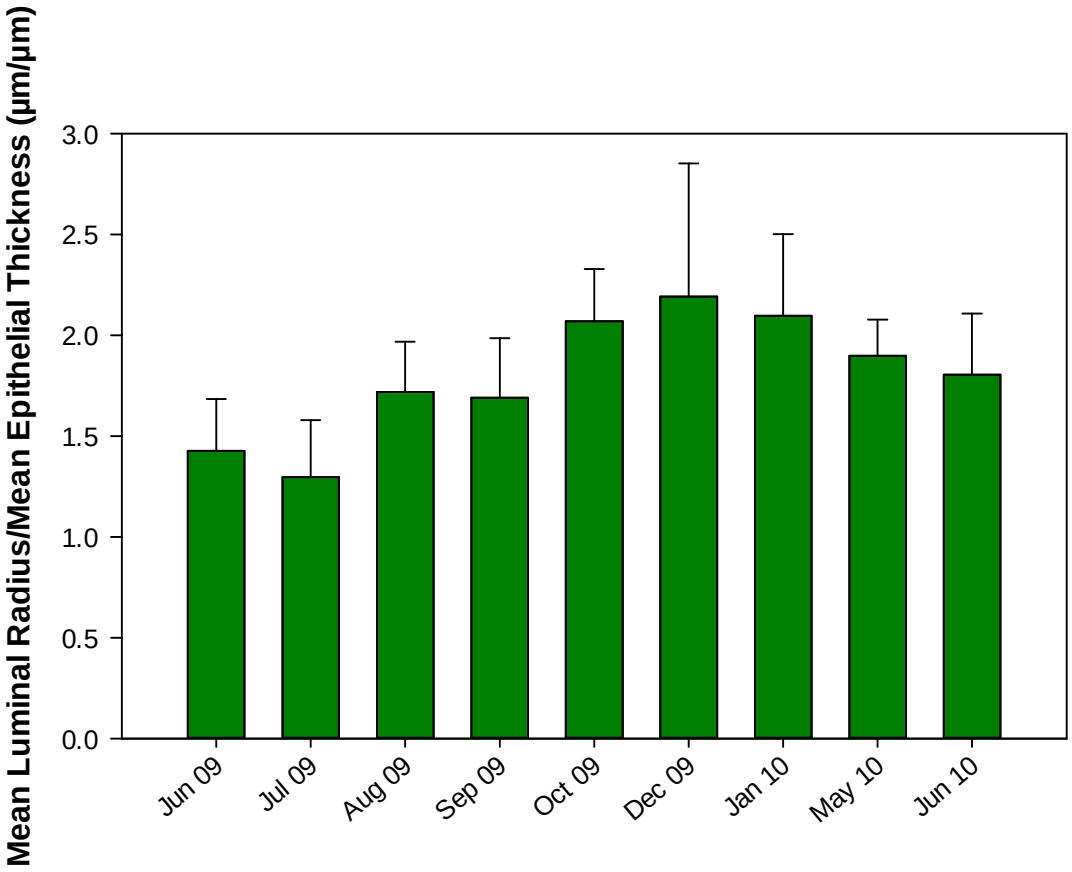

Figure 32 Mean luminal radius/Mean epithelial thickness (MLR/MET) ratio in digestive tubule of *Tapes philippinarum* from Bird Pile, Southampton Water between June 2009 and June 2010. (Mean±SD, n=10 each month)

The relationship between digestive tubule factors and reproductive development are shown in Fig. 33. Epithelial thickness was low in the early developing gonad (developing and late active) and became thicker as the gonad ripened. Digestive tubule thickness was greatest during the spawning phase (an average of 18.76±2.15 µm) and significantly reduced in the spent period (14.40±2.20 µm) (One-way ANOVA, df=4, F=14.96, p < 0.001). Luminal radius appeared to fluctuate throughout gonad development with no statistically significant differences being recorded. The MLR/MET value was high at the beginning of the gonadal cycle (developing stage). A steady, but non-significant, decrease of this ratio was seen from late active stage onwards which declined to a minimum at the spawning phase (an average of 1.43±0.25 µm/µm). The

value (2.03±0.98 µm/µm) later significantly increased in the spent stage clams (One-way ANOVA, df=4, F=7.82, $p < 0.001$). According to the correlation between digestive indices and seawater temperature (Table 8), a weakly positive association was observed between digestive epithelial thickness and temperature, whilst luminal radius and temperature showed no relationships. Furthermore, there was a weakly negative correlation between the MLR/MET and temperature.

Table 8 Estimative of Pearson's product-moment correlation coefficients among digestive parameters and temperature

Variables		r	*P*-Value
Temperature	Digestive epithelial thickness	0.384	< 0.01
	Luminal radius	-0.040	< 0.01
	Mean luminal radius/mean epithelial thickness (MLR/MET)	-0.493	0.021

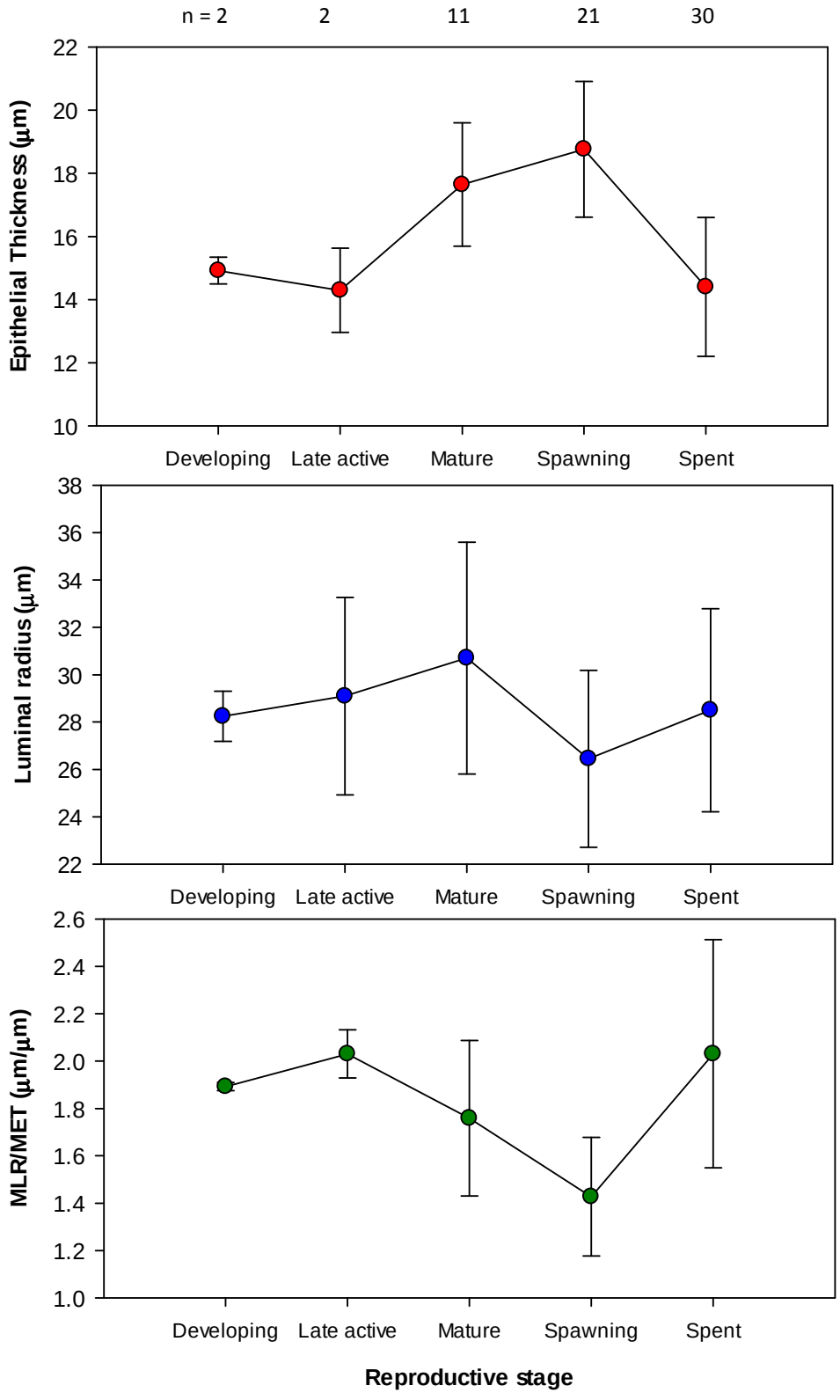

Figure 33 Digestive epithelial thickness, luminal radius and mean luminal radius/mean epithelial thickness (MLR/MET) ratio in digestive tubule of *Tapes philippinarum* from Bird Pile, Southampton Water in each stage of gonad development (Mean±SD, n varies at each point)

3.8 Bivalve larvae abundance

The averaged densities of bivalve larvae in Southampton Water over 9 months of the investigation starting from April 2009 to January 2010 were between 0–0.99 ind.m^{-3} (Fig. 34). Two peaks of bivalve larval densities were observed in summer (June and August 2009) and another peak was in winter (December 2009).

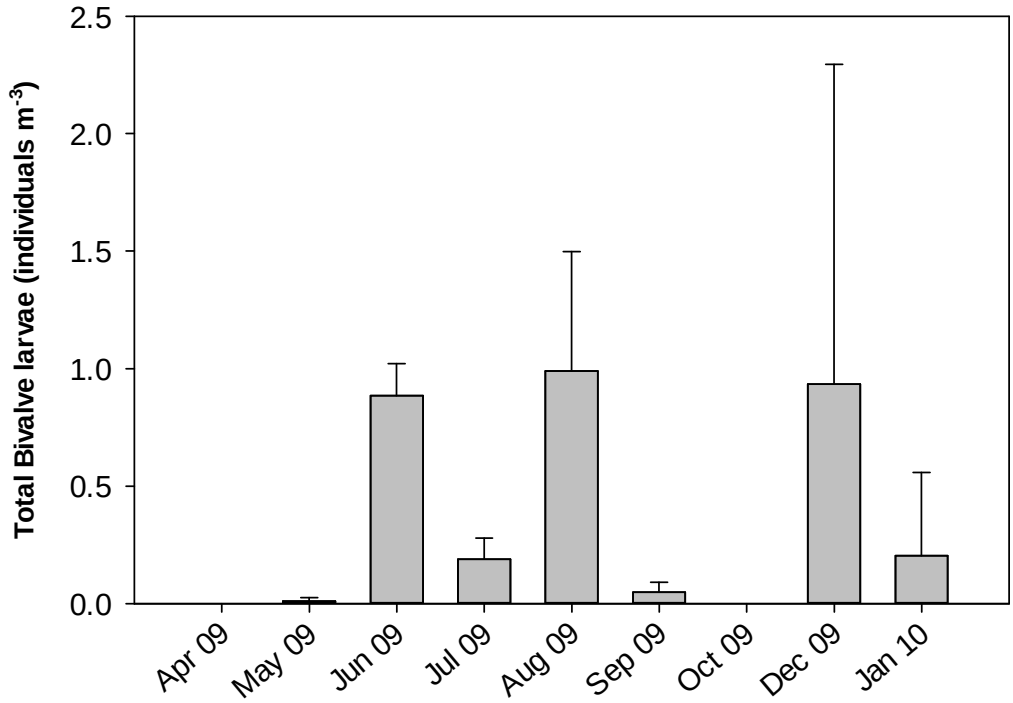

Figure 34 The densities of total bivalve larvae in Bird Pile, Southampton Water between April 2009 and January 2010 (Mean±SD, n = 3 at each point)

This present study attempted to identify species of the bivalve larvae studied based on morphological features. However, it is apparent that such identification is difficult; in particular during the early stages of larval development as D-shaped larvae have a fairly uniform morphology (Abalde *et al.*, 2003; Garland and Zimmer, 2002). Although the bivalve larvae in this current study could not be identified to species level resulting from a lack of expert knowledge and a limited number of bivalve species described in the literature (Chanley and Andrews, 1971; Loosanoff *et al.*, 1966), they were categorised into two main types including

(1) D-shaped larvae (Fig. 35a): The early stage of bivalve larvae was developed from veliger larvae. Hinge is straight and locates at least half of the total shell length.

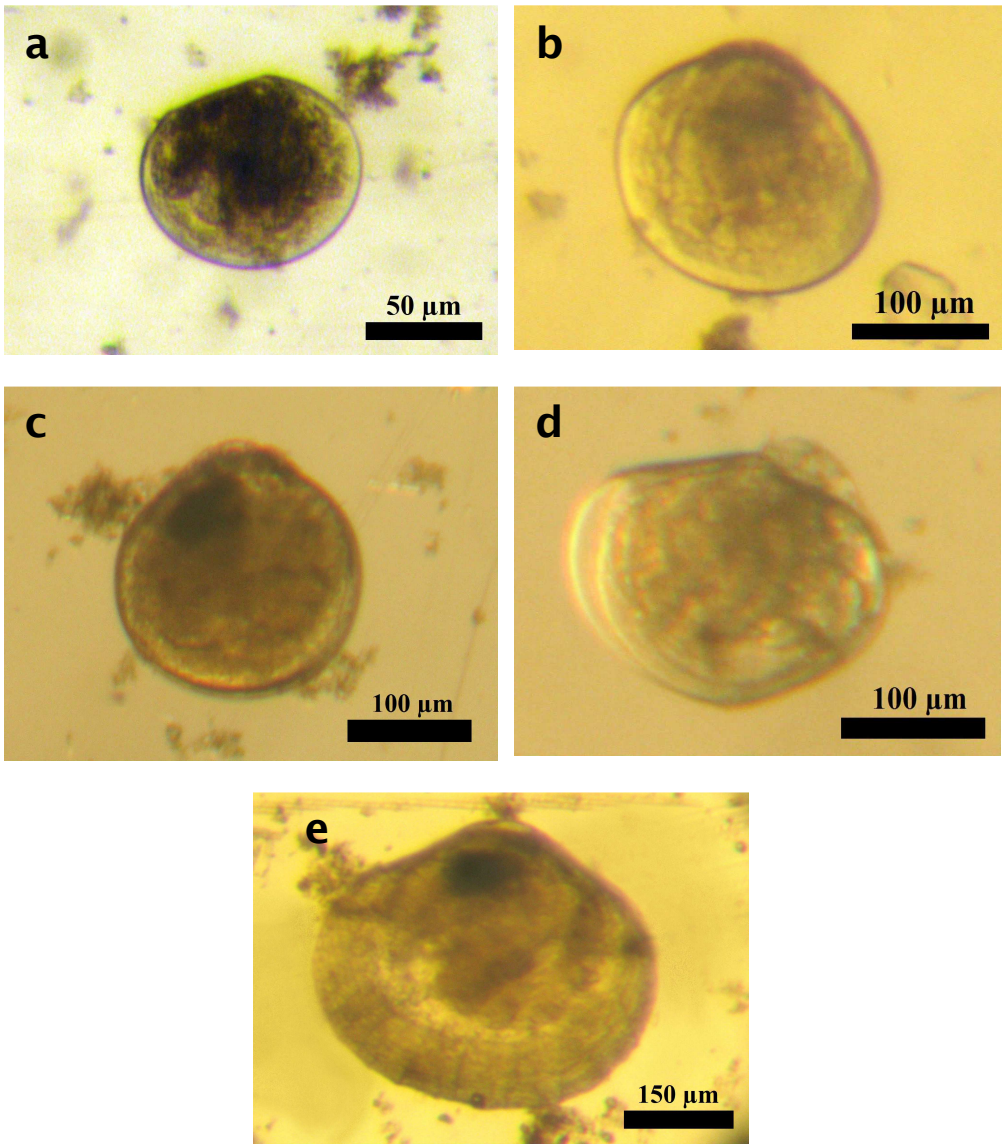

Figure 35 Bivalve larvae from Bird Pile Southampton Water, a: D-shaped larva, b-e: Umbonate larvae, b: type A, c: type B, d: type C, e: type D.

(2) Umbonate larvae: The later stage of bivalve larvae after metamorphosis from D-shaped larvae. Their hinge line is less than half of the total length or umbo is well developed. At this stage, the bivalve larvae can be further divided into four different sub-types;

(2.1) Umbonate larvae type A (Fig. 35b): The shell is equivalve with broadly round in shape. Umbo becomes defined but not much prominent. Shell length is greater than its height. Shoulders are round and anterior and posterior ends also appear rounded. This morphological characteristic is commonly reported in a number of bivalve species, particularly in the early stages for example Veneridae (*Mercenaria mercenaria* and *Tapes semidecussatus*), Mytilidae (*Mytilus edulis*) and Myacidae (*Mya arenaria*) (Chanley and Andrews, 1971; Loosanoff *et al.*, 1966).

(2.2) Umbonate larvae type B (Fig. 35c): The shell is equivalve, discontinuous with obvious umbo (umbos knobby). Shell length is relatively equal to height. Shell shoulders are straight whilst anterior and posterior lengths are almost equal and round. The knobby type of umbo is seen in the larger larvae such as oyster *Ostrea edulis* and *Crassostrea virginica* (Chanley and Andrews, 1971; Loosanoff *et al.*, 1966).

(2.3) Umbonate larvae type C (Fig. 35d): The shell is equivalve and rhomboidal in shape. Shell length is much greater than height. Umbo appears prominent. Both shoulders are straight. Anterior and posterior ends are blunt. Larval shell of family Arcidae, for instance is rhomboidal (Loosanoff *et al.*, 1966).

(2.4) Umbonate larvae type D (Fig. 35e): The shell is equivalve with a prominent umbo. Anterior and posterior ends are blunt. Numerous conspicuous radial ribs were found.

Approximately, 80% of total bivalve larvae were reported as being at the umbonate stage whereas D-shaped larvae accounted for 19.2%. The most abundant type of larva was umbonate larvae type A (69.54%). The umbonate larvae types B, C, and D constituted 2.05%, 3.61%, and 5.62%, respectively of the total bivalve larvae. D-shaped and umbonate larvae type A showed similar trends to total bivalve larvae (two high abundances in summer and one in winter) (Fig. 36). Furthermore, other types of larvae appeared in only spring and summer with very low densities.

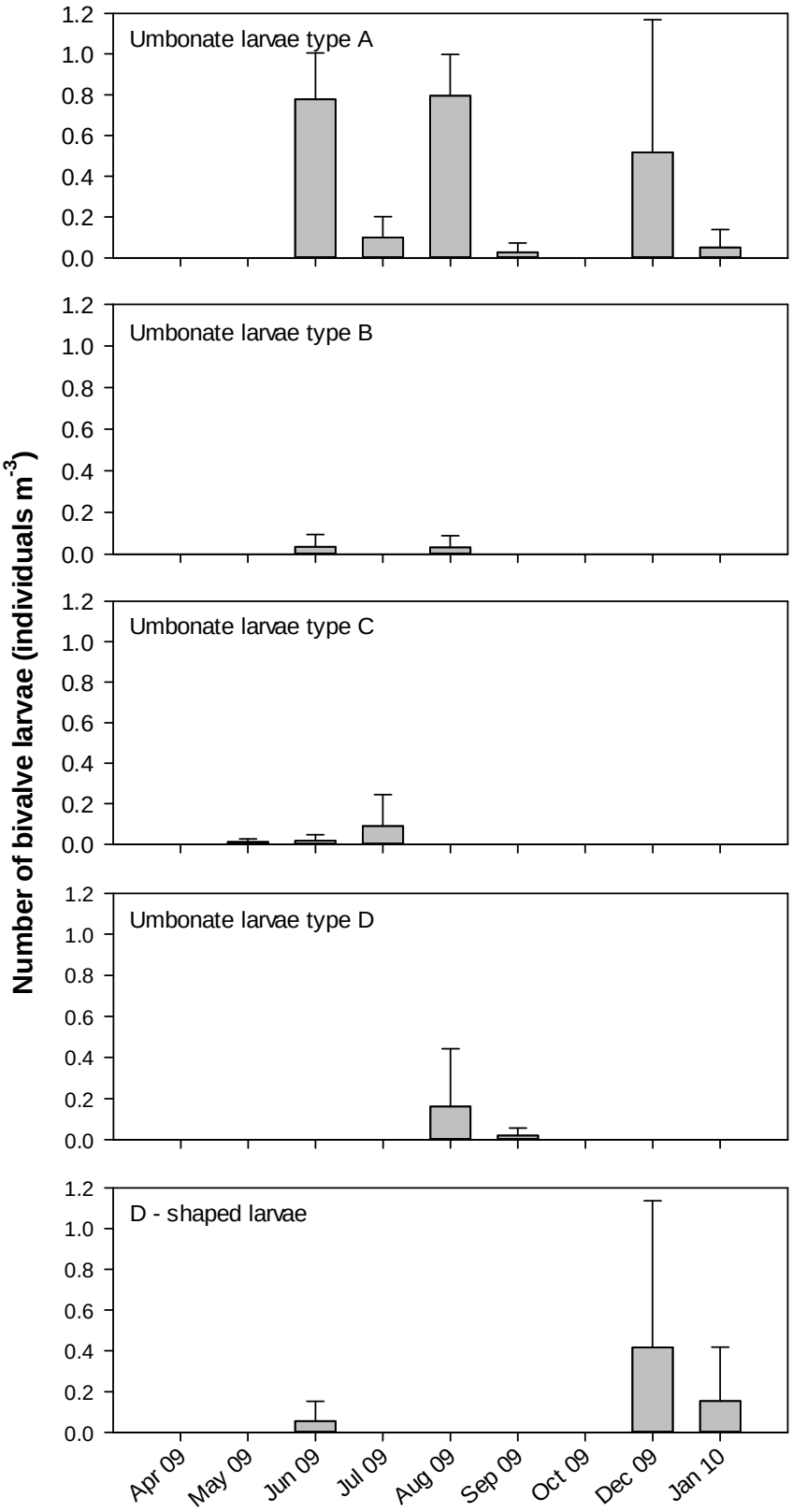

Figure 36 Bivalve larvae densities in each category from Bird Pile, Southampton Water between April 2009 and January 2010 (Mean±SD, n = 3 at each point).

3.9 Spring mortality laboratory experiment

3.9.1 Clearance rate

Clearance rate (CR) of *T. philippinarum* at two different temperatures during 12 weeks and a sample from the wild population is shown in Fig. 37. The results of Two-way ANOVA indicate that CR is affected by temperature and period of study and their interaction (Table 9). After one week a very low CR at 5°C was observed, 0.13±0.16 l g^{-1} h^{-1}. The filtration rate increased at the end of week 4 (0.84±0.85 l g^{-1} h^{-1}) and then decreased in week 8 and 12 (0.40±0.26 and 0.37±0.27 l g^{-1} h^{-1}, respectively) without significant difference. At 10°C, there was no significant difference in CR in the first month studied (week 1; 0.69±0.36 and week 4; 0.87±0.89 l g^{-1} h^{-1}, respectively). A significant increment was seen between week 1 and week 8 (1.64±0.95 l g^{-1} h^{-1}) followed by a distinct decline at week 12 (0.37±0.31 l g^{-1} h^{-1}) (Table 9). The CR of 5°C clam was significantly different from those at 10°C (Table 9). The CR of the wild population was 0.20±0.19 l g^{-1} h^{-1}. No statistically significant differences in CR between both temperatures and wild population were found in week 12.

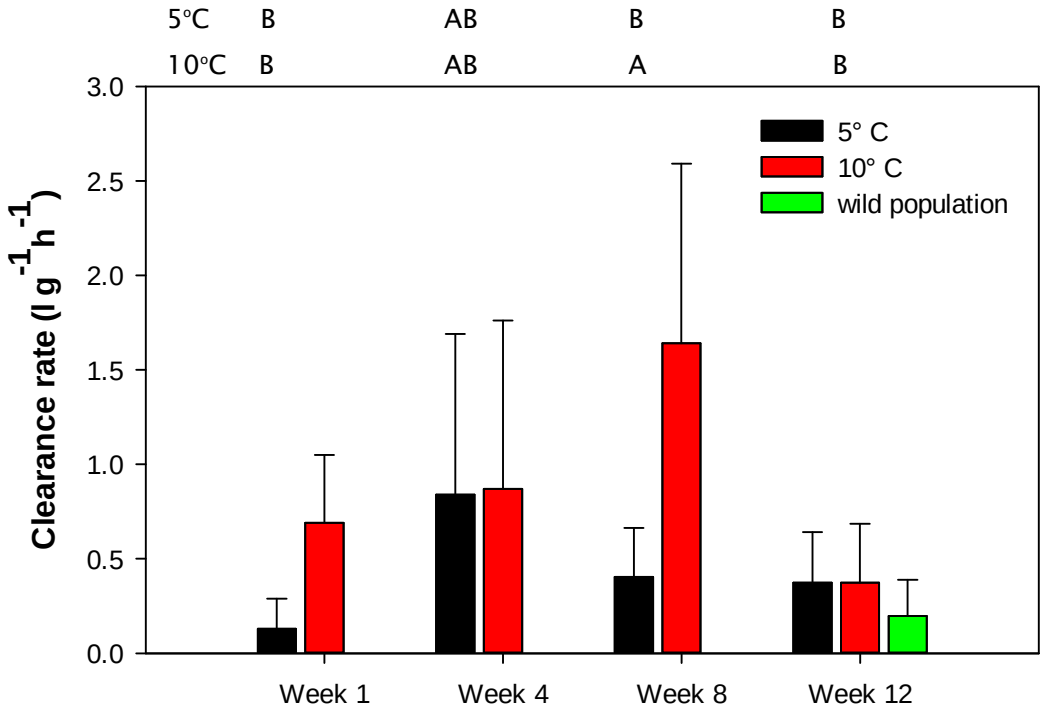

Figure 37 The clearance rate (l g^{-1} h^{-1}) of *Tapes philippinarum* conditioned at 5°C and 10°C and wild specimens sampled at 1, 4, 8 and 12 weeks (Mean±SE, n=12). Shared letters between any temperature-period of study combinations indicate no significant difference.

Table 9 Summary of the results of the Two-way ANOVA performed to determine the effects of temperature, period of study and the interaction of temperature and period of study (Temp x Period) in *T. philippinarum*

Parameter	Factor	df	F	*P*-Value
Clearance rate	Temperature	1	11.84	0.001
	Period of study	3	6.12	0.001
	Temp x Period	3	5.12	0.003
*A*bsorption efficiency	Temperature	1	0.38	0.542
	Period of study	3	2.6	0.062
	Temp x Period	3	4.98	0.004
Respiration rate	Temperature	1	56.63	0.000
	Period of study	3	6.36	0.001
	Temp x Period	3	3.94	0.012
Scope for growth	Temperature	1	0.09	0.767
	Period of study	3	1.32	0.278
	Temp x Period	3	0.51	0.674

3.9.2 Absorption efficiency

Fig. 38 presents absorption efficiency (AE) of *T. philippinarum* conditioned at 5 and 10°C for 12 weeks and in comparison with a sample from the wild population. There is no significant effect of temperature and period of study on the AE, however the interaction of these factors on AE was recorded (Table 9). The AE of the animals at 5°C ranged between 34.83±24.08% and 53.34±11.91%, but no significant difference was seen. The clams at 10°C performed the highest AE at the first week with the value of 70.88±12.61% and then significantly declined at week 8 and week 12 (47.85±19.10% and 36.83±28.44%, respectively) (Table 9). In addition, no significant difference of AE between two temperatures is reported.The wild animals revealed AE at 48.23±20.32% which is not significantly different from that in the clams conditioned at 5 and 10°C at week 12.

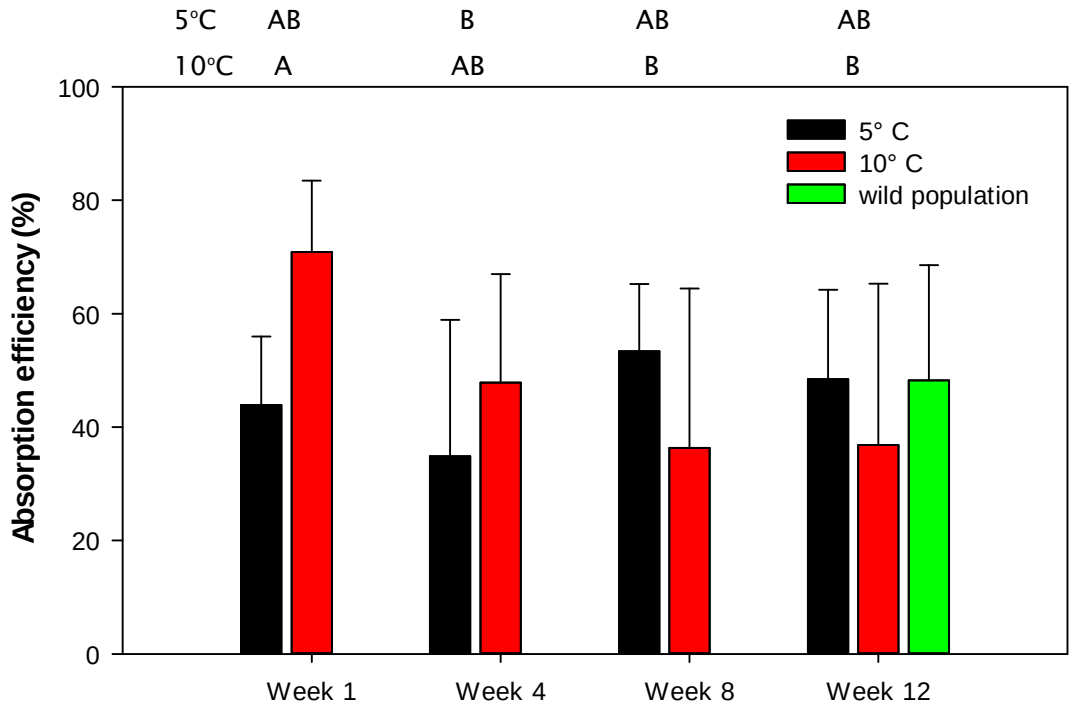

Figure 38 The absorption efficiency (%) of *Tapes philippinarum* conditioned at 5°C and 10°C and wild specimens sampled at 1, 4, 8 and 12 weeks (Mean±SE, n=12). Shared letters between any temperature-period of study combinations indicate no significant difference.

3.9.3 Respiration rate

Respiration rate (RR) of the experimental *T. philippinarum* and the wild animals is shown in Fig. 39. RR is affected by temperature and period of study and their interaction (Table 9). O_2 utilised by 5°C clams was constant throughout the 12 weeks studied, with the consumption varying between 0.03±0.15 and 0.05±0.45 mg O_2 l^{-1} g^{-1} h^{-1} and no significant difference was observed. RR of the animals at 10°C significantly decreased from 0.20±0.67 mg O_2 l^{-1} g^{-1} h^{-1} at week 1 to 0.11±0.05 mg O_2 l^{-1} g^{-1} h^{-1} at week 8 and again declined to 0.09±0.05 mg O_2 l^{-1} g^{-1} h^{-1} at week 12 (Table 9). Only RR of 5 and 10°C clams at week 8 shows significant difference (Table 9). The wild animals consumed oxygen at a lower rate of 0.03±0.02 mg O_2 l^{-1} g^{-1} h^{-1} which is significantly different from the clams at 10°C at week 12 (0.09±0.05 mg O_2 l^{-1} g^{-1} h^{-1}) (One-way ANOVA, df=2, F=5.11, $p < 0.05$).

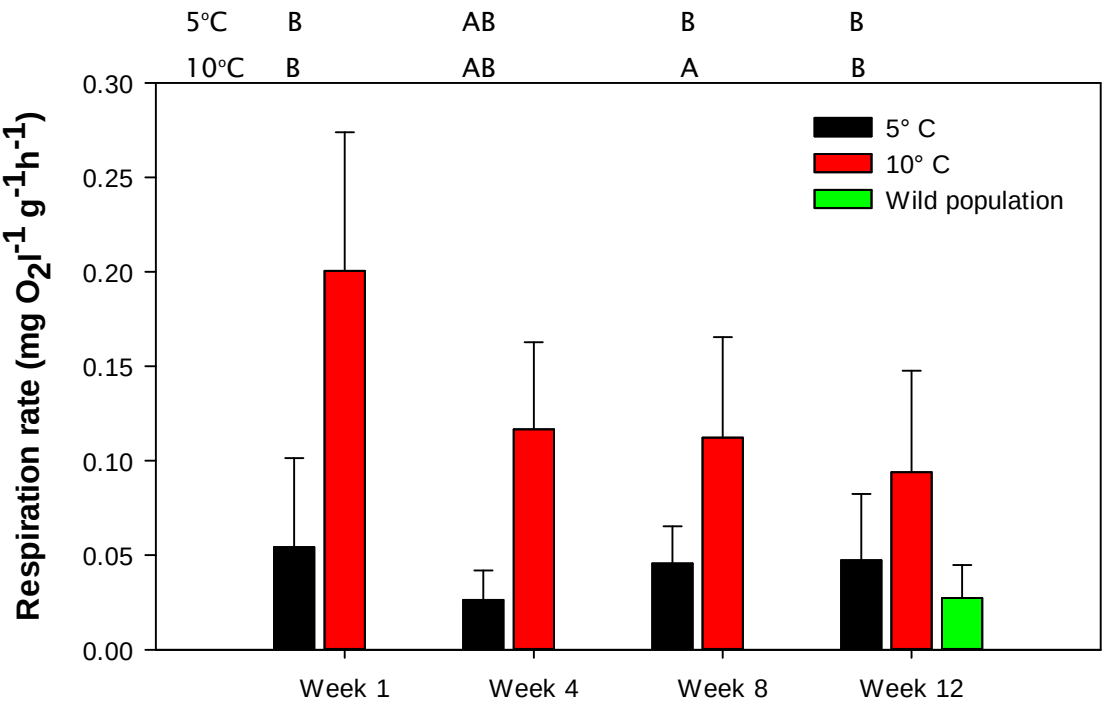

Figure 39 The respiration rate (mg O_2 l^{-1} g^{-1} h^{-1}) of *Tapes philippinarum* conditioned at 5°C and 10°C and wild specimens sampled at 1, 4, 8 and 12 weeks (Mean±SE, n=12). Shared letters between any temperature-period of study combinations indicate no significant difference.

3.9.4 Scope for Growth

Scope for growth (SFG) of *T. philippinarum* conditioned at 5 and 10°C for 12 weeks and compared with samples from Southampton Water is shown in Fig. 40. There is no effect of temperature, period of study and their interaction on SFG (Table 9). The animals reared at 5°C revealed the SFG values close to zero at week 1 (0.07±1.39 J g^{-1} h^{-1}). The SFG increased in week 4 (6.02±7.72 J g^{-1} h^{-1}) followed by declines at week 8 and 12 (3.68±3.25 and 3.10±3.21 J g^{-1} h^{-1}, respectively). Nevertheless, there is no significant difference between the SFG values throughout the period of study. The higher SFG during the first eight weeks in the animals conditioned at 10°C (ranging from 5.59±4.78 to 7.36±3.42 J g^{-1} h^{-1}, respectively) were not significantly different from those at week 12 (1.17±3.08 J g^{-1} h^{-1}). The SFG of the clam conditioned at 5°C is not significantly different from those at 10°C throughout the period of study. The SFG of the wild samples was 1.16±2.13 J g^{-1} h^{-1}. No statistically significant difference in the SFG was found between the animals at 5, 10°C and the wild population at week 12.

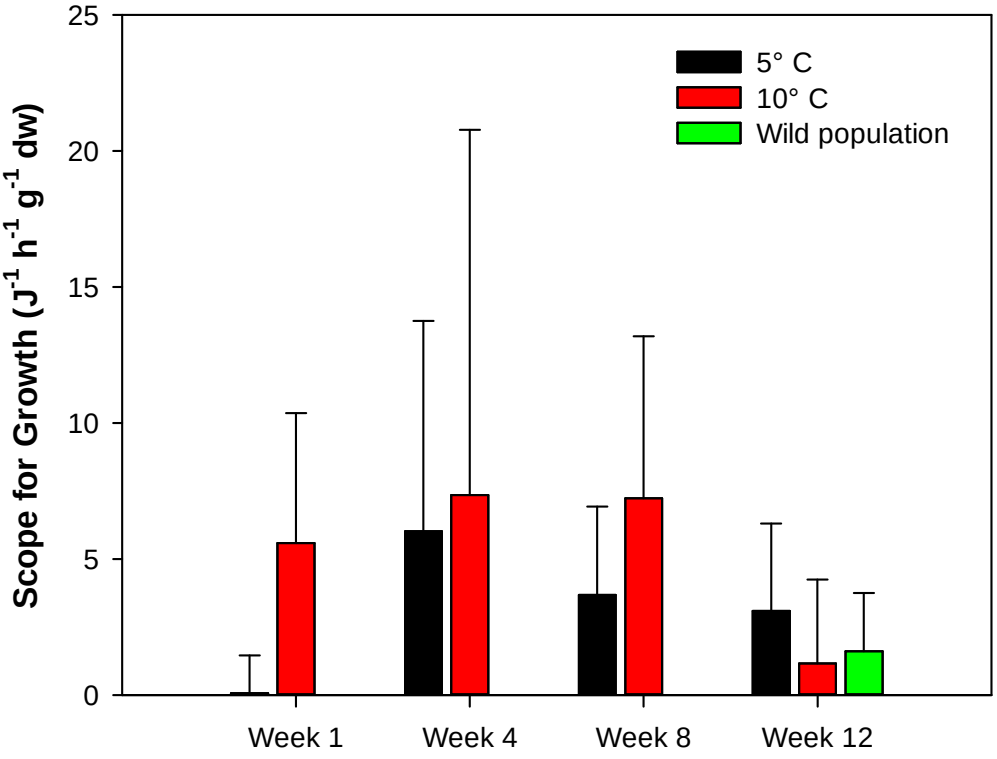

Figure 40 The scope for growth (J g^{-1} h^{-1}) of *Tapes philippinarum* conditioned at 5°C and 10°C and wild specimens sampled at 1, 4, 8 and 12 weeks (Mean±SE, n=12)

3.9.5 Reproductive development

The reproductive development of *T. philippinarum* from the 5 and 10°C experiments investigated for 18 weeks and natural habitat is shown in Fig. 41. During the first eight weeks of the experiment, the gonads of the animals at both 5 and 10°C were in either spent or inactive stage (they were collected at this stage). However, the eventual gonad development of the animal conditioned at 10°C was faster than those at 5°C. During the first 8 weeks SFG experiment, the percentage of inactive gonad of the clams at 10°C ranged between 66.67 and 100% which was higher than those in 5°C ranging from 50 to 66.67%. All gonads (100%) of the animals at 5°C became inactive starting from week 12 onwards whilst the onset of gametogenesis was observed in the clams conditioned at 10°C. The prevalence of early active gonads was only seen in the clams at 10°C (33.33% of gonad at week 12 and 66.67% of gonad at week 18) and the wild population (66.67%).

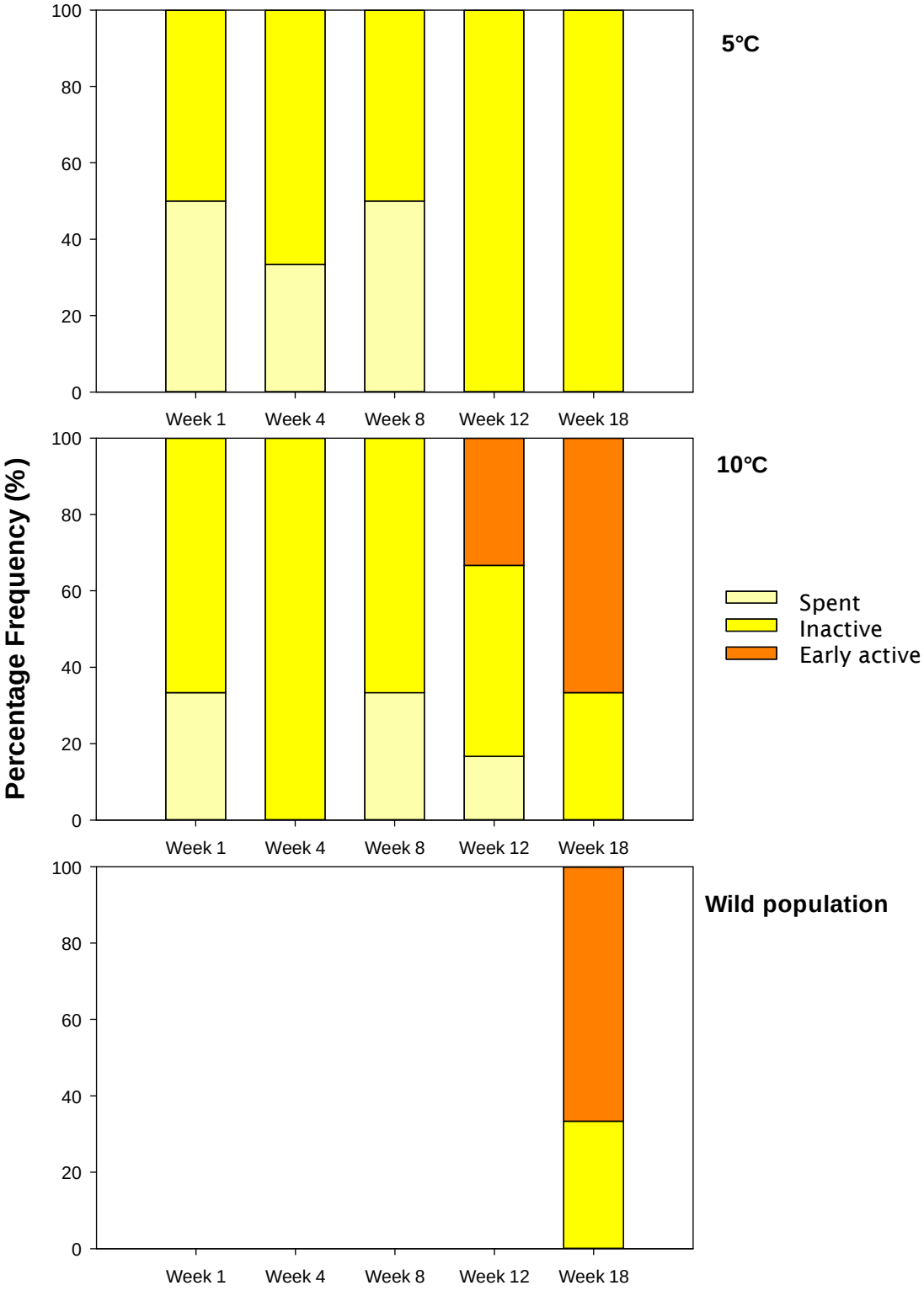

Figure 41 Reproductive development of *Tapes philippinarum* conditioned at 5°C and 10°C and wild specimens sampled at 1, 4, 8, 12 and 18 weeks

3.9.6 Weight loss

Fig. 42 manifested the cumulative growth of *T. philippinarum* from the 5 and 10°C experiments observed for 18 weeks. Two-way ANOVA performed to determine the difference in the cumulative growth rate between two temperatures and time showed that the cumulative growth was affected by temperature (Two-way ANOVA, df=1, F=27.86, p<0.001). However no significant effect of time and interaction between temperature and time was recorded. The wet weight of animals conditioned at 5°C seemed to increase throughout the experiment ranging from 0.01 to 0.26%, but no significant difference was found. Total weight increased from week 1 and became maximal at week 8, but declines were reported at week 12 and 18 without significant difference. At 10°C, wet weight fluctuated in a narrow range between -0.02 and 0.04% without significant difference. The cumulative growth rate of the animals at 5°C was significantly higher than those at 10°C only at week 4 (0.22±0.21 and -0.02±0.10, respectively) and week 12 (0.20±0.16 and -0.03±0.09, respectively) (Two-way ANOVA, df=1, F=27.86, p<0.001).

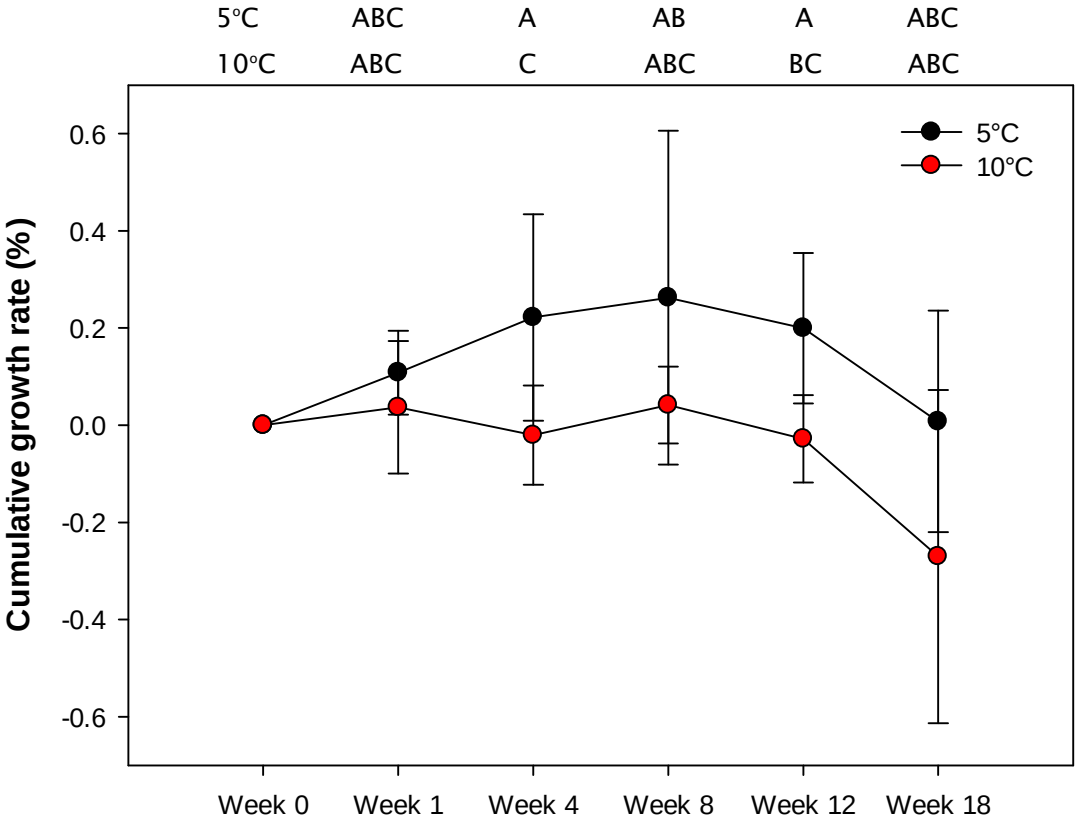

Figure 42 Cumulative growth rate (%) of *Tapes philippinarum* conditioned at 5°C and 10°C sampled at 0, 1, 4, 8, 12 and 18 weeks (Mean±SE, n=12), Shared letters between any temperature-period of study combinations indicate no significant difference.

Chapter 4 Discussions

4.1 Environmental parameters

The seawater temperatures in Southampton Water during this study showed a seasonal variation that was minimal in winter (approximately 5°C) and at a maximum in summer (20°C). The salinity was relatively high (about 30-35) throughout the year and no seasonal pattern was observed. These data are in a good agreement with previous reports describing the same area (Chinnery and Williams, 2003; Hirst *et al*., 1999; Howard *et al*., 1995; Iriarte and Purdie, 1994; Williams and Muxagata, 2006). Chl_a concentrations in Southampton Water peaked in Spring (April 2009; 7.96 µg l^{-1}) prior to a decline to intermediate values (1.76-4.06 µg l^{-1}) in summer. The Chl_a levels then dropped down to the lowest in winter (0.32 µg l^{-1}). A similar pattern was observed by Iriarte and Purdie (1994), Howard *et al*. (1995), and Williams and Muxagata (2006). However, in contrast to the other observations, no summer bloom with its characteristic peak of Chl_a was seen in this study. The summer bloom previously appeared between June and August (Howard *et al*., 1995, Iriarte and Purdie, 1994, Williams and Muxagata, 2006). Furthermore, the peak of Chl_a (7.96 mgm^{-3}) observed by this study was considerable lower than the previous reports in the same area for example 15-18 mgm^{-3} in 1988 (Howard *et al*, 1995), 16-18 mgm^{-3} in 1990 (Iriarte and Purdie, 1994), 8-18 mgm^{-3} from 2001 to 2002 (Williams and Muxagata, 2006), and 32.9 mgm^{-3} in 2003 (Hazeem, 2003).

4.2 Population structure

In the present work population structure has been used not only to quantify population growth but also to reveal the standing stocks of *Tapes philippinarum*. The population structure of *T. philippinarum* in Southampton Water mainly represented a unimodal pattern which is a similar character of the mollusc population with a low share of young and the predominance of animals in the middle size classes (Ponurovsky, 2008). The mean shell length of the clams in this area (31.47$\pm$8.56 mm) is smaller than that reported for the naturalised and farmed populations in Poole Harbour (39.56 and 41.95 mm, respectively) (Grisley, 2003) and the same species in Amursky Bay, South of Japan (38.5$\pm$0.5 mm) (Ponurovsky, 2008). The limits of sampling by dredge possibly resulted in low representation of individuals below 20 mm in shell length. This problem was also reported in the study of *T. philippinarum* in Poole Harbour (Grisley, 2003). A total of 350 *T. philippinarum* were collected from Southampton Water in February 2012 using grab ranging from 5 mm to 47 mm shell length (Jensen,

unpublished). A higher proportion of small clam was recorded as the specimens were numerically dominated by the size class 20-25 mm (34.57% of the total samples) and the clams smaller than 20 mm accounted for 38.57% of the samples (Jensen, unpublished).

A low abundance of larger size classes (>40 mm) in this study possibly revealed the influence of previous fishing activity regulated by the former EU MLS 40 mm. The MLS was changed to 35 mm in 2007 (Dang *et al.*, 2010). Similarly, Grisley (2003) revealed that naturalised *T. philippinarum* (40+ mm) in Poole Habour was seen in a small proportion as a result of fishing impact (MLS 40 mm). In 2012, *T. philippinarum* from Southampton Water sampled using grab recorded only 2.57% of the total specimens (350 individuals) were above MLS of 35 mm shell length (Jensen, unpublished). Dang *et al*. (2010) reported that the majority of population in Arcachon Bay, SW France were clams of 28-40 mm in shell length. The scarcity of adults >40mm and juveniles was obvious. Dang *et al*. (2010) showed that neither reproductive deficiency nor high larval mortality accounted for this phenomenon, but fishing impact did. Increases in proportion of small clams (<25 mm) from August, October and December 2009 in the current study tends to indicate the settlement period of this species. Spawning season of *T. philippinarum* in Southampton Water was between May and September with a single peak from June to August.

4.3 Growth rate

Annual growth rate of adult *Tapes philippinarum* in Southampton Water was remarkably high during summer (June-August 2009; 72.1%-88.9% of annual growth). Growth rapidly rose from June to September (growth rate in shell length 0.12-0.59 mm per month), then slowed down after October (growth rate in shell length 0.02-0.04 mm per month), and finally ceased between November and January with a decline of wet weight. A similar growth pattern was seen in transplanted *T. philippinarum* at age 1-3 years old and a wild population in Jiaozhou Bay, China (Ren *et al.*, 2008). A decline of total weight in the clams from Southampton Water in winter (January 2010) is possibly caused by the combined stress between low temperature and insufficient food during winter (Urrutia *et al*., 1999; Zhang and Yan, 2006). Similarly, juvenile of *T. philippinarum* from a farm in Zhuanghe, Dalian, Liaoning Province, the northern China showed no growth during winter due to the low seawater temperature ranging from -2.7 to 3.0°C (Zhang and Yan, 2006). The final cumulative growth rate of shell length in this study (8.34%) was close to the growth rate of the clam at age 3 years old observed by in Jiaozhou Bay, China (9.7%) (Ren *et al*., 2008). However, Ren *et al.* (2008) recorded that the clams at the age of 1 and 2 years old grew faster in total length

(83.8% and 12.0%, respectively) than the age of 3 years old (9.7%). Moreover, total wet weight of the one-year-old clam increased 123% per year. Gosling (2003) stated that growth of bivalve in the first year of their lives is rapid but progressively slows down with age. It is common that bivalve growth from low latitudes is faster than those in higher latitudes (Gosling, 2003). The growth rate of *T. philippinarum* in the current study (Lat 50°N) which closed to the population in lower latitude (Jiaozhou Bay, China; Lat 36°N) (Ren *et al*., 2008) indicated that the UK marine environment is favourable for this non-native species.

4.4 Reproduction

The sex ratios (male:female) in *Tapes philippinarum* reported in the current study including Bird Pile, Southampton Water (1:0.8) and Poole Habour (1:0.94) are not significant different from the ratio 1:1. The ratio from both areas are similar to the results observed by many authors (1:1) including Grisley (2003) in Poole Habour, Cooke (2009) in Bird Pile, Southampton, and Drummond *et al.* (2006) in North West of Ireland and Ponurovsky and Yakovlev (1992) in the north western part of the Sea of Japan. However the data is not in agreement with the findings obtained by Meneghetti *et al*.(2004) in the Venice Lagoon, Italy and Ponurovsky (2008) in Amursky Bay, Sea of Japan with male/female ratio 1:1.67 and 1:1, respectively.

It is widely known that *T. philippinarum* is a dioecious bivalve and the appearance of hermaphrodite is rare (Drummond *et al*., 2006; Holland and Chew, 1974; Ponurovsky, 2008; Ponurovsky and Yakovlev, 1992). 82.35 % of the sexually unidentified individuals from Bird Pile, Southampton Water were observed during the late autumn until the early spring (September-March). The period is recognised as being the post-spawning, resting, and onset of gametogenesis of the animals. It is difficult to determine the sex of individuals in such a period especially in young/small individuals. Clams smaller than 25 mm shell length (94.11%) formed the majority of undifferentiated individuals.

The annual reproductive cycle of *Tapes philippinarum* has been previously studied (Beninger and Lucas, 1984; Cooke, 2009; Drummond *et al*., 2006; Grisley, 2003; Laruella *et al*., 1994; Ponurovsky and Yakovlev, 1992; Sbrenna and Campioni, 1994; Shpigel and Spencer, 1996, Toba *et al*., 1993; Toba *et al*., 2007; Uddin *et al*., 2010). The onset of gametogenesis of *T. philippinarum* from Southampton Water in this study was observed in February and March. This is followed by a period of gamete development, and reaching maturity in May. The spawning commences in May or June and continues until September. Spawning appeared to be continuous between June and August. From September onwards, most of sexual products had been released and the

gonads were entering the winter resting stage (December-February). However, two spawning peaks were observed in the Po River Delta, Italy (Sbrenna and Campioni, 1994) and Tokyo Bay (South eastern Japan) (Toba *et al*., 1993). In contrast to the study carried out by Meneghetti *et al*. (2004), gametogenesis of *T. philippinarum* in the Venice Lagoon, Italy began earlier (January) than those reported here. According to Grisley (2003) and Sbrenna and Campioni (1994), gonad of the animals was developing later in Poole Habour, UK and Canarin and Scardovari Lagoon, Italy (April), respectively in comparison to the present investigation in Southampton Water (February and March). The gonad of *T. philippinarum* from Poole Habour in this study started developing in March and matured in June. Gamete release was observed between July and September. Reproductive inactivity then occurred from November to January. The gametogenesis of population from Poole Habour commenced a little later than those in Southampton. In contrast to Southampton Water, the gonads in females from Poole Harbour had developed earlier than those in males. These are in agreement with the observation by Grisley (2003) in Poole Harbour.

T. philippinarum in Southampton seems to have adapted to the lower water temperature (compared to its native range) of the UK. The lower temperature limits is 10°C for initiating gonadal activity (Millican and Williams, 1985) and 14°C for maturing and spawning (Holland and Chew, 1974; Ohba, 1959). In addition, based on the minimum threshold (10°C) for gametogenesis, *T. philippinarum* has been shown to successfully spawn after being conditioned for 480 D° (Utting and Doyou, 1992), 500-600 D° (Beninger and Lucas, 1984), 460 D° (Kersuzan, 1989), and 560 D° (Lovatelli, 1985). In contrast, the gonads of *T. philippinarum* from Bird Pile were active in February 2009 when the seawater temperature remained much lower (4.8°C) than the assumed limit for gametogenesis (10°C). Furthermore, the water temperature in May when clams were observed the maturing and spawning was 13.3°C, calculated as about 64.27 D°. This shows that *T. philippinarum* are able to develop gametes and spawn below the assumed 10°C limit previously reported. Similarly, the gonads of population in North West Ireland were active in February (the average bimonthly water temperatures were 7.3°C and 5.8°C). Minor spawning was seen at the temperature 13.1°C in May (Drummond *et al*., 2006). Using the number of day degrees alone to predict the spawning period of bivalves might be inappropriate. Firstly, this is because gametogenesis is affected by not only temperature but also other exogenous factors such as food availability and endogenous factors e.g. energy reserves (Utting and Millican, 1997). For example, the day degrees of *Ostrea edulis* provided with microalgae supplements was 330-390 D°, whilst longer duration for spawning (385-540 D°) was found in those without supplements. The day degree is also affected by the extent of gonad development when conditioning takes place. The spawning of *T.*

philippinarum started conditioning at 15, 18 and 20°C in November (early resting stage) took place in 17 weeks (595 D°), 13 weeks (728 D°), and 11 weeks (847 D°), respectively (Mann, 1979). However more rapid day degree was seen in *T. philippinarum* commenced the experiment in February (late resting stage) (Delgado and Camacho, 2007). Ripening and spawning in individuals conditioned at 14 and 18°C was shown at day 78 (equivalent to 312 D°) and day 32 (256 D°), respectively (Delgado and Camacho, 2007). Secondly, the relationship between temperature and bivalve gametogenesis is complex and gametogenesis is a function of absolute temperature and duration of exposure rather than the achievement of a critical temperature (Mann, 1979). Additionally, spawning could be triggered by even short-lived temperature increases (Gosling, 2003).

Gamete atresia or gamete resorption was found in *Tapes philippinarum* from Poole Harbour in this study. It is a physiological process occurring in the gonads of both invertebrates and vertebrates associated with programmed cell death (Reunov and Crawford, 2010). Atresia is a common process of destruction of unspawned mature gametes in post-spawning gonads (Tyler *et al*., 1982). However it can takes place as a mechanism to provide space for the growth of gonadal products (Devine *et al*., 2000; Mermillod *et al*., 1999), as a consequence of egg competition for blood supply (Boyle and Chevis 1992), as a reaction to infection (Avarre *et al*., 2003), and as a factor of fecundity regulation (Kurita *et al*., 2003). In addition, atresia can be influenced by environmental stresses e.g. nutritional deficiency, inappropriate temperature (too low or high), environmental contamination (oils, polycyclic aromatic hydrocarbons (PAHS), DDT, and alkylphenols) (Aarab *et al*., 2004; Bayne, 1976; Binelli *et al*., 2001; Lowe, 1988; Lowe and Pipe, 1987; Newell, 1989; Ortiz-Zarragoitia and Cajaraville, 2006; Ortiz-Zarragoitia and Cajaraville, 2010; Ortiz-Zarragoitia *et al*., 2011; Vaschenko *et al*., 1997). In reproduction, the atresia is a mechanism the animals use to save energy rather than waste it because of unfavourable conditions for spawning. With regard to the short life span of oocytes, if they are unable to be spawned, they will be lysed in order to supply energy for new oocytes (Dorange and Le Pennec, 1989; Le Pennec *et al*., 1991; Morvan and Ansell, 1988; Pazos *et al*., 1996; Pipe, 1987a; Pipe, 1987b). Pollution events may cause the gametes to undergo massive lysis by the pollutants or be degenerated to provide the energy to deal with the metabolic demands of detoxification (Dorange and Le Pennec, 1989; Ortiz-Zarrogoitia, 2011). Atresia acts as a buffer process to transfer energy away from the gonads in unfavourable energetic condition (Pazos *et al*., 1996). This reproduction impairment could lead to a possible decline in gamete quantity and consequently cause ineffective recruitment to the stock of animals.

The atresia in this study was mainly found in *T. philippinarum* from Poole Harbour throughout the 2009 spawning season (50%-83.33%), but was presented at much lower level in 2010 (0.00%-16.67%). Interestingly, low occurrence of spatfall in Poole Harbour was observed in 2009, but a higher density of the spatfall was evident in 2010 (Matthew Harris, pers.comm.). It is likely that limited settlement of *T. philippinarum* in some years has been caused by gamete atresia. However, no prevalence of atresia in the population from Poole Harbour observed between 2000 and 2001 (Grisley, 2003). The gonad resorption therefore seems uncommon for the population in the area. It is likely that the oddity could be related to adverse environmental condition prevailing in the summer of 2009. As now that various factors could cause the atresia, the actual reason of this event needs further investigation.

The minimum size at sexual maturity of *Tapes philippinarum* observed in this study (17.9 mm in males and 20.2 mm in females) seems to be at the higher end of minimum size of maturity reported in the literature. Holland and Chew (1974) report gamete development in the 5-10 mm shell length range at Big Beef Harbor and Misery Point located in Hood canal, Washington. Mature and spawned individuals, were found in animals larger than 15 – 20 mm and 20-25 mm respectively. Ko (1957) revealed that ripe gonads were found in *T. philippinarum* from Japan with a shell length of 12 mm and many individuals about 15 mm spawned. A few clams between 7.5 and 8.6 mm were recorded as mature in Vostok Bay, Sea of Japan (Ponurovsky and Yakovlev, 1992). In Possjet Bay and Vostok Bay, Sea of Japan, males within the shell length range 10-15 mm and females within 15-20 mm were ripe (Holland and Chew, 1974; Ponurovsky and Yakovlev, 1992). Similar findings were recorded by Cooke (2009) in *Tapes philippinarum* from Bird Pile which showed that the lowest size at maturity in female was 19 mm with an averaged oocyte diameter of 39.96$\pm$5.88 μm. Even though most of the reports point out that the size at first sexual maturity in *T. philippinarum* is about 15-20 mm, not all individuals reached full maturity and complete spawning at this size. As indicated in this study, a high percentage of individuals smaller than 25 mm shell length were found to be immature. Poor development of gonads in clam less than 20 mm shell length was reported by Yap (1977). In the Sea of Japan, all individuals in Melkovodnay and Loga Bays became mature at the length of 30-35 mm. Furthermore, no mature gametes were found in some individuals as large as 40-45 mm from Vladimir Bay (Ponurovsky and Yakovlev, 1992). Cooke (2009) described that the smallest spawning individuals (as against mature) found were all > 20 mm (22 mm in male and 27 mm in female).

The linear relationship is seen between oocyte sizes of *T. philippinarum* in Southampton Water and time during the spawning season. The mature eggs from

Southampton's samples were first measurable in May (early spawning season) and had the smallest diameter in comparison to the following months. The egg diameter increased in June and July (early spawning season) and became the largest in August (late spawning season). Similarly, the smallest mature ooctyes from Poole Harbour clams were present for the first time in June and July and were significantly larger in September (late spawning season). The same pattern was reported by Laruelle *et al.* (1994), a mean oocyte diameter of *T. philippinarum* in the Bay of Brest, France rose from 27.79 μm in early May to 52.21 μm in early July as well as at Morbihan Gulf from 31.32 μm in early April to 57.84 μm in early July. Cooke (2009) recorded the first ripe eggs in *T. philippinarum* from Bird Pile, Southampton in June 2008 with a mean diameter of 35.9±0.5 μm. The oocyte size then continued to increase until approaching the maximum diameter in September 2008 (43.3±0.2 μm) (Cooke, 2009).

Uniquely, this study has produced data suggesting a relationship between oocyte diameter and shell length of *T. philippinarum*. It is believed that a larger oocyte diameter enhances the fertilization success and larval viability of marine invertebrates (Luttikhuizen *et al*., 2004; Luttikhuizen *et al*., 2011). In fact, lipid (Gallager and Mann, 1986) or protein content (Li *et al*., 2011) in oocyte of *Mercenaria mercenaria*, *Crassostrea gigas* and *Mactra chinensis* which play the role as energy reserve for the marine planktotrophic larvae increase with the oocyte diameter. There are significant correlations ($p < 0.01$) between the initial oocyte lipid content and survival of both D-shaped larvae (24 h) and pediveliger larvae of both *Mercenaria mercenaria* and *Crassostrea gigas* (Gallager and Mann, 1986). Whilst not reported elsewhere for *T. philippinarum* the relationship between size of eggs and shell length of animals was shown in *Coecella chinesis* (Kang *et al*., 2007) and *Yoldia notobilis* (Nakaoka, 1994). Conversely, no differences in oocyte diameter between size classes were seen in *Mya arenaria* (Brousseau, 1987), *Macoma balthica* (Honkoop and van der Meer, 1998), *Mactra venerformis* (Chung and Ryou, 2000) and *Callista chione* (Martinez-Pita *et al*., 2011). Kang *et al.* (2007) pointed out that physiological adaptation of the clam *Coecella chinesis* to poor food availability could result in small oocytes. This is also confirmed by Toba and Miyama (1994). They found that egg diameter did not significantly differ between size classes in conditioned *T. philippinarum* fed with sufficient algae. This would suggest that in 2009 the reproductive potential of *T. philippinarum* in Southampton Water was reduced by a reduction in the availability of food. A peak of Chl_a concentrations from Southampton Water in 2009 was 7.96 mgm^{-3} which was considerable lower than the previous reports for example 15-18 mgm^{-3} in 1988 (Howard *et al*., 1995), 16-18 mgm^{-3} in 1990 (Iriarte and Purdie, 1994), 8-18 mgm^{-3} from 2001 to 2002 (Williams and Muxagata, 2006), and 32.9 mgm^{-3} in 2003 (Hazeem, 2003).

Currently the Minimum Landing Size (MLS) for *T. philippinarum* is set at 35 mm shell length by current EU legislation. Recent suggestions by English fishermen that the 35 mm MLS could be reduced to say, 20 mm are not supported by this study which suggests that the MLS must not drop below 34 mm if all clams are to be allowed to reproduce at least once before harvest. At 29.7 mm all clams sampled during the spawning season were ripe and none of the clam below 29.8 mm shell length had the average oocyte larger than 44 µm despite the range of average egg diameter in all individuals being between 31.13±6.31 and 55.74±6.20 µm. Furthermore oocyte size of *T. philippinarum* in Southampton Water was positively related to the shell length. Therefore retaining clams above 30 mm shell length in the fishery would ensure the success in reproduction and oocyte size large enough that viable larvae would be produced. If the clams at 30 mm shell length are to be allowed to reproduce once before harvest, it will grow to approximately 34 mm shell length within one reproductive cycle based on the annual growth rate of *T. philippinarum* (12%) (Ren *et al*., 2008). In addition, the possibility of 'atresia' as observed in Poole Harbour needs to be taken into consideration; meaning that not all years will provide favourable environmental conditions for a successful reproductive cycle. 'Spring mortality', the death of notable percentage (up to 90% in some years, Wordsworth pers. comm.) of adult clams in spring thought to be caused, at least in part, (Grisley, 2003) by starvation induced by a late spring phytoplankton bloom following a 'warm' winter, will also occasionally reduce the numbers with the spawning population. It is believed that the current 35 mm MLS acts to provide a viable, sustainable stock of clams in the existing conditions on the South coast of the UK and this should be maintained. Bald *et al*. (2009) evaluated the *T. philippinarum* stock in Arcachon Bay, France using a model and pointed out that the best management measures are, in order of efficiency: (i) the maintenance of the MLS (35 mm), (ii) a reduction the harvest season, and (iii) an increase of no-fishing zones. They noted that the allowance of fishing the clam below MLS (10%, between 21-34 mm) resulted in a decrease of the stock about 13%.

4.5 Immunological parameters

4.5.1 Haemocytes

Bivalve haemocytes are part of the defence mechanism against pathogens and foreign materials and are susceptible to environmental changes. They can, therefore, be used as an indicator to monitor the status of bivalve 'health'. This study revealed the effect of season and physiological status on haemocyte parameters of *Tapes philippinarum.* Total haemocyte count, granulocyte and hyalinocyte showed seasonal variations. The concentrations of haemocyte were high in summer and low in winter in both 2009 and

2010 but there was only a weak positive correlation between haemocyte count and seawater temperatures. Previous studies of *T. philippinarum* indicated that THC, granulocyte, and hyalinocyte were positively related to the temperatures in laboratory experiment (Paillard *et al.*, 2004) and in the field studies (Flye-Sainte-Marie *et al.*, 2009; Soudant *et al.*, 2004).

Paillard *et al.* (2004) explored the effect of temperature on THC. The clams incubated in 8°C and 14°C presented lower THC than those in 21°C. The authors suggested a direct relationship with metabolic rate, since this species is poikilothermic, and so the metabolism of the animal would have increased as temperature rose. An increase in concentrations of circulating haemocytes could be caused by an elevated metabolism through either raising the rate of haemocyte production or mobilisation of haemocyte from tissues towards the haemolymph. Soudant *et al.* (2004) revealed that the THC of *T. philippinarum* from four rearing sites along the French coast was greatly affected by a seasonal variation. High THC was observed in spring-summer and low number of THC was seen in autumn-winter (Soudant *et al.*, 2004). A positive correlation between sea temperature and both granulocyte and total haemocyte counts was seen in *T. philippinarum* from Gulf of Morbihan, France (Flye-Sainte-Marie *et al.*, 2009). Nevertheless, Brooks (1994) in a contrasting study suggested that the effect of temperature on these immunological parameters was minimal at coastal salinity (33) conditions.

In addition, this study has revealed a possible relationship between the reproductive cycle of *T. philippinarum* and THC. High THC value ($23.90 \pm 6.88 \times 10^6$ cells ml^{-1}) was found in June during the pre-spawning period and continuously decreased to the minimum in September ($6.36 \pm 2.68 \times 10^6$ cells ml^{-1}). These data support the previous investigation by Matozzo *et al.* (2003) in *T. philippinarum* in the Venice Lagoon where the highest THC occurred in January and the lowest was seen in September in synchrony with gametogenesis. It appears that haemocytes typically move from haemolymph towards the gonad in order to degrade the residual gamete in the post-spawning stage (Matozzo *et al.*, 2003). However, this apparent relationship has not been found in every study, a decrease in THC could not be associated with the gonadal cycle of *T. philippinarum* from Gulf of Morbihan, France (Flye-Sainte-Marie *et al.*, 2009).

4.5.2 Phagocytosis

The total haemocyte count is a basic and simple assay which reflects the 'well-being' of bivalves, it is unable to quantify or qualify immunocompetence. Phagocytosis is widely known as an indicator demonstrating the functional capacity of haemocytes.

Observation reveals that phagocytosis of *Tapes philippinarum* in Southampton Water remained relatively constant and showed no seasonal trends throughout the study period (June 2009-September 2010). There was also no significant relationship to seawater temperature or gonadal cycle. Unlike total haemocyte count, both phagocytic activity and capacity are not easily susceptible to environmental and physiological changes (Flye-Sainte-Marie *et al*., 2009; Matozzo *et al*., 2003; Soudant *et al*., 2004).

This is in agreement with the study in *Crassostrea gigas* that the phagocytosis indices between pre- and post-spawning were not different (Li *et al*., 2009b). Despite, up to date, no report regarding the impact of reproductive cycle on the phagocytosis of *T. philippinarum*, other bivalves species such as *Crassostrea gigas* (Li *et al*., 2009a), *Placopecten magellanicus* (Pichaud *et al*., 2009), and *Mytilus* spp. (Lemaire *et al*., 2006) showed a decline of phagocytosis during spawning periods.

Matozzo *et al.* (2003) showed great variation in phagocytic index of *T. philippinarum* in the Venice Lagoon, Italy throughout the year (September 2000-July 2001). According to Flye-Sainte-Marie *et al.* (2009), phagocytosis significantly varied temporally, but no correlations with any environmental factors (temperature, salinity, POM, POC and PON) were found from the clams in Gulf of Morbihan, France. This does contradict the in vitro study of Allam *et al*. (2002) where phagocytosis was related to temperature. An increase in phagocytosis of *T. philippinarum* according to the elevated temperature from 8°C to 21°C was apparent (Allam *et al*., 2002).

In contrast to phagocytosis in *T. philippinarum*, the changes in phagocytosis in other bivalves are well documented where phagocytosis varies with temperature (Alvarez *et al*., 1989; Ballarin *et al*., 2003; Carballal *et al*., 1997; Chen *et al*., 2007; Chu and La Peyre, 1993; Donaghy and Volety, 2011; Fisher and Tamplin, 1988; Foley and Cheng, 1975; Tripp, 1992). Low temperature generally suppresses phagocytosis (Alvarez *et al*., 1989; Carballal *et al*., 1997; Fisher and Tamplin, 1988; Foley and Cheng, 1975). The number of phagocytosed haemocytes of *Mytilus galloprovincialis* at 10°C was lower than those in 20°C and 30°C (Carballal *et al.,* 1997). A similar phenomenon was also reported in *Mercenaria mercenaria* and *Crassostrea virginica* (Alvarez *et al*., 1989; Chu and La Peyre, 1993; Fisher and Tamplin, 1988; Foley and Cheng, 1975; Tripp, 1992). Chen *et al*. (2007) evaluated an acute temperature challenge effect in Zhikong scallop (*Chlamys farreri*). The percentage of phagocytosed haemocyte was significantly lower in the scallop introduced to the higher temperatures (from 17°C to 23°C and 28°C for a period of 72 hours). However, there was no negative effect on those transferred to the lower temperature (10°C) (Chen *et al.,* 2007). Data from green mussel (*Perna viridis*) indicated that the phagocytic activity was minimal at 10°C and

gradually increased with temperature (20°C and 30°C) (Donaghy and Volety, 2011). Ballarin *et al.,* (2003) observed the temporal variation in a phagocytic index in the Venus clam (*Chamelea gallina*) from the western coastline of the Adriatic Sea, Italy. It was typically higher in summer than in winter showing a significantly positive correlation between phagocytic activity and the temperatures.

In *C. virginica*, rates of locomotion of haemocytes were positively related to temperature (Fisher and Tamplin, 1988). It is considered that the increment of phagocytosis index at high temperatures was affected by an increase of haemocyte locomotion which enhanced the haemocyte number confronting the foreign materials. However, haemocyte stress can be caused by an extremely high temperature above a certain threshold (25°C for *C. virginica*) (Chu and La Peyre, 1993). This could lead to restraint phagocytosis at high temperature (Fisher and Tamplin, 1988; Fisher *et al.*, 1989; Chu and La Peyre, 1993).

4.5.3 Digestive tubule

The digestive gland is involved in digestive enzyme production, digested food absorption, pollutant accumulation, detoxification and elimination. It can be used to monitor the health of bivalves, but the majority of the previous studies using digestive gland of bivalves mainly focused on the effect of pollutants. However, this study has underlined the impact of seasonal and physiological changes on the digestive gland index of *Tapes philippinarum*. The digestive epithelial thicknesses of *T. philippinarum* from Southampton Water were between 13.36-19.39 µm which are in the same range as the result obtained by Daros and Nesto (2005). The ranges of epithelial height of the population from the Venice Lagoon, Italy in two 'clean' sites were 17.16-17.53 µm and 14.21-16.43 µm, whereas those 'exposed to pollutants' had epithelial thickness ranging from 12.81 to 16.39 µm (Daros and Nesto, 2005). Mean luminal radius/Mean epithelial thickness (MLR/MET) in this finding (1.30$\pm$0.28-2.19$\pm$0.66 µm/µm) is similar to those observed in the mussel *Mytilus galloprovincialis* with the ratio from 0.7 to > 1.5 µm/µm (Marigomez *et al.,* 2006) and from 0.7 to > 1.6 µm/µm (Garmendia *et al.,* 2011).

The seasonal trends in digestive epithelial thickness and the mean luminal radius/mean epithelial thickness (MLR/MET) of *T. philippinarum* from Southampton Water during June 2009–June 2010 were obvious even though those factors showed only weakly positive and weakly negative correlations with the seawater temperatures, respectively. The epithelial thickness was significantly higher in summer (Jun-July 2009 and June 2010) than in winter (December 2009-January 2010). The MLR/MET, on the contrary, were low in summer (June-July 2009) followed by an increase to the maximum

value in autumn-winter (October 2009-January 2010). Similar to data from the mussel *M. galloprovincialis* in the Bay of Biscay, Spain (Garmendia *et al.,* 2011), the MLR/MET were 0.7 and 1.2 µm/µm in spring-summer and autumn-winter, respectively. The MLR/MET of *M. galloprovincialis* in the Bay of Biscay, Spain in April was higher than 1.5 µm/µm and decreased to approximately 1.4 µm/µm in September (Marigomez *et al.,* 2006). No clear evidence of seasonality in luminal radius was found in this observation because it seemed to be stable throughout the year. The epithelial thickness and the MLR/MET of *T. philippinarum* in Southampton Water were possibly affected by physiological changes in particular gonad development. Indeed, a decrease of the epithelial thickness and an increase of the MLR/MET were recorded during pre and post-spawning stages. This was the first record of relationship between digestive indices and reproductive activities in *T. philippinarum.*

The results of digestive gland measurements, particularly epithelial thickness and the MLR/MET, indicated that *T. philippinarum* in Southampton Water experienced stress in winter. Both parameters are tissue-level responses which are susceptible to environmental and physiological changes and more sensitive than luminal radius. They therefore could be used as biomarkers to evaluate the stresses in *T. philippinarum* in which case the influences of seasonality and the reproductive cycle need to be taken into consideration. As described by Weinstein (1997) in the study of oyster *Crassostrea virginica* from Marina pipe and the neighbouring areas, there were different trends in the temporal pattern of mean digestive epithelium. These might be caused by other additional stressors rather than fluoranthene or total PAHs.

4.6 Bivalve larvae abundance

The averaged densities of bivalve larvae in Southampton Water examined between April 2009 to January 2010 were relatively low (maximum 0.99 ind. m^{-3}) in comparison to other observations (Green, 2004). Green (2004) reported zooplankton distribution between June 2003 and January 2004 from three stations in Southampton Water including Marchwood, NW Netley and Calshot representing the head, middle and mouth of the estuary, respectively. The highest densities of bivalve veliger were between 13.58 ind.m^{-3} at NW Netley and 110.35 ind. m^{-3} at Marchwood. Such difference in bivalve larvae density between this study and the finding from Green (2004) is possibly because of the different sampling equipment and methods employed. From the study conducted by Green (2004), bivalve larvae were collected using a plankton net with a mesh size of 200 µm beneath sea surface whilst, a 120 µm mesh size net was used to sample the larvae at one metre above sea floor in this study. De Bernardi (1984) stressed that a decline of the sampling efficiency of a plankton net might be

caused by net clogging. In fact, clogging quickly occurs when using a finer mesh size plankton net in the high load of suspended matter like Southampton Water (Sharples, 2000). Moreover, the area of Bird Pile is in the middle of the estuary which is documented as having lower zooplankton production area in comparison to elsewhere in Southampton Water and the Solent at the same season (Conover, 1957; Hirst *et al.*, 1999; Lucas, 1993) with an exception from the observations of Zinger (1989) and Muxagata *et al*. (2004). Zooplankton in NW Netley (the middle of the estuary) was lower than in Marchwood and Calshot which are the upper and lower estuary respectively throughout the sampling (Green, 2004). A great difference in the highest bivalve veliger densities was seen between Bird Pile (5.04 ind. m^{-3}) and the Solent (588 ind. m^{-3}) (Samolis, 2010).

Two peaks of bivalve larvae in June and August 2009 observed in the present study closely matched the spawning season of *Tapes philippinarum* which is a dominant bivalve species in the vicinity of Bird Pile, Southampton Water. *T. philippinarum* in this area spawned from May to September with a single peak between June and August. Nevertheless, it is most likely that the third peak of the larvae seen in December 2009 is attributable to another bivalve species as most of the *T. philippinarum* gametes were released by September and all gonads became inactive in December. Similarly, Pelizzato *et al*. (2011) revealed that the recruitment of the zero-year cohort of *T. philippinarum* in the Venice Lagoon, Italy during 2002-2007 occurred in late spring (May and June) and some continued for the entire summer. The highest density of the seed was detected in September every year. This fits the spawning season (May to September/October) of the clam in the area (Daros *et al*., 2005; Meneghetti *et al*., 2004) and abundance of post larvae (between June and August) (Caberlotto *et al*., 2003). Larvae of *T. philippinarum* could be a potential candidate species in the umbonate larvae type A because the broadly round umbo is a common characteristic well illustrated in venerupid larvae. Nevertheless, this morphology is also seen in the young larvae of many other bivalve species (Chanley and Andrews, 1971; Loosanoff *et al*., 1966), therefore it is difficult to tell to what species they belong. Adult bivalves of 20 species from 11 families have been found in Southampton Water (Hibbert, 1976; Leggatt, 1996; Vourdoumpa, 2007).

Population dynamics of *Tapes philippinarum* in the current observation cannot be completed as a consequence of the limitation in taxonomic tools to distinguish from those of other bivalve species and so accurately quantify the larvae of this species. The developmental phases in the larvae of some invertebrate species such as crustaceans are distinguishable. Bivalve larvae, conversely, remain relatively uniform in morphology

throughout the entire development until metamorphosis to an adult (Garland and Zimmer, 2002). An accuracy of precise species identification varies the reliability of the larval data. Several techniques have been suggested in order to identify bivalve larvae to species level. Garland and Zimmer (2002) reviewed three techniques for bivalve larvae identification. Firstly, morphological method relies on a direct microscopic observation (light/electron) requiring comparison between sampled larvae and a voucher collection. The collection could be either as preserved specimens, drawings, or photographs of larvae of known origin that have been identified by specialists. The morphological criteria for larval identification include shape, dimension, hinge-line length, hinge structure, umbo character and colour. In spite of the simplicity and low cost to develop and employ, the main drawback of this technique is that it requires years of experience by the taxonomists to accurately identify species. In case of *T. philippinarum* larvae, the hinge morphology (Le Pennec, 1980) and the morphology of Venerupid bivalve larvae in the western coastal Korea (Hur *et al.,* 2005) and in Sea of Japan (Semennikhina *et al.,* 2006) were described. The combination between image analysis technique with special software (e.g.NIRECO Co Ltd) and morphological data (such as aspect ratio, round shape factor and unevenness shape factor) was applied to distinguish larvae of *T. philippinarum* from other bivalve species and zooplankton (Terazaki *et al*., 2001)

Secondly, molecular methods (e.g. immunological and DNA-based markers) are widely accepted to be a powerful tool to generate more accurate and precise information than the morphological technique as well as low subjectivity (Garland and Zimmer, 2002). Antibody probes developed from protein or portions thereof in the target species have been used as immunological markers providing species-specification. However the hindrances of the probe are (i) protein alteration by intrinsic (physiology) and extrinsic factors (environmental condition) and (ii) highly conserved protein in larvae of closely related species resulting in no species-specific marker. To study the dispersal and recurrence of *T. philippinarum* larvae in Tokyo Bay, the monoclonal antibody technique was developed to identify the target species (Hamaguchi *et al*., 2004). Garland and Zimmer (2002) described that oligonucleotide markers represent a DNA-based method. At the initial stage of the method development, a combination of cytogenetics (examination of chromosome and nuclei at the cellular level) and cytology (staining of structures inside cells) was used to discriminate the larvae species. Over the last two decades, the method has consequently focused on the fundamental information code in the cell, called deoxyribose nucleic acid (DNA). Apart from species-specification, an advantage of the DNA-based technique is the conservative character of DNA from physiological and environmental changes. The technique has expanded from a single primer to multiple random primers. Nevertheless, the method applied for field

specimens' identification has not been fully developed. *In situ* hybridisation coupled with fluorescent detection (FISH) is fortunately designed to target species level of *Ostrea edulis, Macoma balthica, Crassostrea gigas and Cerastoderma edule*, but only family level of Veneridae can be determined (Le Goff-Vitry *et al.,* 2007). A SYBR Green real-time PCR assay, containing TPHI16S1F and TPHI16S2R primers (specific to *T. philippinarum* female mtDNA), was developed to provide a rapid method for the larval quantification (Quinteiro *et al*., 2011).

A similarity of immunochemical and oligonucleotide probes is to explore the signature molecules unique to a particular species (Garland and Zimmer, 2002). Despite being able to generate informative and accurate data, the molecular techniques still have some disadvantages. Firstly, they are expensive and time-consuming in developing suitable probes and optimising appropriate conditions. Secondly, bivalve larvae need to be manually sorted from plankton samples and individually analysed via gel electrophoresis which is laborious. Lastly, the specimens (wholly or partly) will be completely destroyed during a DNA isolation process so the specimens will not be available for other studies. Ideal marker technique for the field samples are (1) no requirement of sub-sampling, (2) effective for intact, the specimens can be preserved for other analyses, (3) producing a detectable result through image analysis, (4) relatively inexpensive to develop probes for a large number of species, (5) relatively inexpensive to produce once developed and (6) producing accurate and repeatable results (Garland and Zimmer, 2002).

Finally, acoustic and other remote-imaging technologies are referred to as optical methods. So far, a species discrimination for bivalve larvae using this technique is limited; therefore only higher taxa can be identified by this approach. However, identification to species level may be accomplished in the future using this technique in conjunction with molecular tagging procedures (Garland and Zimmer, 2002).

4.7 Spring mortality laboratory experiment

Energetics of *Tapes philippinarum* during winter is rather complex because a number of endogenous and exogenous factors are involved. Clearance rate, food absorption efficiency, respiration rate, scope for growth and gametogenesis of this species were examined in order to investigate the combined effects of temperatures and reproductive cycle on those parameters under nutritive stress during a long period of time.

Even though the physiological responses of *T. philippinarum* conditioned to 5°C were not significantly different over 12 weeks observed, its metabolism seemed to be affected by low temperature. The CR, RR and AE in the first week were low. The SFG value was close to zero as a consequence of an energy input shortage. However, an increase of SFG was seen in week 4 because an increment of CR (energy input increasing) and a decrease of RR (energy cost decreasing). This process might be used to balance the energy budget of animal if food is insufficient. However, neither CR increase nor RR reduction would help to maintain the energy budget over a long (months) duration under nutritive restriction. This can be seen since week 8 that the clams at 5°C had suppressed the mechanical activities including CR decreasing and RR sustaining in order to save the energy. This reaction to conditions is known as energy conserving (Gillmor, 1982). The CR of scallop *Argopecten irradians irradians* decreased when the Chl_a concentration reduced to below 12% of the natural levels (Rheault and Rice, 1996). Albentosa *et al.* (2007) recorded the energy conservation in *Ruditapes decussatus* and *Venerupis pullastra.* After 15 days of starvation, RR of both species declined and remained at basal levels for the rest of the experimental period (84 days) (Albentosa *et al.,* 2007). An increase or a decrease in metabolic rate usually occurs as a response to nutritive stress according to whether food is present or not. Under a prolonged absence of food, metabolic rate declines to the minimum level and is then stabilised throughout the starvation period (standard metabolism). This strategy enables the animal to reduce its energy usage at a time of starvation or nutrition deficiency-when no energy (or only a very small amount) is gained (Bayne and Newell, 1983).

Such strategy observed in *T. philippinarum* at 5°C from week 8 to week 12 seemed to help the animals coping with the stresses of both low temperature and food restriction. Even though a decrease of SFG was seen at week 8, it still remained positive and was maintained at this level until week 12. A positive energy balance in the clams controlled at 5°C was reflected by a given SFG and wet weight production for the entire experiment. Fernández-Reiriz (2007) reported that, without nutritive stress, an accumulative reserve in *T. philippinarum* was produced and sustained in the lowest temperature of the experiment (14°C) while the energy store of the animal in the higher temperature (18°C) was exploited. Meat growth (tissue dry weight) of *T. japonica* at 12°C was higher than those at 14, 16 and 18°C (Mann and Glomb, 1978). Fortunately, low temperature (5°C) in the current study preserved the clams from gonad activity which demands a lot of energy. Otherwise a negative energy budget would occur and potentially bring about mortality.

The SFG values of *T. philippinarum* maintained at 10°C in the current study possibly revealed the sign of a negative energy balance at the end of the experiment (week 12). High SFG had been seen since the beginning until week 8, but a marked decline appeared in week 12 which coincided with the onset of gametogenesis in the 10°C clams. Clams generally balance their energy budget by increasing energy input and decreasing energy expenditure (Gillmor, 1982) however the food ration was at winter levels and so the energy input might not have been sufficient for the energy demand of gametogenesis. During the first 8 weeks, CR continuously increased whilst the AE decreased. Once a slight energy input was noticed, the animals reduced the respiration rate (RR) presumably to lower energy expenditure. In spite of energetic stress for 12 weeks, the 10°C animals were most likely to survive on the grounds of limited positive energy budget prior to gametogenesis. As shown in this study, the onset of gametogenesis (starting about week 12), a sudden decrease of SFG and body weight loss between week 12 and 18 probably indicated a negative energy balance.

The likely cause is that gonad development typically requires a lot of energy, but clams possibly have low energy reserve. This phenomenon (neutral or negative energy balance occurring after months with a low level food supply) is one which might provoke mortality in clams. Delgado and Camacho (2003) revealed that the gonad of *Ruditapes decussatus* could develop even under at negative energy balance (daily food ration of 0.025% algal dry weight per clam live weight). The clams fed 0.025% ration of food lost 20% of their DW at day 70 of the experiment period. However the extent of gonadal development was directly related to the amount of food available. The food ration resulting in the positive energy balance (0.24%, 0.48% and 0.96%) gave the value of Gonad Occupation Index (GOI representing the degree of maturity) of 30-40% in females and 60-75% in males by between day 12 and day 45. The clams with zero (0.05% ration) and negative energy balance reached as same GOI as the positive energy balance group at day 70.

Mann (1979) showed similar anomalies in *T. philippinarum* maintained at 12, 15, 18 and 21°C for a period of 19 weeks and an apparent increase in mortality appeared in the animals kept at 21°C in week 11 and 13. This matched the onset of spawning and a very distinct decrease in dry meat weight. The author suggested that the energetic demandeds post spawning were thought to be high, due to not only the constant high conditioning temperature but also the energy cost associated with histolysis and histogenesis of tissues immediately after the spawning. The death of the experiment animals at the higher temperatures from week 11 onwards was possibly induced by this high stress. Moreover, Zwarts (1991) reported the lesser weight losses (tissue weight) in winter at lower temperatures in four bivalves from the Dutch Wadden Sea

including *Macoma balthica, Scrobicularia plana, Mya arenaria* and *Cerastoderma edule*. This was due to a decline of energy expenditure when the bivalves were inactive in the lower temperatures (Zwarts, 1991).

The explanation in this study might not be appropriate to describe spring mortality in juvenile bivalves since there is no energy cost for sexual development. Zarnoch and Schreibman (2008) revealed the mortality in juvenile *Mercenaria mercenaria* in Jamaica Bay, New York during winter and spring from 2001 to 2005. They suggested that the mortality in the young *M. mercenaria* was attributed to severe winter temperatures. Reduction in *M. mercenaria* water pumping was induced by low water temperatures (<5°C) and by a subsequent loss of energy stores to maintain standard metabolism. A substantial decrease in physiological condition i.e. carbohydrate content resulted in mortality of the juveniles in spring. This was due to; (i) temperature increases, (ii) insufficient food levels and (iii) high metabolic demand. Zarnoch and Schreibman (2008) recorded low mortalities (0.0%-0.2% per day) of *M. mercenaria* in both mild winters 2001 and 2002. Similarly, a negligible mortality was observed in a spring after a severe winter (2004-2005) because of the coincidence of seawater temperature increase and, additionally, a rise in food abundance (Chl_a; 36-64 μg l^{-1}) was seen that spring. A spring after experiencing a cold winter, conversely, revealed a significant mortality (up to 0.99% per day). At the time, food supply was low (Chl_a < 3 μg l^{-1}) when temperature was rising. A decline of carbohydrate content was noticed during that period. Furthermore, the animals living in the critical low temperature possibly demands greater energy to maintain their standard metabolism (Zarnoch and Schreibman, 2008).

According to Laing and Child (1996), unfed juveniles of *T. philippinarum* and *T. decussatus* conditioned at 3, 6 and 9°C showed significant differences in survival rate. All *T. decussatus* and more than 50% of *T. philippinarum* at 3°C died after 3 weeks of the experiment. No mortality occurred in all clams kept at 6 and 9°C up to 11 weeks. The biochemical reserves, weights and conditions of the juveniles all reduced during starvation. Even though the clams attempted to balance their energy during a temperature decrease through the reduction in food consumption and respiration, at temperature outside their normal limits of tolerance might cause the mortality (Wilson and Elkaim, 1991). For example, Laing and Child (1996) proposed that at temperature of approximately 6°C seemed to be a critical temperature for *T. decussatus* because their oxygen consumption was higher at 3°C than 6 and 9°C. The temperature below a critical temperature might cause additional stresses on the normal metabolic function of *T. decussatus* (Laing and Child, 1996).

The results obtained here indicate that there are no significant differences of CR, AE, RR, and SFG values between the clam conditioned at 5°C and 10°C during 12 weeks of experiment except CR and RR at week 8. Even though a number of reports describing that filtration and respiration rates in *T. philippinarum* are related to temperature (Brooks, 1994; Fernández-Reiriz *et al.*, 2007; Han *et al.*, 2008; Laing and Child, 1996). This is because an elevated temperature raises metabolic rate of a number of bivalves such as *T. philippinarum* (Fernandez-Reiriz *et al.*, 2007; Han *et al.*, 2008; Laing and Child, 1996; Moschino and Marin, 2006), *T. decussatus* (Laing and Child, 1996), S*pisula subtruncata* (Rueda and Smaal, 2004) and *Brachidontes pharaonis* (Sara *et al.*, 2008). However, Brooks (1994) who examined the physiological responses of *T. philippinarum* at 5, 10, 15, 20 and 25°C reported that the filtration rate, absorption efficiency, respiration rate and scope for growth of the clam at 5°C and 10°C were not significant different. The difference was seen between low temperatures (5 and 10°C) and higher temperatures (15, 20 and 25°C). The author suggested that the optimum temperature for *T. philippinarum* was 15-20°C because the highest values in all physiological parameters were recorded.

Similar physiological responses of *T. philippinarum* conditioned at 5 and 10°C in the present study, on one hand, reflects that the seawater temperature in some winters (about 5°C) can be categorised as 'warm winter' and its effect is most likely to differ from the 'cold' winter (temperature below 5°C for a given period). On the other hand, it is well known that is native to subtropical to low boreal of the western Pacific and its optimum temperature range is 13-21°C, however its range of tolerance is 0-25°C (Bernard, 1983). *T. philippinarum* conditioned at 5°C was able to maintain positive SFG for long period of time (3 months) under food limitation and showed no significant differences in any physiological responses from those conditioned at 10°C. This capability might infer the wide physiological tolerance of this non-indigenous species.

The findings from the current study can be used to explain the spring mortality of *T. philippinarum* in Poole Harbour, although the temperatures applied in the present experiment were different from the actual seawater temperatures during winter (severe winter<5°C). The collected wild population experiencing a warm/mild winter 2011-2012 (a minimum water temperature of approximately 5°C) showed similar reproductive characteristic to the animals kept at 10°C for 18 weeks despite the seawater temperature being below the minimum temperature of gametogenesis (10°C). The SFG of the wild clams was not significantly different from those kept at 5 and 10°C at week 12. Energy balance behaviour was expressed through not only low CR and RR but also moderate AE. This observation possibly reflects the negative energy balance in the clams encountering a 'warm' winter in Southampton water. Nevertheless the

factors controlling the energy budget of the animals in the natural habitat are possibly more complicated than the combination of temperature, trophic resource and gonad activity.

4.8 Future of *Tapes philippinarum*

The results obtained from this study point out that the environmental condition of Southampton Water including temperature and salinity regime as well as food availability (Chl_a) provided *T. philippinarum* with a favourable habitat. This is shown through normal growth rate and good health status reflected by haemocyte number, phagocytosis and digestive tubule structures. Furthermore, *T. philippinarum* can complete its life history as seen in the success of the gonad development, fertilization and larval development (bivalve larvae) and settlement (population structure of juvenile; 10-20 mm shell length). Hence it can be concluded that *T. philippinarum* has successfully naturalised in Southampton Water.

There is growing concern about the spread of this non-native species along the south coast and northwards. According to this current study, it is apparent that *T. philippinarum* has great potential to establish if it is deliberately introduced to similar isotherm areas such as south & west coast of the UK (up to Lancashire) and south & west coast of Ireland (Hiscock, 1998). The possibility of the distribution of *T. philippinarum* via larval dispersal seems to be low because until now the establishment of the clam adjacent to Poole Harbour and Southampton Water has not been recorded in spite of the naturalization in these areas for about 20 and 7 years, respectively. It is likely that the larval dispersal has been prevented by hydrodynamics of the water mass and/or hydrographical and geographical barriers (Hiscock, 2004). Nonetheless, uncommon jetstream currents or storms at spawning period may take larvae across a barrier (Hiscock, 2004); consequently the distribution of *T. philippinarum* through larval dispersal may occur.

To assess the northern extension of *T. philippinarum* in European Watesr, the ambient seawater temperature regime in summer and winter needs to be taken into consideration. Generally, the northern limit will be set by winter temperature being too low for the clam to survive and summer temperature being too low for growth and/or reproduction (Hiscock, 2004). For examples, the temperature regime in some areas in the UK and Ireland such as Northern Ireland, Scotland, east coast of England and north & west coast of Ireland is 12-15^{o}C in summer and 0-9^{o}C in winter (Hiscock, 1998). Regarding the growth and reproduction of adult *T. philippinarum*, Grisley (2003) recorded that in the following spring after winter 2002 when temperature dropped

down nearly 0°C, *T. philippinarum* in Poole Harbour could survive and successfully develop their gametes. The present study and Xie and Burnell (1994) also confirm that this bivalve species is able to develop their gametes (4.8 and 5.8°C, respectively) and spawn (13.3 and 13.1°C, respectively) below the temperature limit for gametogenesis (10°C) and spawning (14°C). Despite the optimum temperature for larval development of *T. philippinarum* being 23-25°C (Helm and Bourne, 2004), the evidence to date from both laboratory (Lovatelli, 1985) and field-based studies (Grisley, 2003; Xie and Burnell, 1994) shows that larvae are successfully developed at temperatures between 15-20°C. The temperature ranges in those areas seem to be in the tolerance range of juvenile and adult *T. philippinarum*, but summer temperature are possibly too low for the success of larval development. However the fact that this bivalve species has great potential to extend its temperature tolerance below published observations and global warming (temperature rising) should be taken into the assessment. Austin *et al*. (2001) predicted that the UK seawater temperature will be raised a further 2°C or more by the 2050s. If it is true, increasing seawater temperature would allow a northward establishment due to the isothermal extension. However, apart from temperature, the spread of *T. philippinarum* is also influenced by other environmental factors such as salinity, water quality, suitable substrate and food availability.

Chapter 5 Conclusions

1. Temperature, salinity and Chl_a concentration were the environmental parameters examined in the current study. The annual variation in temperatures was between 4.7 and 19.7°C, The salinities were high and relatively stable throughout the year (29.64-35.20). Both parameters seemed to provide *T. philippinarum* with a favourable environment as indicated by their growth, reproductive condition and immunocompetence. The Chl_a concentration (0.32–7.96 mgm^{-3}), representing the food availability during the sampling period (April 2009-January 2010) was low. The low Chl_a concentration might result in the positive relationship between oocyte diameter and shell length of *T. philippinarum*.

2. The middle size classes of 30–35 mm and 35–40 mm were numerically dominant (52.7%) in the population structure of *T. philippinarum* in Southampton Water. A low abundance of larger size classes (>40 mm; 11.67%) possibly reflected the use of fishing gear developed when the former EU MLS of 40 mm shell length was in force. The proportion of small clams (<25 mm) which increased from August, October and December 2009 was probably showing the the settlement period of this species to be late summer.

3. Increases in all growth measurements including shell length, shell height, shell width, and total live wet weight indicate that *T. philippinarum* did grow well in Southampton Water. Growth was rapid in summer (June–August 2009) accounting for more than 70% of annual growth, whilst growth ceased in winter. The annual growth rate in shell length, shell height and shell width in this study were 2.93$\pm$1.23, 2.80$\pm$1.25, 2.73$\pm$0.95 mm and 4.43$\pm$1.64 g, respectively.

4. The sex ratios (male:female) of *T. philippinarum* from Southampton Water and Poole Harbour in this study was 1:0.8 and 1:0.94 which were were not significantly different from the ratio 1:1 (χ^2=3.02; df=1; p=0.08 and χ^2=0.06; df=1; p=0.81, respectively). Hermaphrodite clam was not recorded.

5. The reproductive pattern of both males and females was synchronised. Gametogenesis of *T. philippinarum* from Southampton Water occurred from February to October and gonad maturation was seen from May onwards. Spawning was detected between May and September with a single peak from June to August. The gametogenesis of population from Poole Habour

commenced a little later than those in Southampton. The gonad started developing in March and matured in June. Gamete release was observed between July and September. Reproductive inactivity then occurred from November to January.

6. The onset of gametogenesis of *T. philippinarum* in Southampton Water began in February when the seawater temperature was 4.8°C. Gonad ripening and spawning were seen in May when the temperature had reached 13.3°C, calculated as 64.27 D°. *T. philippinarum* in Southampton seems to have adapted to the lower water temperature (compared to its native range) of the UK.

7. The oocyte diameter of *T. philippinarum* from Southampton Water had a significant linear relationship with the shell length of the clam (Multiple regression, df=1, F=8.5636, r^2=0.63, p<0.01) and time during the spawning season (May-August) (Multiple regression, df=1, F=38.9251, r^2=0.63, p<0.001). None of the clam below 29.8 mm shell length produced oocyte larger than 44 µm despite the average egg diameter of all specimens were between 31.13±6.31 and 55.74±6.20 µm. The oocyte diameter of *T. philippinarum* from Poole Harbour also significantly increased over time from July to September (One-way ANOVA, df=3, F=7.98, r^2=0.63, p<0.01).

8. The minimum size at sexual maturity of *T. philippinarum* in Southampton Water was 17.9 mm in male and 20.2 mm in female. Even if the clams within 15-20 and 20-25 mm shell length size classes became mature, not all individuals in those size classes did reach sexually maturity. Only 22.22% and 28.57% of the clam within 15-20 and 20-25 mm shell length size classes, respectively were mature. In contrast, a high percentage of maturity (87.50%-100%) was recorded in clams larger than 25 mm. In fact, all *T. philippinarum* above 29.7 mm shell length were mature during breeding season.

9. Atresia or gamete resorption was detected in *T. philippinarum* from Poole Harbour during the 2009 spawning season, affecting between 50% and 83.33% of all mature clams in the population, but was present at much lower level in 2010 (0.00% - 16.67%).

10. Haemocytes are important components in the defence mechanism of bivalves. Haemocytic indices of *T. philippinarum* in Southampton Water, including total haemocyte count (THC), granulocyte and hyalinocyte number, showed a similar pattern of seasonal dependence; high in summer and low in winter in both 2009 and 2010. However this pattern was not seen in phagocytosis, because

both phagocytic activity and capacity remained relatively constant throughout the year. These responses possibly reflected that the defence system of *T. philippinarum* in Southampton Water was competent. This is because although the numbers of haemocytes were compromised by season, leaving the clams more vulnerable to disease challenge in winter, phagocytic activity and capacity were temporally stable allowing the clam to compensate for any defence deficiency.

11. THC was found to decline after spawning. There was no significant difference between phagocytosis and gametogenesis of *T. philippinarum* from Southampton Water, indicating that the phagocytosis was not affected by gonad activity.

12. A seasonal variation was seen in digestive tubule indices, in particular digestive epithelial thickness and mean luminal radius/mean epithelial thickness (MLR/MET), but not in luminal radius. The stress of winter conditions on *T. philippinarum* was showed as a decline in epithelial thickness and an increase of MLR/MET. The average epithelial thickness decreased from 19.02-19.39 µm in summer 2009 (June-July) to 13.36-13.59 µm in winter 2009-2010 (December 2009-January 2010), however again increased to 18.85 µm in summer 2010 (June) (One-way Anova; df=8, F=12.61, p <0.001). MLR/MET increased from 1.3-1.4 µm/µm in summer 2009 (June-July) to 2.07-2.19 µm/µm in autume and winter (October 2009-January 2010) (One-way ANOVA, df=8, F=5.59, p<0.001).

13. Stress related with physiological change, particularly reproductive development, was noticed when a reduction in the epithelial thickness from 18.76$\pm$2.15 µm to 14.40$\pm$2.20 µm (One-way ANOVA, df=4, F=14.96, p < 0.001) and an increment of the MLR/MET from 1.43$\pm$0.25 µm/µm to 2.03$\pm$0.98 µm/µm (One-way ANOVA, df=4, F=7.82, p < 0.001) was seen after spawning.

14. The averaged densities of bivalve larvae from the waters around Bird Pile, Southampton Water obtained in this study were between 0.00-0.99 ind.m^{-3}.Two peaks of bivalve larvae observed in June and August 2009 closely matched the spawning season of *T. philippinarum*. The numbers of bivalve larvae peaked again in December 2009 but this is attributable to another bivalve species as most of the *T. philippinarum* gametes were spent by September and all gonads became inactive in December.

15. Physiological responses of *T. philippinarum* including clearance rate (CR), assimilation efficiency (AE), respiration rate (RR), and scope for growth (SFG), accumulative growth rate, and gametogenesis were examined at different

temperatures (5°C and 10°C) for 12 weeks in order to investigate the synergistic effects of temperatures and reproductive condition under nutritive restriction (Table 9). CR was affected by temperature and period of study and their interaction. At 5°C, there was no significant different in CR over 12 weeks of experiment, but a significant increment was seen in CR at 10°C at the end of week 8 (1.64 ± 0.95 l g^{-1} h^{-1}) followed by a distinct decline at week 12 (0.37 ± 0.31 l g^{-1} h^{-1}) (Table 9). Only CR at 5°C significantly differed from that at 10°C at week 8 (Table 9). No statistically significant differences in CR between both temperatures and wild population were found in week 12.

16. There was no significant effect of temperature and period of study on the AE, however the interaction of these factors on AE was recorded (Table 9). The AE of the animals at 5°C ranged between 34.83 ± 24.08% and 53.34 ± 11.91% without significant difference. The clams at 10°C performed the highest AE at the first week with the value of 70.88 ± 12.61% and then significantly declined at week 8 and week 12 (47.85 ± 19.10% and 36.83 ± 28.44%, respectively) (Table 9). In addition, no significant difference of AE between two temperatures is reported (Table 9). The wild animals revealed AE at 48.23 ± 20.32% which is not significantly different from that in the clams conditioned at 5 and 10°C at week 12.

17. RR was affected by temperature and period of study and their interaction (Table 9). O_2 utilised by 5°C clams was constant throughout the 12 weeks studied (0.03 ± 0.15 and 0.05 ± 0.45 mg O_2 l^{-1} g^{-1} h^{-1}) and no significant difference was observed. RR of the animals at 10°C significantly decreased from 0.20 ± 0.67 mg O_2 l^{-1} g^{-1} h^{-1} at week 1 to 0.11 ± 0.05 mg O_2 l^{-1} g^{-1} h^{-1} at week 8 and again declined to 0.09 ± 0.05 mg O_2 l^{-1} g^{-1} h^{-1} at week 12 (Table 9).. Only RR of 5 and 10°C clams at week 8 shows significant difference (Table 9). The wild animals consumed oxygen at a lower rate of 0.03 ± 0.02 mg O_2 l^{-1} g^{-1} h^{-1} which is significantly different from the clams at 10°C at week 12 (One-way ANOVA, df=2, F=5.11, $p < 0.05$).

18. There is no effect of temperature and period of study and their interaction on SFG (Table 9). The SFG at 5 and 10°C showed no significant difference throughout the period of study. Moreover, the SFG of the clam conditioned at 5°C is not significantly different from those at 10°C throughout 12 weeks. The SFG of the wild samples was 1.16 ± 2.13 J g^{-1} h^{-1}. No statistically significant

difference in the SFG was found between the animals at 5, 10°C and the wild population at week 12.

19. The eventual gonad development of the animal conditioned at 10°C was faster than those at 5°C. All gonads (100%) of the animals at 5°C became inactive starting from week 12 onwards whilst the onset of gametogenesis was observed in the clams conditioned at 10°C. The prevalence of early active gonads was only seen in the clams at 10°C (33.33% of gonad at week 12 and 66.67% of gonad at week 18) and the wild population (66.67%) at week 18.

20. The cumulative growth rate of the animals at 5°C was significantly higher than those at 10°C only at week 4 (0.22$\pm$0.21 and -0.02$\pm$0.10, respectively) and week 12 (0.20$\pm$0.16 and -0.03$\pm$0.09, respectively) (Two-way ANOVA, df=1, F=27.86, p<0.001). No significant difference was recorded in the cumulative growth rate of the animals at 5°C and 10°C over 18 weeks of study.

21. The positive SFG of 5°C clams throughout 12 weeks of study and an increase in body weight revealed the positive energy balance of the clams. A possible negative energy balance was seen when a marked decline of SFG in the clams kept at 10°C appeared in week 12 (1.17 J g^{-1} h^{-1}) and body weight losses were detected at week 4, 12 and 18, coinciding with the onset of gametogenesis.

22. Similar reproductive development was recorded in the collected wild population experiencing a warm/mild winter 2011 (a minimum water temperature of approximately 5°C) and the clams kept at 10°C at week 18. The SFG of the wild *T. philippinarum* was low and not significantly different from those maintained at 5 and 10°C at week 12. This observation possibly reflects a negative energy balance in the clams encountering warm winter in Southampton water.

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Appendix

Appendix 1 The volume of water sample through plankton net for bivalve larvae study

Sampling date	Replicates	Seawater volume (m^3)
07/04/2009	1	62.62
	2	9.76
	3	27.81
08/05/2005	1	93.32
	2	70.97
10/06/2009	1	38.63
	2	23.87
	3	23.11
17/07/2009	1	39.10
	2	14.88
	3	44.16
17/08/2009	1	41.16
	2	33.34
	3	27.93
21/09/2009	1	37.51
	2	31.75
	3	40.69
28/10/2009	1	9.41
	2	11.58
	3	13.29
03/12/2009	1	34.22
	2	13.11
	3	12.82
21/01/2010	1	13.05
	2	9.82
	3	13.35