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335

Anne Thonig

The Effect of Variation in Developmental Mode on the Population Dynamics of a Spionid Polychaete (*Pygospio elegans*) in a Heterogeneous Environment

Roskilde Universitet
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Editors
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Pekka Olsbo, Ville Korkiakangas
Open Science Centre, University of Jyväskylä

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ABSTRACT

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Yhteenveto: Kehitysmuotojen variaatio ja sen vaikutus *Pygospio elegans* -monisukasmadon populaatiodynamiikkaan heterogeenisessä ympäristössä.

There is a great diversity in larvae of marine invertebrates. To understand the causes and consequences of different modes of development on population dynamics, study of poecilogonous species that show a polymorphism in developmental mode might be more useful than are comparisons between species, since no confounding effects due to speciation arise. In this study, I documented the population ecology and genetics of the poecilogonous polychaete *P. elegans* and investigated the impact of abiotic and biotic variables on population dynamics. Four focal populations from the Isefjord-Roskilde-Fjord estuary complex, Denmark were sampled over one year. I observed highly dynamic population structure in both size cohort data and population genetic data that is possibly explained by the short life span of *P. elegans* and sweepstakes reproductive success. Additionally, stochastic events, such as rain storms, can lead to abrupt drops in salinity which can be detrimental for *P. elegans* and hence introduce further changes in population structure. Seasonal dynamics, including sexual reproduction, were correlated with temperature, whereas spatial differences in density, size and reproductive activity of *P. elegans* as well as species diversity of the benthic invertebrate community, were related to sediment structure. A positive correlation between species and allelic richness of *P. elegans* might indicate that environmental impacts are of greater importance in shaping population dynamics than are species interactions. Switches in developmental mode could reflect a strategy for coping with life in an unpredictable, heterogeneous habitat. Although switches in developmental mode were correlated with the appearance of genetically differentiated size cohorts, environmental or epigenetic effects cannot be ruled out.

Keywords: Benthic invertebrates; Isefjord-Roskilde-Fjord estuary complex; life history; poecilogony; population ecology and genetics; *Pygospio elegans*.

Anne Thonig, University of Jyväskylä, Department of Biological and Environmental Science, P.O. Box 35, FI-40014 University of Jyväskylä, Finland & Roskilde University, Department of Science and Environment, P.O. Box 260, DK-4000, Roskilde, Denmark.

Author's address Anne Thonig
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä
Finland
anne.a.thonig@student.jyu.fi

Department of Science and Environment
P.O. Box 260
DK-4000, Roskilde
Denmark
athonig@ruc.dk

Supervisors Lecturer Dr. K. Emily Knott
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä
Finland

Associate Professor Dr. Gary T. Banta
Department of Science and Environment
P.O. Box 260
DK-4000, Roskilde
Denmark

Professor Dr. Benni Winding Hansen
Department of Science and Environment
P.O. Box 260
DK-4000, Roskilde
Denmark

Dissertation at Roskilde University

Committee

Professor Dr. Hans Ramløv
Department of Science and Environment
P.O. Box 260
DK-4000, Roskilde
Denmark

Dr. Lisa N. S. Shama
Alfred-Wegener-Institute
Hafenstraße 43
DE-25992, List/Sylt
Germany

Dr. Dorte Bekkevold
Technical University of Denmark (DTU AQUA)
Vejlsøvej 39
DK-8600, Silkeborg
Denmark

Dissertation at University of Jyväskylä

Reviewers

Dr. Lisa N. S. Shama
Alfred-Wegener-Institute
Hafenstraße 43
DE-25992, List/Sylt
Germany

Dr. Dorte Bekkevold
Technical University of Denmark (DTU AQUA)
Vejlsøvej 39
DK-8600, Silkeborg
Denmark

Opponent

CNRS Research Director Dr. Frédérique Viard
Station Biologique of Roscoff (UPMC/CNRS)
Place Georges Teissier
FR-29680, Roscoff
France

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I–IV.

I Thonig, A., Knott, K.E., Kesäniemi, J.E., Winding Hansen, B. & Banta, G.T. 2016. Population and reproductive dynamics of the polychaete *Pygospio elegans* in a boreal estuary complex. *Invertebrate Biology* 135: 370–384.

II Thonig, A., Banta, G.T., Winding Hansen, B. & Knott, K.E. 2017. Seasonal genetic variation associated with population dynamics of a poecilogonous polychaete worm. *Ecology and Evolution, in press*.

III Knott, K.E., Thonig, A., Heiskanen, S., Winding Hansen, B. & Banta, G.T. 2017. Seasonal variation in diversity of marine benthic invertebrates leads to a positive species-genetic diversity correlation. Submitted manuscript.

IV Thonig, A., Banta, G.T., Winding Hansen, B. & Knott, K.E. 2017. Acute and chronic response to changes in salinity of the euryhaline polychaete *Pygospio elegans*. Manuscript.

The table shows the contributions to the original papers.

	I	II	III	IV
Original idea	KEK, JEK, GTB, BWH, AT	KEK, GTB, BWH, AT	KEK, GTB, BWH	AT, BWH, GTB
Data	AT, KEK, JEK, GTB, BWH	AT, SH, KEK	AT, SH	AT
Analyses	AT, GTB, BWH	AT, KEK	KEK, AT, SH, GTB	AT, KEK, GTB
Writing	AT, GTB, KEK, JEK, BWH	AT, KEK, GTB, BWH	KEK, AT, GTB, BWH	AT, KEK, GTB, BWH

AT = Anne Thonig, KEK = K. Emily Knott, GTB = Gary T. Banta, BWH = Benni Winding Hansen, JEK = Jenni E. Kesäniemi, SH = Siru Heiskanen

1 INTRODUCTION

1.1 Larvae of marine invertebrates

Marine invertebrates exhibit a wide diversity of reproductive strategies that can differ in gametogenesis, gamete release and, particularly, in the type of larvae they produce (Llodra 2002, Heyland *et al.* 2011, Henshaw *et al.* 2014). Larvae are an ancient characteristic of metazoans, and among marine invertebrates, larvae show a great variety with diverse structures to facilitate swimming, feeding, settlement and for defense against predators, which has raised the question of the evolution and the consequences of different larval forms (Strathmann 1985, Wray 1995). Just to name a few, veliger larvae are found in gastropods and bivalves, while some other molluscs have trochophora larvae, as do annelids and platyhelminths; nauplius and zoea larvae are characteristic for crustaceans; and echinoderms exhibit pluteus larvae, a complex version of the dipleurula larvae (Fig. 1, Levin and Bridges 1995).

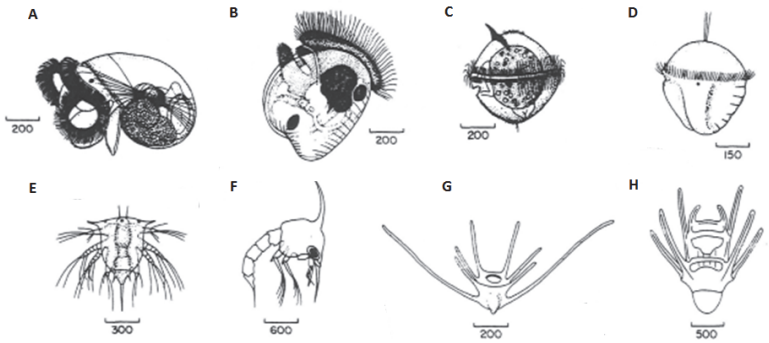

FIGURE 1 Different types of larvae of marine invertebrates (adapted from Levin and Bridges 1995). A) gastropod veliger, B) bivalve veliger, C) polychaete trochophora, D) polyplacophoran trochophora, E) crustacean nauplius, F) crustacean zoea, G) ophiuroid ophiopluteus, H) echinoid echinopluteus.

Ecologically, larvae can be categorized according to their site of development (planktonic, demersal, benthic) or their nutritional mode. Planktotrophic larvae swim and feed in the plankton and for that purpose exhibit efficient ciliary structures and a digestive system (Levin and Bridges 1995, Wray 1995). Larvae can also acquire their nutrients maternally via yolk deposited in the egg (lecithotrophy) or by feeding on other eggs or siblings within the brood (adelphophagy). Facultative planktotrophic larvae can complete their development without feeding (like lecithotrophic larvae), but have feeding structures and a planktonic phase like planktotrophic larvae and can feed if needed (McEdward 1997). Moreover, larval forms that gain nutrients from their mothers (translocation), uptake dissolved organic matter from the seawater (osmotrophy) or synthesize nutrients themselves (autotrophy) have also been described (Levin and Bridges 1995). Planktotrophy and lecithotrophy are present in most marine invertebrate phyla and within phyla, several evolutionary transitions from one mode to the other have occurred. In some taxonomic groups, planktotrophic larvae are thought to be the ancient form (e.g. echinoids, asteroids), while in other groups (e.g. polychaetes, gastropods) they are proposed to be the derived form (Strathmann 1993, Levin and Bridges 1995, Wray 1995, Rouse 2000, Collin *et al.* 2007).

Although transition in larval form is common among species, larval form is likely to be conserved within species. Only a few species of marine invertebrates are able to produce different types of larvae, resulting in a polymorphism in developmental mode called poecilogony. Many species that were initially thought to be poecilogonous, however, turned out to be cryptic species (Hoagland and Robertson 1988). So far, five poecilogonous species have been described among sacoglossan sea slugs, (*Costasiella ocellifera*, *Elysia chlorotica*, *Elysia zyleica*, *Elysia pusilla* [Vendetti *et al.* 2012], and *Alderia willowi* [Krug *et al.* 2012]); two species have been described among caenogastropods, (*Calyptrea lichen*[McDonald *et al.* 2014] and *Buccinum undatum* [Smith and Thatje 2013]); and seven species have been described among spionid polychaetes (*Streblospio benedicti* [Levin 1984b, Levin and Huggett 1990], *Pygospio elegans* [Söderström 1920, Hannerz 1956, Rasmussen 1973], *Boccardia proboscidea* [Blake and Kudenov 1981, Gibson *et al.* 1999], *Boccardia polybranchia* [Duchêne 1984], *Polydora cornuta* [Rice and Rice 2009], *Polydora hoplura* [David *et al.* 2014], and *Polydora cf websteri* [David *et al.* 2014]). Although all of these species are described as poecilogonous, their developmental mode can vary to different degrees (McDonald *et al.* 2014): between populations (e.g. in *Elysia chlorotica* and *Costasiella ocellifera* [Vendetti *et al.* 2012]), between females within the same population (e.g. *Streblospio benedicti* and *Pygospio elegans* [Söderström 1920, Hannerz 1956, Rasmussen 1973, Levin 1984b]), between broods of the same female (e.g. *Polydora cornuta* and *Calyptrea lichen* [Rice and Rice 2009, McDonald *et al.* 2014]) or even within broods (e.g. *Boccardia proboscidea* and *Buccinum undatum* [Blake and Kudenov 1981, Smith and Thatje 2013]).

1.2 Life history strategies

Size and number of larvae are important life history traits of marine invertebrates, as are characteristics of adult stages, such as maturity, age and size specific fecundity and mortality, and longevity. Therefore, larvae are major fitness components of the life history of an organism (Braendle *et al.* 2011). The life history of an organism is affected by environmental conditions and subject to natural selection that optimizes the reproductive value, i.e. the amount of expected future reproductive success of an individual (Fischer 1930, Edward and Chapman 2011). However, the investment into one fitness component can lead to reduced investment into another one, or trade-offs, leading to a negative correlation between them (Roff and Fairbairn 2007). Therefore, the combination of certain life history traits is restricted, due to genetic, physiological, developmental and phylogenetic limits. Developmental properties and historical contingencies can lead to constraints on certain traits. For example, among echinoderms feeding structures were lost many times, and once lost, feeding structures were not reacquired again (McEdward 2000). Genetic trade-offs can arise due to linkage disequilibrium and pleiotropy, whereas physiological trade-offs can arise due to the allocation of limited resources to competing functions, such as maintenance, growth and reproduction. Examples of physiological trade-offs are growth vs. reproduction, current reproduction vs. future reproduction and number vs. size of offspring (Levin and Bridges 1995, Llodra 2002, Braendle *et al.* 2011, Edward and Chapman 2011).

Different models have been developed to investigate the trade-offs between growth, reproduction and longevity (Cole 1954, Lewontin 1965, Charnov and Schaffer 1973, Grime 1977, Stearns 1992). A classic example is the model of r- vs. K-selection, describing strategies with high colonizing ability vs. high competitive ability (Wilson and MacArthur 1967, Pianka 1970). An extension of this, the pace-of-life syndrome hypothesis, suggests that certain physiological and behavioural traits co-evolved along with particular life history strategies in response to environmental conditions (Ricklefs and Wikelski 2002, Réale *et al.* 2010). In conclusion, several factors have been identified as important for shaping life history traits, namely abiotic factors (resource limitation and density-independent factors) and biotic factors (competition and predation), species specific factors (metabolic rates and mating systems), and whether variation in the environment is predictable or stochastic (Rockwood 2015).

Among marine invertebrates, the most commonly studied trade-off is the trade-off between fecundity and size of offspring. This trade-off might represent only a different way of packaging resources, not necessarily a difference in energy allocation, but it is important, since it affects other aspects of demography, such as developmental time, age-dependent survivorship and dispersal (Jaeckle 1995, Pechenik 1999, Llodra 2002). This trade-off is apparent when comparing marine benthic invertebrate species that either produce many

small eggs resulting in larvae that feed in the plankton (planktotrophic) versus few large eggs resulting in larvae that feed on yolk in the egg (lecithotrophic). Mathematical models that relate reproductive energetic efficiency to egg size in marine benthic invertebrates taking into account planktonic mortality, developmental time and egg number were derived by Vance (1973) and extended by Christiansen and Fenchel (1979) and McEdward (1997). They proposed that only the extremes of egg sizes are favoured. Hence, highest fitness was obtained when fecundity was maximized (planktotrophic larvae) or when developmental rate was maximized (lecithotrophic larvae). The former strategy is favoured when food is abundant and mortality is low in the plankton, while the latter strategy is favoured in opposite conditions (Vance 1973, McEdward 2000).

Yet, the presence of intermediate sized eggs of planktotrophic larvae, facultative planktrophic larvae and also frequent transitions between larval modes indicate that also other factors need to be taken into account (McEdward 1997, 2000, Allen and Pernet 2007, Collin 2012). For example, if conditions are unpredictable, variance in fitness can be reduced in the long term via bet-hedging strategies that buffer stochastic events. These include putting higher investment into offspring (conservative bet-hedging) or increasing offspring variation (diversified bet-hedging) (Collin 2012, Marshall and Burgess 2015). Furthermore, events in one life-stage can affect fitness in another one, hence provisioning of eggs needs to optimize performance in larval, juvenile and adult stages (Marshall and Keough 2006). Accordingly, larger eggs of lecithotrophic larvae might increase juvenile fitness, not larval fitness (Armstrong and Lessios 2015). Einum and Fleming (2004) propose that small egg size in salmon serves to increase maternal, not offspring fitness. For that reason Roughgarden (1989) integrated the larval phase in the entire life cycle, hence including pre- and post-settlement selection and proposed five stable life cycles based on his model, including a typical planktotrophic and a lecithotrophic one (Havenhand 1995).

However, the selection pressures favouring one life-history strategy over another in marine invertebrates are not yet fully understood (Marshall *et al.* 2012). According to Stearns (1992), to understand variation in a life history trait we need to know i) the phenotypic and genotypic variation, ii) the effects on a population and iii) the developmental and phylogenetic constraints. One study by Armstrong and Lessios (2015) used reciprocal hybrid crosses of two echinoderm species with different modes of development to disentangle the impacts of maternal investment and hormonal and genetic regulation on the mode of development. Another study by Collin (2001) investigated the consequences of different modes of development on population dynamics, i.e. gene flow, population structure and species distribution, in congeneric *Crepidula* species of the same geographical range using genetic markers. The advantage of using true poecilogonous species instead of differentiated or even sibling species to investigate variation in life-history traits is, however, that developmental and phylogenetic constraints can be disregarded (Knott and McHugh 2012).

1.3 Environmental impact and natural selection (CAUSES)

The evolution of phenotypic and genotypic variation of an organism and its life history is affected by the environment they experience, including abiotic (temperature, food, space, precipitation, etc.) and biotic factors (competition, predation, parasites, diseases). The performance of a population in an environmental gradient is described by its tolerance range, which is usually illustrated as a bell-shaped curve. Populations with broad ranges of medium performance are referred to as generalists, while populations with narrow ranges of high performance are specialists (Fig. 2). The tolerance range can be categorized according to the ecological performance of the organisms. At the optimum, organisms are able to grow and reproduce and hence maintain the population, while further to the edge of the tolerance range organisms are only able to survive, and even further they can only survive for a limited amount of time (Ricklefs 2001, Frederich and Pörtner 2000).

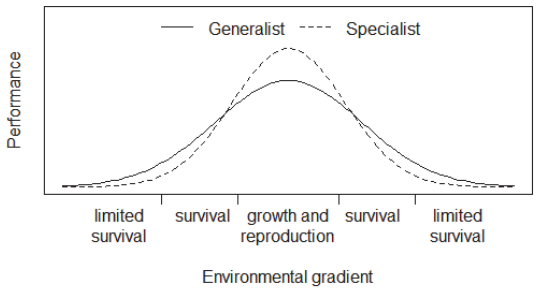

FIGURE 2 Tolerance curve displaying the performance of a population in a specific environmental gradient (according to Ricklefs 2001).

Provided that populations show variability in phenotypes and that phenotypes have a genetic basis, they are subject to natural selection that can change the tolerance range of a population (adaptation) to increase the mean individual fitness via changes in the genetic composition of the population. Depending on the environmental conditions, selection can be directional, balancing or disruptive; furthermore, it can be frequency- or density-dependent (Hamilton 2009). In species with complex life cycles, such as marine invertebrates with planktonic larvae, different life stages occupy different niches and are hence subject to different selection pressures, pre- and post-settlement selection. To optimize performance of both life stages, uncoupled evolution of both phases is necessary (Wray 1995).

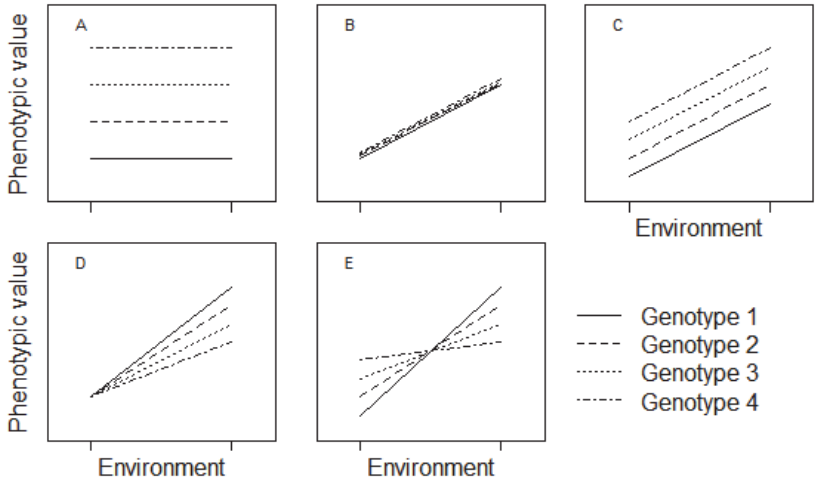

FIGURE 3 Reaction norms displaying the phenotype produced by a particular genotype in different environments. The different effects of genotype (A) and environment (B) as well as an additive effect of both (C) and their interactions (D, E) are illustrated according to Hamilton (2009).

Reaction norms describe the relationship between a phenotypic trait produced by a particular genotype and how it varies in response to variation in the environment. They are commonly used in quantitative genetics to illustrate the contribution of the environment and genotype to a particular phenotypic trait. Hereby, different genotypes can lead to different phenotypes regardless of environmental influences, which is called genetic polymorphism (Fig. 3A). In contrast, if the trait is environmentally controlled, different genotypes could lead to the same phenotype that shows similar changes according to the environment (Fig. 3B). More commonly, genotypic and environmental effects can be additive (Fig. 3C) or show interactions. In the latter case genotypes can differ in phenotypic variance (Fig. 3D) or the rank order of phenotypic values can change ranks across environments (Fig. 3E).

Genotype-by-environment interactions are also called phenotypic plasticity, or in case of discrete traits, polyphenism (Agrawal 2001, Hamilton 2009, Forsman 2015). Phenotypic plasticity can be divided into developmental plasticity and phenotypic flexibility. Developmental plasticity describes irreversible phenotypic variation due to developmental alterations, and is hence quite similar to genetic polymorphism. Phenotypic flexibility, in contrast, refers to reversible intra-individual plasticity in physiology, morphology or life history traits (Forsman 2015). Hence, mapping a genotype on a phenotype, i.e. disentangling genetic and environmental impacts, is difficult, since a single genotype can produce different phenotypes, and similarly, several genotypes can result in the same phenotype (Pigliucci 2010, Braendle *et al.* 2011). The interpretation of genotypic contribution is furthermore hampered because

reaction norms, although often displayed as such, are probably non-linear (DeWitt 2016). Furthermore, Pigliucci (2010) criticises that the genotype is only one of several factors determining the phenotype and proposes to also investigate RNA folding, protein functioning and gene networks.

Genetic polymorphism (local adaptation) or phenotypic plasticity can be responsible for variation in phenotypic traits. Which one will be favoured by selection depends on the benefits and costs of both, environmental heterogeneity, reliability of potential environmental cues, and a genetic basis of plasticity (Berrigan and Scheiner 2004). The advantage of plasticity is that an organism can thrive in a broader range of environments, yet there are probably costs to it, as a plastic organism rarely achieves the same performance level as a specialist at that range. Costs of plasticity can supposedly arise due to the maintenance of sensory structures to gather information about the environment and the process of plasticity itself as well as the genetic architecture (DeWitt *et al*. 1998). However, different studies propose that costs are rarely observed and limits to plasticity might rather be set by the unpredictability /unreliability of the environment, not enough time to adapt, or simply developmental constraints (Berrigan and Scheiner 2004). Also evolutionary transitions between monomorphism, polyphenism and genetic polymorphism could happen through multiple routes (Schwander and Leimar 2011). Plasticity could be the initial state, and only later be fixed as a genetic polymorphism. Likewise, a genetic cue for variants could be exchanged for an environmental one (Leimar and Schwander 2011).

In stable environments genetic polymorphism will be favoured (Leimar 2009). Costs of plasticity, environmental fluctuations that occur too quickly for an organism to detect or respond to, as well as environmental unpredictability decrease the probability that plasticity will evolve. For example, a model by Reed *et al.* (2010) predicted that populations with strong plasticity encounter higher extinction probabilities when no reliable cues were present, while population size could be buffered in variable environments when cues were reliable. Quantitative genetic models with continuous environmental variation proposed that spatial environmental heterogeneity in general leads to genetic polymorphism, while temporal fluctuations, within and between generations leads to plasticity. However, discrepancies between models exist. In a metapopulation, with high migration rates, spatial heterogeneity might be translated into temporal heterogeneity, and hence favour plasticity as well (Berrigan and Scheiner 2004). Predictability of environmental fluctuations favours plasticity due to the genetic load of fixed polymorphisms (Leimar 2009). However, there is an ongoing discussion about whether phenotypic plasticity has a genetic basis and is, hence, the target of selection or whether the different phenotypes are under selection and plasticity is just a by-product (Windig *et al.* 2004).

In respect to poecilogony, genetic polymorphisms as well as environmentally cued polyphenisms are known as underlying mechanisms resulting in the variation in developmental mode observed within species (Collin 2012, Knott and McHugh 2012). According to mating experiments

poecilogony in *Streblospio benedicti* is based on a genetic polymorphism (Levin *et al.* 1991, Levin and Bridges 1994). Comparative transcriptome analyses of *S. benedicti* with lecithotrophic and planktotrophic developmental modes, however, revealed recent gene flow between them and suggest that the genetic basis of poecilogony in *S. benedicti* is either due to differences at developmentally important loci or modest allele frequency differences at many loci, or alternatively, that recurrent ecological diversification has occurred (Zakas and Rockman 2015). A well-studied example of polyphenism as a mechanism of poecilogony is represented by the sea slug *Alderia willowi* (Krug *et al.* 2012). In this case, temperature and salinity are the cues that in some populations trigger the onset of planktotrophic clutches in winter by the same females that produce only lecithotrophic ones in summer. Since planktotrophic larvae lose their dispersal advantage in closed systems and the higher survival chances of lecithotrophic larvae prevail, this plasticity serves as an adaptation to the seasonal cycle of estuary opening and closing (Krug *et al.* 2012). Developmental mode can also be influenced by the phenotype or environment of the parents (parental effects) as shown for *Polydora cornuta* where sperm limitation leads to the production of lecithothrophic larvae (Badyaev and Uller 2009, Rice and Rice 2009). Diversified bet-hedging might be represented by the different types of larvae emerging from the same egg capsule in *B. proboscidea* (Gibson 1997, Oyarzun and Strathmann 2011).

1.4 Population ecology and population genetics (CONSEQUENCES)

The life history of an organism is directly linked to the dynamics of a population, since population growth is determined by the age-specific birth and mortality rates as well as the population´s age structure. Incorporating only these parameters would result into an exponential or geometric growth rate depending on whether reproduction occurs continuously or is restricted to certain periods in the year (Ricklefs 2001). The growth rate is, however, also subject to environmental impacts, both abiotic and biotic. These are categorized into density-dependent and density-independent factors. The availability of density-dependent resources decreases with increasing densities due to competition, e.g. food, area or hiding places. Moreover, at high densities diseases and parasites are easily spread and predators are attracted. Hence, population growth is self-limited resulting in logistic growth curves rather than exponential ones, and it approaches zero when the maximum number of individuals that can be supported under these environmental conditions is reached, i.e. the carrying capacity (Ricklefs 2001). Cyclic or chaotic oscillations in population size can also be observed as a result of time delays between the response of birth and mortality rates to changes in the environment. Density-independent factors including temperature, precipitation, or catastrophes such

as storms or sudden freeze do not regulate population growth but can influence it and decrease population size below its carrying capacity. Environmental conditions can vary temporally and spatially as well as be predictable or stochastic. For example, seasonal changes in temperature and food availability are predictable temporal fluctuations that have great impact on the timing of reproduction. Sudden rain storms or other catastrophes, on the other hand, are rather unpredictable (Ricklefs 2001).

Particularly important for populations of marine invertebrates is, however, that they resemble open populations. Due to the great dispersal potential of planktonic larvae and high connectivity of marine habitats, immigration and emigration also play an important role in local population dynamics (Gaines and Lafferty 1995). Consequently, marine invertebrate populations should rather be viewed as metapopulations that are composed of local subpopulations as done by Roughgarden and Iwasa (1986). The dynamics of metapopulations are determined by local extinction and recolonization of empty habitat patches (Levins 1970, Hanski 1998). Of further importance is the spatial heterogeneity of landscapes leading to habitat patches of different size, quality, and connectivity to other patches. Large, high quality patches can support large populations that in turn can serve as sources of individuals to smaller populations on poor quality patches and hence prevent them from potential extinction (Pulliam 1988, Ricklefs 2001). The concept and assessment of metapopulations, however, is more studied with terrestrial populations since there arise several problems when dealing with marine populations. Firstly, the large population sizes and high dispersal potential during the larval stage leads to weak genetic structure. Additionally, marine metapopulations occur on large spatial scales making it impossible to sample every subpopulation and they are subject to extreme temporal variability which can lead to stochastic migration patterns (Gaggiotti 2017). Gaggiotti (2017) suggested an integrated approach including genetic data, microchemical fingerprinting of larvae and biophysical modelling of larval dispersal using a Bayesian framework to solve these issues.

As mentioned earlier, complex life-cycles of marine invertebrates imply that different life stages are subject to different selection pressures, and that events in one life stage can affect performance in other life stages. Hence, population dynamics might be predicted more appropriately by models taking into account specific processes occurring in the benthos (competition during settlement, reproduction, benthic mortality) and the water column (dispersal via diffusion and advection, larval mortality) (Eckman 1996, Possingham and Roughgarden 1990). For example, considering that larval mortality is about 14 % higher than post-larval mortality for marine invertebrates in the inner Danish waters (Pedersen *et al.* 2008), a combinatorial approach might be needed.

Population structure and dynamics can be analysed via life tables that describe fecundity and survival rates at certain ages, but also via allele frequencies of genetic loci in the population. For example, with such data we can gain information about migration and differentiation between populations. High connectivity and migration rates between subpopulations that translate into gene flow will lead to homogeneity in allele frequencies between these

subpopulations. In contrast, limited gene flow due to a geographical, temporal or behavioural barrier can result into population structure, i.e. heterogeneity in allele frequencies between subpopulations. Such population differentiation can be caused by natural selection or random effects. Genetic drift describes the random change in allele frequency from one generation to the next and will eventually lead to the fixation/loss of one allele and a change in genotype frequency. Genetic drift resembles a sampling effect and is dependent on the number of individuals contributing gametes to the next generation, the effective population size. Hence, the effect of genetic drift is more pronounced in small populations. The founder effect, when a small number of individuals establish a new population, can lead to population differentiation for the same reason (Hamilton 2009).

Migration and subsequent gene flow between populations of marine invertebrates can occur via drifting of adult specimens but mainly occurs during the larval stage. The dispersal potential of larvae has been described as proportional to the time the larvae spend in the plankton (Weersing and Toonen 2009). Accordingly, subpopulations with planktotrophic larvae are expected to show high gene flow leading to homogenous allele frequencies between subpopulations and low fluctuations in population density, while subpopulations with lecithotrophic larvae are expected to be genetically differentiated due to lack of dispersal (Havenhand 1995, Eckert 2003). Based on their models, Palmer and Strathmann (1981) proposed that population growth increases with distance of larval dispersal, although the increase slows until reaching an asymptote with increasing dispersal distances. Moreover, using population genetics, a discrepancy between potential and realized dispersal was detected (Gaines and Lafferty 1995, Weersing and Toonen 2009). Weersing and Toonen (2009) suggested that there might not be a correlation between pelagic larval duration and population differentiation, because the time spent in the plankton can be a plastic trait and dispersal is also dependent on mesoscale oceanographic currents (Weersing and Toonen 2009). Moreover, larvae can detect environmental cues and actively position themselves in the water column, so that local recruitment to a favourable habitat might be preferred (Strathmann *et al.* 2002).

Population genetic models for metapopulations, additionally include the probability of subpopulations to go extinct and become re-colonized. Although there are different types of metapopulations, in general, depending on the mixture of larvae from different subpopulations re-colonization events can either decrease or increase differentiation between subpopulations (Harrison and Hastings 1996, Hamilton 2009). When larvae are not randomly mixed, i.e. when there is collective dispersal, a founder effect can be introduced, increasing differentiation between subpopulations (Broquet *et al.* 2013). Likewise, population structure can arise due to sweepstakes reproductive success (SRS), when variation of reproductive success in highly fecund organisms leads to only a small subsample of the population contributing to the next generation. This variation can be a result of stochastic oceanographic processes acting on fertilization, spawning, larval survival and settlement (Hedgecock 1994). Both

effects, collective dispersal and SRS, can lead to chaotic genetic patchiness (CGP), which describes temporal and spatial population structure and differentiation on small scales where dispersal should enable gene flow and homogenization (Johnson and Black 1982).

In respect to poecilogonous species, although different life history traits were observed, the population dynamics of the spionid *Streblospio benedicti* were similar regardless whether planktotrophic or lecithotrophic larvae were predominant (Levin *et al.* 1987, Levin and Huggett 1990). Higher larval and juvenile survivorship of lecithotrophic larvae was counterbalanced by higher fecundity of individuals with planktotrophic larvae. Moreover, sites dominated by lecithotrophic larvae had higher densities, while planktotrophic larvae had higher colonization abilities, and hence, were more common among disturbed areas (Levin *et al.* 1987, Levin and Huggett 1990). Planktotrophic larvae are typical for opportunistic species and might be advantageous in temporally varying environments to dampen population fluctuations and re-colonize empty habitat patches, but are also characterized by high mortality and poor competitive ability (Thorson 1950, McEdward 2000, Marshall and Burgess 2015). Lecithothrophic larvae, in contrast, might be advantageous in environments with high predation in the plankton and spatially heterogeneous habitat quality, to ensure larvae are recruited locally into favorable habitats (Pechenik 1999). The evolution of phenotypic polymorphisms might be promoted by environmental heterogeneity (Chia *et al.* 1996). Yet, a dispersal polymorphism, as observed in *S. benedicti*, might simply be the result of asymmetric dispersal commonly present in metapopulations due to their source-sink dynamics (Zakas and Hall 2012). Regardless of the underlying evolutionary and mechanistic basis, polymorphisms show similar effects on population dynamics such as increased niche breadth and colonization ability as well as decreased population fluctuations and vulnerability to environmental change. However, similar effects due to developmental plasticity, genetic polymorphism and bet-hedging are achieved via different mechanisms and based on certain assumptions, e.g. that plasticity has a genetic basis (Wennersten and Forsman 2012).

1.5 Aims of the thesis

The ultimate goal of this thesis was to contribute to a better understanding of the selective processes that favour a certain type of larvae in a certain environment, and consequently, lead to the evolution of larval diversity in marine invertebrates (Strathmann 1985). The type of larvae is a result of the trade-off between size and number of offspring and, as an important life history trait, has major consequences on the dynamics of a population. Advantages of one type of larvae or the other have been attributed to higher dispersal potential and avoidance of predation (Pechenik 1999), but are likely more complex since, for example, a greater dispersal potential of planktotrophic larvae is not always

realized (Weersing and Toonen 2009). Studies investigating the presence of different modes of development in poecilogonous species have the advantage that they are not confounded by developmental and phylogenetic constraints as are studies comparing developmental modes between different species (Knott and McHugh 2012). Therefore, the focus of this thesis were populations of the poecilogonous spionid polychaete *Pygospio elegans* in the Danish Isefjord-Roskilde Fjord estuary complex, since these populations are known to produce different types of larvae (Rasmussen 1973, Kesäniemi *et al.* 2014a).

In order to understand the evolution of a certain mode of development and the consequences of developmental mode on a population level, the ecology of the species in its particular environment must be known. Hence, the aim of study I in the thesis was to describe the structure and dynamics of four geographically close populations, particularly focusing on their reproduction, including larval type. Furthermore, certain abiotic environmental parameters were documented and their impact on the population dynamics of *P. elegans* investigated.

Population structure and dynamics might not only be the result of environmental conditions. The plasticity of an organism´s response to environmental conditions can be genetically determined. Hence, different degrees of plasticity might lead to different underlying mechanisms of poecilogony. Accordingly, in the sea slug *Alderia willowi* poecilogony was attributed to an environmentally cued polyphenism, while in the polycheate *Streblospio benedicti* the variation in developmental mode is based on a genetic polymorphism (Levin *et al.* 1991, Krug 2007). An interaction between environmental and genetic impacts on poecilogony could also occur via epigenetic effects. The aim of study II was to investigate the spatial and temporal genetic structure of populations characterized in study I and to relate genetic structure to the previously observed population dynamics and environmental parameters. The results of study I and II were expected to help clarify the mechanisms and consequences of poecilogony in *P. elegans*.

The genetic diversity within a species but also the species diversity of the whole community affects the dynamics of a population. Diversity at both of these levels can in turn be influenced in a similar way by ecological and evolutionary processes due to the carrying capacity of the habitat or environmental conditions (Vellend 2003). The aim of study III was to characterize the species diversity of the benthic fauna community at the same locations where the population dynamics and genetic diversity of *P. elegans* were assessed previously. Furthermore, patterns among locations and across seasons were compared to determine whether genetic diversity of *P. elegans* and species diversity correlate and, if so, which environmental parameters appear to affect diversity measures on both levels. While specific to these populations and locations, this study contributes to a general lack of study of such species-genetic diversity correlations (SGDC) in marine environments.

Heterogeneous environments promote metapopulations, which in turn could maintain dispersal polymorphisms, such as poecilogony, due to assymmetric dispersal (Chia *et al.* 1996, Dias 1996, Zakas and Rockman 2015).

Estuaries are generally described as heterogeneous environments due to temporal (tidal, seasonal and stochastic) and spatial fluctuations in salinity as well as other environmental parameters such as sediment structure, dissolved oxygen content, and temperature (Kaiser *et al.* 2011, Whitfield *et al.* 2012). *Pygospio elegans* is a euryhaline species and common in estuaries, and populations from estuaries show variation in developmental mode more often than do populations of *P. elegans* from more stable environments (Rasmussen 1973, Gudmundsson 1985, Morgan 1997, Bolam 2004, Kesäniemi *et al.* 2014b). The aim of study IV was to investigate how *P. elegans* copes with salinity changes at different time scales, measuring physiological and ecological responses, to clarify whether salinity fluctuations might be related to variation in developmental mode. Understanding the consequences of salinity stress might help explain why poecilogony is more common in estuaries and whether it can be related to metapopulation dynamics.

2 MATERIALS AND METHODS

2.1 Study species *Pygospio elegans*

Pygospio elegans is a small tube-dwelling spionid polychaete that commonly occurs on intertidal mudflats in the circumboreal region. *Pygospio elegans* is very versatile in many respects: it occurs in densities from several hundred up to 600,000 individuals per m^2 (Linke 1939, Morgan 1997); it exhibits wide habitat tolerances, and for example, is present in salinities down to 5 (Anger 1984). It also exhibits a variety of feeding strategies and can act as a deposit-, suspension- as well as filter-feeder (Fauchald and Jumars 1979). Most interesting for this study, however, is its variable life history (Söderström 1920, Hannerz 1956, Gudmundsson 1985, Rasmussen 1973, Kesäniemi *et al.* 2012b). Specimens of *P. elegans* can reproduce asexually by fragmenting into three to four pieces that subsequently remain in the sand tube and regenerate (Rasmussen 1953). Moreover, it was confirmed as a truly poecilogonous species via morphological and genetic analysis (Morgan 1997, Kesäniemi *et al.* 2012c). Females lay one egg string that is composed of several egg capsules within their tube and ventilate it (Fig. 4B). One egg capsule contains the eggs produced in one segment (Söderström 1920). Depending on the ratio of fertile eggs to nurse eggs, larvae will emerge as planktotrophic/planktonic or adelphophagic/benthic larvae (Fig. 4A). Planktonic larvae hatch at a 3 setiger stage from egg capsules that contain more than 10 fertile eggs and no or only few nurse eggs. They possess swimming setae and remain in the plankton for 4–5 weeks to feed before they settle at a size of 12–16 setigers (Fig. 4C, D). Benthic larvae, on the other hand, develop within the egg capsules that contain up to three fertile eggs and many nurse eggs until they hatch at 14–20 setiger stage and are immediately ready to settle (Fig. 4E). Also intermediate types of larvae have been described that spend short time in the plankton before settling (Söderström 1920, Hannerz 1956, Rasmussen 1973).

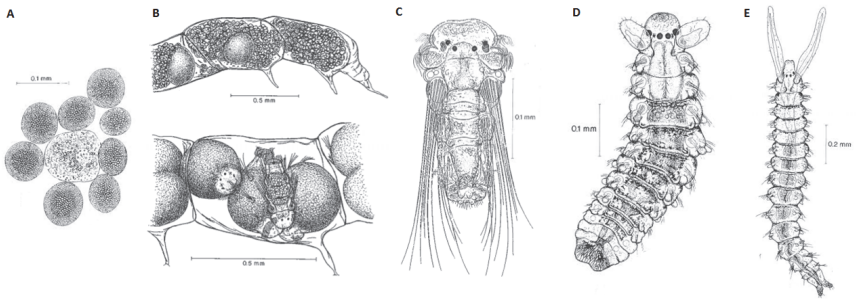

FIGURE 4 Drawings of egg capsules and larvae of *Pygospio elegans* by Rasmussen (1973): A) one fertile egg with nucleus and several nurse eggs. B) three egg capsules of an egg string showing benthic developmental mode with few fertile eggs and a typical hunchback shape of the embryos at a later stage, C) small pelagic larvae from plankton, D) pelagic larvae at the benthos after metamorphosis, E) benthic larvae shortly after hatching.

Other life history traits, such as maturity and longevity for populations of *P. elegans* with planktonic larvae were described by Anger *et al.* (1986). Larvae reproduce for the first time 15–17 weeks after hatching and mature individuals were usually larger than 35 setigers, and in most cases even larger than 45 setigers (Gudmundsson 1985, Anger *et al.* 1986). When specimens carry gametes, males and females are easily distinguished, but sex cannot be determined from live individuals at other times. In laboratory cultures the average life span of *P. elegans* was 9 months, however the oldest specimen survived for almost 2 years (Anger *et al.* 1986). Reproduction was described to be seasonal with sexual reproduction occurring from late autumn to early spring and asexual reproduction happening throughout the year but peaking in spring after sexual reproduction (Rasmussen 1973, Gudmundsson 1985, Bolam 2004). However, populations can differ in timing of reproduction with sexual reproduction also noted as occurring during summer (Söderström 1920, Hannerz 1956, Morgan 1997). Not only variation in timing but also differences in developmental mode could be observed even between geographically close populations (Morgan *et al.* 1999, Kesäniemi *et al.* 2014a). Accordingly, the population in Kiel Bight was solely maintained via asexual reproduction, while other populations showed no signs or only low degree of it (Anger 1977, Bolam 2004). Likewise, only benthic and intermediate larvae or only planktonic larvae could be observed in some populations, while others showed a seasonal switch from planktonic to benthic larvae or multiple types of larvae were present simultaneously (Anger 1977, Gudmundsson 1985, Morgan *et al.* 1999, Bolam 2004). Population genetic analyses of *P. elegans* revealed a pattern of isolation by distance from the Baltic to the North Sea and temporal variation (Kesäniemi *et al.* 2012c, Kesäniemi *et al.* 2014b).

2.2 Field survey

2.2.1 Isefjord-Roskilde Fjord complex and sampling scheme

The Isefjord-Roskilde Fjord estuary complex is the second largest estuary in Denmark (Fig. 5). Isefjord with its 280 km² surface area and a mean depth of 7 m does not represent a typical estuary, as salinities are higher in the interior part than at the entrance or outside of the estuary because evaporation exceeds freshwater inflow. Its salinity is highest in winter and lowest in summer and it is mainly shaped by oceanic inflow, wind and river runoff. Roskilde Fjord, in contrast, shows the typical salinity gradient with higher salinities at the entrance compared to the interior part of the estuary and reaches its highest salinities during summer. It has a surface area of 117 km² and consists of a shallow broad interior part (max. depth 6 m) and a long narrow upper part that connects it with Isefjord. Both estuaries are microtidal estuaries, with tidal ranges of < 20 cm, but wind-driven changes in water levels can be as large as 100 cm (Rasmussen 1973).

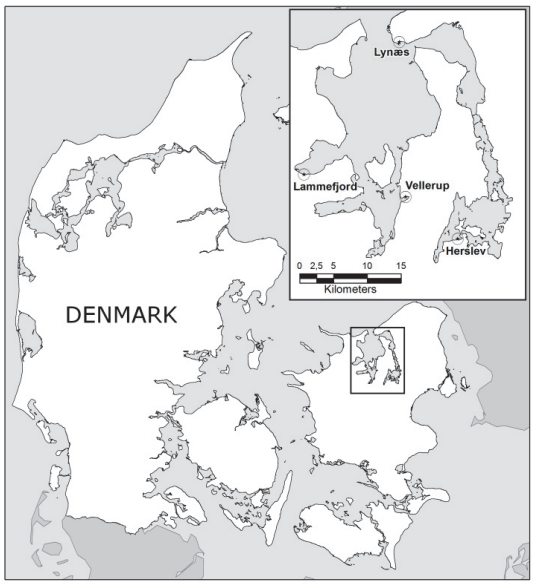

FIGURE 5 Location of the four sampling sites in Isefjord-Roskilde-Fjord estuary complex.

Pygospio elegans was described to reproduce asexually as well as produce benthic and planktonic larvae either seasonally or simultaneously in the Danish Isefjord-Roskilde Fjord estuary complex (Rasmussen 1973). Its population genetic structure in this estuary was described as patchy with temporal

variation (Kesäniemi *et al.* 2014a, b). For that reason, a field survey was conducted from March 2014 until February 2015 at four sites within the estuary complex including Lynæs located at the entrance, Lammefjord and Vellerup within Isefjord, and Herslev within Roskilde Fjord. Different sampling schemes were applied for the different parameters measured. Salinity and temperature were measured continuously, sediment characteristics and macrofauna were determined in March, May, August and November, population dynamics of *P. elegans* were monitored once per month and allele frequencies within *P. elegans* populations were analyzed from samples in March, May, August, October, November and February.

2.2.2 Abiotic environmental parameters (I+III)

The impacts of the environmental parameters salinity, temperature and sediment, including structure and nutrients were investigated, since these were described as important abiotic parameters that shape the distribution of species in estuaries (Kaiser *et al.* 2011). Salinity and temperature at all four sites were documented every 10 minutes via data loggers (HOBO U24-002-C salinity logger, 100–55,000µS cm-1). Reference seawater samples were taken monthly to determine salinity with a salinometer to account for potential drift of the loggers. Sediment characteristics, including water content and porosity, organic content, carbon and nitrogen content, and median particle size and sorting were determined from three replicate kajak cores (5 cm diameter, at least 15 cm length). For that purpose the top 1 cm of the cores was pooled and mixed and sediment characteristics were determined in three analytical replicates except for particle size. Water content and porosity were derived from the wet weight and dry weight (24h at 105° C) of 5 cm3 sediment. Organic content [%] was determined from the dried sediment via loss on ignition (2h at 550° C). Carbon and Nitrogen content was measured in dried and ground as well as pre-combusted and ground sediment samples (30–50 mg) to account for high amounts of shells using an element analyzer (Flash 2000 NCS-Analyzer and FlashEA® 1112 CHNO Analyzer, Thermo Scientific). Particle size was determined to calculate the median particle size and sorting of the sediment (Gray and Elliott 2009). For that purpose the proportion of the dry weight of each size fraction of the Wentworth size scale (8 mm, 4 mm, 2 mm, 1 mm, 0.5 mm, 0.25 mm, 0.125 mm, 0.063 mm) was determined for 50–150 g wet sediment.

2.2.3 Species diversity (III)

Species distributions are furthermore affected by interactions with the biotic environment via intra- and interspecific competition, predation, parasites and diseases. The number of species, i.e. species richness, of a habitat can give valuable information in that respect. Species richness can hereby be measured at different spatial levels. Alpha diversity describes the number of species in a small homogeneous habitat, gamma diversity in all habitats of a geographic area and beta diversity describes the difference in species from one habitat to

another. However, comparisons of species richness can be problematic since species richness can be standardized to area or individuals (Gotelli and Colwell 2001). Standardizing species richness to area results in species density and somewhat assumes that species occur in similar densities, which is obviously not the case. When standardizing species richness to number of individuals more taxa will be detected the more individuals are sampled, with species richness eventually reaching an asymptote. To compare species richness among samples differing in total number of individuals sampled, rarefaction methods can be applied, which randomly draw equal sized subsamples from the total sample (Gotelli and Colwell 2001). However, species richness neglects the fact that also abundance of a species plays an important role for its function in the community (Ricklefs 2001). Different diversity indices, e.g. Shannon-Wiener or Simpson´s index, include both species richness and abundance, such that common species contribute to diversity more than rare ones. Hereby, evenness describes how equal the distribution of sampled individuals is among the different species, i.e. J´ = 1, when individuals are equally distributed among species and J´ will be close to zero when almost all individuals belong to one species.

For study III macrofauna were collected from three replicate sediment cores by sieving them with a 1 mm mesh. The material that remained on the sieve was fixed with 5 % buffered formaldehyde and sorted in the laboratory. Species were identified according to Barnes (1994) and Hayward and Ryland (1995). Subsequently, alpha-diversity was calculated to compare environmental influence on species and allelic richness. Furthermore, Shannon-Wiener index and Pielou´s evenness were calculated with PRIMER-E v. 6.1.13 according to the following formulas:

Shannon-Wiener index: $H' = -\sum P_i \log_e (P_i)$,
P_i - proportion of individuals of i^{th} species from total number

Pielou´s evenness: $J' = H'/\log_e S$,
S - species richness

From these same samples also the density of *Pygospio elegans* for study I was derived.

2.2.4 Population ecology (I+II)

Population dynamics of *P. elegans* were analyzed by determining their size, gender, reproductive activity as well as mode of development from samples collected monthly. For that purpose, surface sediment was collected randomly, sieved with a 1 mm mesh and sand tubes typical for *P. elegans* were collected. Gender and reproductive activity were determined from a subsample of at least 50 individuals. Males can be recognized by an additional pair of soft appendages at the second setiger and sperm in the coelom (Fig. 6A), while females carry eggs in the coelom (Fig. 6B). When several small individuals with

regenerating ends were found in the same tube it was noted as asexual reproduction (Fig. 6C). When egg strings were found in the sand tubes, the mode of development of larvae was categorized according to the number of larvae in the capsules into benthic (1–3) (Fig. 6D), intermediate (4–10) or planktonic (>10) (Fig. 6E) (Kesäniemi *et al.* 2012b). A size distribution of the population was obtained by measuring the length from the eye to the beginning of the gills for at least 30 individuals (Fig. 6F). This procedure was preferred because many individuals lost their tails during sampling. The measurement was performed on narcotized specimens using a camera mounted on a dissecting microscope and the software NIS BR v. 4.2 (Nikon, RAMCON A/S Birkerød, DK). The size distributions were used to subsequently identify size cohorts using Bhattacharya´s method implemented in FiSatII (FAO-ICLARM Stock Assessment Tools II User´s guide, Bhattacharya 1967, see Fig. 6G).

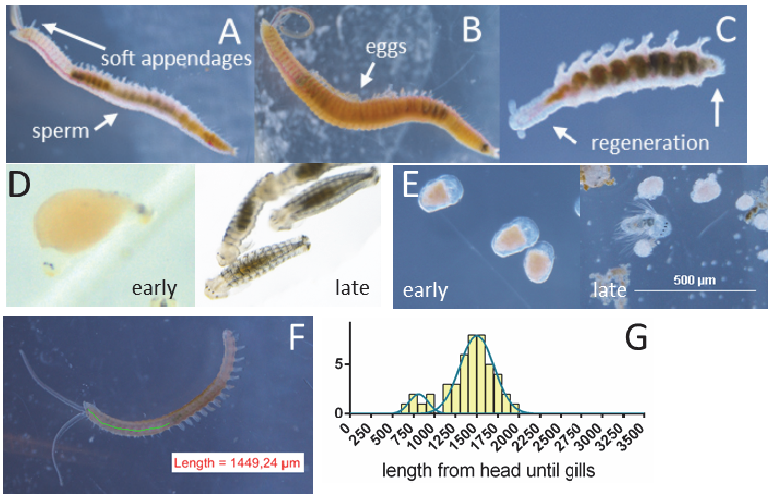

FIGURE 6 Specimens of *Pygospio elegans* were characterized according to the illustrated characteristics as male (A), female (B), performing asexual reproduction (C), producing benthic (D) or pelagic larvae (E). Size was determined from head until gills (F) and the size distributions used to determine average cohort size (G). Source of photographs: Anne Thonig.

2.2.5 Population genetics (II+III)

The genetic structure of *P. elegans* populations was investigated using microsatellite markers, which are useful to explore the most recent evolutionary changes in a population. DNA was extracted from complete specimens using the Qiagen DNeasy Blood & Tissue Kit. Subsequently, ten microsatellite loci were amplified using 1x Qiagen Multiplex PCR Master Mix. Fragments were separated on an ABI PRISM 3130xl and analysed with GeneMapper® v.5 Software (Applied Biosystems). Due to high percentage of missing data and null alleles in three of the loci, the population genetic analysis was continued with only seven loci Pe307, Pe385, Pe6, Pe19, Pe234, Pe294 and Pe369.

Descriptive measures of genetic diversity, such as observed and expected heterozygosity, gene diversity and FIS were calculated in Arlequin v.3.5.2 (Excoffier and Lischer 2010). Rarefied allelic richness was determined using Fstat v. 2.9.3.2 and HP-Rare v1.1 (Goudet 1995, Kalinowski 2005) and relatedness was calculated with the triadic likelihood estimator in Coancestry v.1 (Wang 2007, Wang 2011). The temporal and spatial population genetic structure was analysed using analysis of molecular variance (AMOVA) implemented in Arlequin v.3.5.2 (Excoffier and Lischer 2010). Furthermore, the differentiation between samples was calculated using the indices G´ST and Jost´s D with the R package diveRsity (Hedrick 2005, Jost 2008, Keenan *et al.* 2013). The number of genetic clusters present in the complete sample as well as assignment of individuals to the respective clusters was performed with three different programs: Structure v.2.3.4, InStruct and Flock (Pritchard *et al.* 2000, Gao *et al.* 2007, Duchesne and Turgeon 2012).

2.3 Salinity experiments (IV)

In order to investigate the effect of changes in salinity on *P. elegans* different physiological responses were measured after an acute exposure that more closely represents the fluctuating situation experienced in an estuary. Moreover, also ecological responses were examined after a long-term exposure to changed salinity that specimens occurring in the northern Baltic Sea might experience. Specimens for these experiments were collected in Herslev during summer 2015 and 2016. Hence, they originated from a salinity around 15 and were subsequently exposed to seawater of salinity 5 and 30. Acute exposures lasted for up to four hours and then changes in body volume, tissue water content and RNA expression of seven genes of interest in *P. elegans* were measured.

To document potential changes in body volume, a camera mounted on a dissection microscope combined with the respective NIS BR v. 4.2 software (Nikon, RAMCON A/S Birkerød, DK) was used to record a time lapse video of *P. elegans* after exposure. Additional measurements were taken once a day for the following week. For simplification, a cylindrical shape of the worms was assumed and hence the volume was calculated from measurements of length and width at the fifth setiger.

Tissue water content and RNA expression were measured 45 min and 4 hours after exposure. Tissue water content was determined as the percentage of weight loss between wet weight and dry weight (60°C for 2 hours) from a pool of about 30 specimens. For RNA expression RNA was extracted from whole specimens using Ambion RNaqueous Microkit and transcribed into cDNA using iScript cDNA synthesis Kit (BioRad). Primers for genes included in energy and amino acid metabolism, ion transport, cell signalling and construction of the cytoskeleton were designed based on transcriptome data (Heikkinen *et al.* 2017). Absolute RNA expression was measured using the

digital droplet PCR (ddPCR) technology, where the PCR mix is divided into an emulsion of several thousands of droplets that either contain no or a single or more copies of the template of interest. Amplification will lead to a positive fluorescence signal due to the incorporation of EVAGreen dye, so that the initial concentration of template of interest in the PCR mix can be derived from the ratio between positive and negative droplets.

For the long-term experiment, six groups of 30 specimens were exposed to salinity 5, 15, and 30. Three groups per treatment were sacrificed after 3 weeks, the rest was sampled after 6 weeks. Survival, mean length, and reproductive status including ripe individuals, presence of egg strings as well as signs of asexual reproduction were recorded.

2.4 Statistical analyses

2.4.1 Statistical techniques

Throughout these studies different statistical methods were applied including linear models and multivariate analyses. The basic concepts of both are explained in the following sections.

2.4.2 Linear models

Linear models (LM) are linear regressions with a single response variable and multiple predictor variables of the form:

$$y_i = a + \beta_1 x_{i1} + \beta_2 x_{i2} + \ldots + \beta_p x_{ip} + \varepsilon_i,$$

with y_i - value of Y of the i^{th} observation, x_{i1} - value of first predictor variable of the i^{th} observation, a and β - regression coefficients (intercept and slope), ε - residual error (Quinn and Keough 2002). The total variation is partitioned into variation explained by the regression coefficients (β's) and residual variation (ε). The significance of a regression coefficient can be evaluated by using F or t-statistics to test whether the coefficient is significantly different from zero. The importance of a predictor variable is then described by the p-value and the estimate of the respective regression coefficient. Hereby, predictor variables can be continuous or categorical. To include categorical predictor variables they need to be converted into numerical variables, so called indicator or dummy variables. For example, when including gender a dummy variable could encode all male individuals with 0 and all females with 1 or vice versa. Using dummy variables planned contrasts can be performed, which allow comparisons between groups that are of interest. Such comparison should be decided on (a priori) before data inspection.

Linear models assume normal distribution of the residual errors and therefore also of the response variable, as well as variance homogeneity

between treatments, and independence of observations (Quinn and Keough 2002). Whether a variable is normally distributed can be assessed via Shapiro or Kolmogorov-Smirnov tests or visually via QQ-plots. Variance homogeneity can be checked via Levene´s test or a residual plot, plotting fitted values against residuals. The probability distributions of certain types of data are, however, not expected to follow a normal distribution, such as count data or binary data. Count data, e.g. species abundances, are better described by a Poisson distribution where the mean equals the variance. If over-dispersion occurs and the variance is greater than the mean, e.g. when species distributions are patchy, then a negative binomial distribution describes count data best. For binary data, e.g. presence and absence data, the probability distribution resembles a logistic function. When the probability distribution of the response variable is not a normal distribution the models are termed generalized linear models (GLM). In GLMs the residual error will be modelled by the respective probability distribution. Moreover, a link function is needed that connects the response variable and the predictor variables. In the case of LMs this link function is an identity link function, for Poisson and negative binomial regressions it is a log link function and for logistic regressions a logit link function is needed.

Certain experimental designs do not allow for independence of observations, such as e.g. hierarchical sampling, time series or repeated measures. In generalized linear mixed models (GLMM) random factors can be included in addition to fixed factors to account for such lack of independence. The specific models used are described in more detail in the manuscripts.

2.4.3 Multivariate statistics

In contrast to linear models, multivariate statistics considers multiple response variables at the same time (Manly and Navarro Alberto 2016). Multivariate statistics distinguishes between R- and Q-mode, the former is based on the association between variables (covariances and correlations) including methods such as PCA. The latter is based on resemblance measure between objects including methods such as NMDS and cluster analysis. For this study, multivariate analyses based on resemblance matrices were used and were performed in PRIMER-E v.6 (Clarke and Gorley 2006). Resemblance matrices can describe similarities, dissimilarities or distances and are the basis for many analyses, however, since every response variable will be weighted equally, pre-treatment of the data is necessary in some cases. For example, variables that are on different scales, as is often the case for environmental parameters (temperature, organic content) need to be normalized (Clarke and Gorley 2006). Likewise, e.g. abundance data might need to be transformed to avoid the possibility that highly abundant taxa dominate the results (Clarke and Gorley 2006). Depending on the nature of the data different methods to create similarity or dissimilarity matrices exist. The most common ones are Euclidian distance, which describes a straight line between two points and is geometry based (e.g. environmental parameters, morphometric) and Bray-Curtis

dissimilarity, which is typically used for count data as it gives less meaning to zero abundances.

One way to visualize a resemblance matrix is by ordination using non-metric multi-dimensional scaling (NMDS). NMDS translates relative dissimilarities into relative distances between samples represented as points in a 2D space, i.e. similar samples are close together while different samples are far apart from each other (Clarke and Gorley 2006). To test whether predefined groups of samples differ from each other, the permutation based routine PERMANOVA can be applied. It performs an analysis of variance for several response variables using the resemblance matrix of the samples. For that purpose it partitions the total sums of squares into within and among group sums of squares to calculate the Pseudo F statistics. The distribution of the Pseudo F statistics is obtained via a permutation procedure and the p-value can be derived from it for a specific Null hypothesis (Clarke and Gorley 2006). Hence PERMANOVA does not assume normal distribution of the residuals, but independent and identical distribution of observations (the equivalent of the assumption of variance homogeneity in ANOVAs). While PERMANOVA tests the difference between groups, the routine SIMPER identifies which variables contribute to similarity within a defined group or dissimilarity between groups by decomposing the similarity/dissimilarity matrix (Clarke and Gorley 2006).

The focus of this study was to relate environmental parameters and population dynamics or species abundance. For that purpose the routine RELATE can be used, which measures the rank correlation (Spearman´s or Kendall) between all the elements of two resemblance matrices (one derived from the environmental parameters, the other one derived from the response variables, population dynamics in this case) (Clarke and Gorley 2006). This method is similar to a Mantel test that in contrast uses Pearson correlation. Note, RELATE gives a correlation not a cause-effect relationship between the two multivariate data sets. It is assumed, however, that the environment affects population dynamics and species abundances, hence, also a directional relationship can be investigated, e.g. using distance-based linear models (DistLM) and distance-based redundancy analysis (dbRDA). Similar to a regression, these routines aim to model the relationship between a matrix of predictor variables and a matrix of response variables via partitioning of variation (Clarke and Gorley 2006). Models including different predictor variables are tested and evaluated using various information criteria, such as Akaike or Bayesian information criteria (AIC, BIC). Constrained ordination via the dbRDA routine is used to visualize the variation explained by a specific model (scores) (Clarke and Gorley 2006). The overlaid vectors in a dbRDA plot indicate the importance (loadings) of the predictor variables (the longer the more important) and the relationship between predictor variables (rectangular vectors resemble independent predictor variables; vectors in opposite directions resemble predictor variables with opposite effects).

3 RESULTS AND DISCUSSION

3.1 Field survey

3.1.1 Aim of the field survey

Life history traits such as maturity, longevity, size and number of offspring, as well as number of broods can be genetically fixed due to selection or alternatively, be plastic. This study tried to disentangle the effects of genotype (3.1.2) and environment (3.1.3) on the population dynamics of the poecilogonous polychaete *P. elegans* (3.1.1) with special focus on developmental mode. For that purpose population ecology and genetics were correlated to several environmental parameters.

3.1.2 Population ecology (I)

Sexual reproduction of *P. elegans* occurred mainly from September until May in the Isefjord-Roskilde-Fjord estuary complex. During these times, the highest percentages of gravid females and ripe males were observed followed by the occurrence of egg strings one month later. In contrast, the lowest percentages of gravid females and ripe males were observed during summer, when egg strings were also absent. At Lynæs, Lammefjord and Vellerup two peaks of gravid females and ripe males were observed, while at Herslev only one plateau of sexual competent worms was detected. Additionally, at the three former mentioned sites a switch in developmental mode of the larvae within the egg strings was observed: mostly intermediate and benthic larvae were observed in spring 2014, whereas planktonic larvae were predominant in winter 2014/15. Additionally, benthic larvae occurred again at Vellerup in February 2015. At Herslev, in contrast, there was no switch in developmental mode and primarily benthic and intermediate larvae were found during the whole period. Asexual reproduction occurred throughout the year, but peaked slightly from April to June when sexual reproduction was declining. Similar patterns of sexual and asexual reproduction were observed by Rasmussen (1973, also in the same

estuary system), Gudmundsson (1985) and Bolam (2004), however, Söderström (1920) Gudmundsson (1985), and Morgan (1997), described sexual reproduction also occurring during summer. A seasonal switch in mode of development was described by Rasmussen (1973) in Horsens Fjord and Isefjord and by Gudmundsson (1985) in Blyth estuary (UK), whereas Armitage (1979), Morgan (1997), and Bolam (2004) observed only planktonic larvae year round and Gudmundsson (1985) only benthic ones at Cullercoats (UK). It is unclear whether *P. elegans* is truly iteroparous as suggested by Gudmundson (1985). During this study, gravid females were found within tubes that contained egg strings, however, it is possible that they switched tubes during the sampling and sorting procedure. Also, because the mode of development could be determined only from larvae within the egg strings, it remains uncertain whether a single female could switch the mode of development between broods.

Combining the density and cohort data of *P. elegans* observed in our study, recruitments of new individuals in spring and fall to the studied populations seems likely. At Lammefjord and Herslev new cohorts represented by smaller individuals appeared in spring and fall. At Lynæs the high percentage of asexual reproduction might have led to additional recruitments. In contrast, at Vellerup no cohorts could be distinguished; instead, small individuals seemed to reach adult sizes within a month. At Vellerup sampling of small specimens, however, might have been hampered due to the coarse sediment. Additionally, a mesh size of 1 mm was used, which was probably too coarse to sample the smallest specimens accurately (Gudmundsson 1985, Bolam 2004). Thus, according to the estimated growth rates, the arrival of new recruits might have been missed by about one month at every sampling site. Highest population densities were observed in May, except at Lynæs. No second density peak was observed in fall, which might be due to the fact that density was only quantified in March, May, August and November. The increases in density might be the result of overlapping cohorts: a new cohort arrived while the oldest cohort was still present (Beukema *et al.* 1999). Peaks in density in spring or fall were observed by Gudmundsson (1985) and Bolam (2004), but Morgan (1997) described a more stable population. In general, the densities observed in this study (75–7847 individuals m^{-2}) are in the range of other populations described in Denmark and the English Channel (Muus 1967, Morgan *et al.* 1999) but remain far below the values described at other sites, namely 50,000 to 500,000 individuals m^{-2} (Armitage 1979, Bolam 1999, Morgan *et al.* 1999).

Besides seasonal changes also differences between the sampling sites were observed. Vellerup and Herslev seemed to represent higher quality habitats, since at these sites large specimens (Vellerup: 1496–1848 µm, Herslev: 1343–1818 µm), relatively high densities (Vellerup: 132–7847 ind m^{-2}, Herslev: 189–4791 ind m^{-2}), high percentages of gravid females (maximum, Vellerup: 26 %, Herslev: 33 %) and ripe males (maximum, Vellerup: 33 %, Herslev: 42 %), and a high normalized number of egg strings (maximum, Vellerup: 0.28, Herslev: 0.44) were observed. In contrast, Lammefjord and Lynæs were characterized by small specimens (Lynæs: 1139–1731 µm , Lammefjord: 1074–1648 µm), lower

densities (Lynæs: 0–377 ind m^{-2}, Lammefjord: 75–4357 ind m^{-2}), and lower numbers of gravid females (Lynæs: 10 %, Lammefjord: 22 %), ripe males (Lynæs: 13 %, Lammefjord: 19 %) and egg strings (Lynæs: 0.09, Lammefjord: 0.12), hence, indicating poor habitat quality. This pattern was, however, not reflected in the growth rates of cohorts at the different sites: Lynæs: 3.31–6.41 µm d^{-1}, Lammefjord: 3.61–4.52 µm d^{-1}, Vellerup: 0.88 µm d^{-1}, Herslev: 1.52–4.20 µm d^{-1}. The discrepancy could be due to difficulties in distinguishing cohorts by size and different prevalences of asexual reproduction at the different sampling sites.

3.1.3 Population genetic structure (II)

The analysis of molecular variance (AMOVA) revealed that the population genetic structure of *P. elegans* shows spatial and temporal differentiation. Accordingly, the fixation indices G_{ST} and Jost´s D as well as the cluster analysis with the program Structure indicated that the populations at Lynæs and Lammefjord are genetically similar as well as the populations at Vellerup and Herslev, but that there is genetic structure between the two pairs of study sites. Moreover, seasonal genetic variation was observed in Lammefjord and Vellerup. Specimens sampled at Lammefjord in August and October differed genetically from specimens sampled during other months, and at Vellerup, worms differed in August, October and to some degree also in November. Likewise, highest allelic richness and expected heterozygosity as well as lowest relatedness were observed during August and October at all sampling sites, but these trends were most distinct at Lammefjord and Vellerup.

The change in the genetic composition of the populations at Lammefjord and Vellerup was associated with the arrival of small individuals in spring and fall noticed from the cohort data (see 3.1.1). When comparing genetic composition of the different cohorts identified at each site, it showed that cohorts differed genetically. At Herslev, cohort 2 and 3 differed significantly according to G_{ST}, at Lynæs cohort 3 differed significantly from all other cohorts, and at Lammefjord cohort 2 differed from the other cohorts (cohorts could not be defined based on size at Vellerup, see 3.1.1). These genetic differences are interesting, since they indicate a seasonal turnover of the populations due to the arrival of genetically differentiated cohorts and disappearance of older cohorts. Because there was little or no seasonal genetic differentiation at Lynæs and Herslev, recruitments at these sites might have been predominantly local or from genetically undifferentiated sites. At Lammefjord and Vellerup the arrival of new cohorts in fall 2014 (leading to genotyped adults sampled in November and February) could be the result of local recruitment from the egg strings laid in spring 2014 (by adults genotyped in March and May). Similarly, the spring recruitment 2014 (leading to genotyped adults sampled in August and October) might be the result from egg strings laid in winter 2013/14 (no samples or genetic data available). Since egg strings were observed from March until June at some sampling sites and new recruitments were registered from September on, this would result in a developmental time from egg capsule to juvenile of 2–

6 months. However, according to Anger *et al.* (1986), in the laboratory, planktonic larvae of *P. elegans* spend about 4–5 weeks in the plankton from hatching until settlement. Although not known with certainty, developmental time of benthic larvae is expected to be faster than that of planktonic larvae. Hence, the fall recruitment (leading to the genotyped adults sampled in November and February) more likely originate from egg strings laid in early fall 2014 (by adults genotyped in August and October). And the spring recruitment (leading to genotyped adults sampled in August and October) might emerge from egg strings laid in early spring 2014 (by adults genotyped in March and May). This would indicate that the spring and fall recruitment at Lammefjord and Vellerup occurred from one or more genetically differentiated sites located in Isefjord or even Kattegat since neither genetic cluster two persists through August and October nor cluster three persists through November and February. Interestingly, the genetically differentiated cohorts appear to be transient, but our sampling did not cover an additional recruitment event in spring 2015.

A pattern of chaotic genetic patchiness (CGP), when temporal and spatial variation is present even at small scales where dispersal should be able to homogenize the allele frequencies, was previously observed in this estuary complex by Kesäniemi *et al.* (2014a, b). In the present study, also seasonal variation in allele frequencies was observed that, furthermore, differed between sampling sites. Variance in reproductive success (Eldon *et al.* 2016) could be one explanation for the pattern of CGP observed for *P. elegans* in Isefjord-Roskilde-Fjord estuary complex. Hedgecock (1994) termed the process sweepstakes reproductive success (SRS), when only a small proportion of the population contributes to the next generation due to stochastic oceanic conditions. Indeed, at all study sites only 20–60 % of individuals in the populations produced gametes. Moreover, neither gravid females nor ripe males represent in equal proportions the genotypes present in the populations. For example, the genetic cluster dominating at Lynæs and Lammefjord in October and November, does not contribute to sexual reproduction. Such variance in reproductive success results in low effective population size and low genetic diversity of offspring per population. However, the effects of variation in reproductive success can be diminished if larvae from different populations are mixed during their dispersal phase. When larvae from the same population disperse together, a process termed collective dispersal, there can be reduced gene flow between occupied habitat patches and genetic bottlenecks when empty habitat patches are re-colonized, hence, leading to increased genetic diversity between populations (Broquet *et al.* 2013, Eldon *et al.* 2016).

Different cohorts were composed of different genotypes, which was very distinct at Lammefjord. Hence, not only different cohorts, but also different genotypes might explain the switch in mode of development observed at some sites. Indeed, the genetic composition of gravid females and ripe males showed a switch between March, October and February at some sites. Herslev, where predominantly benthic larvae were found, was dominated by the first genetic cluster. At Lynæs, Lammefjord, and Vellerup gravid females only or

predominantly belonged to the first and second genetic cluster from March until August, to the third genetic cluster in October and November, and from November on the number of gravid females belonging to the first or second genetic cluster increased again. Egg strings were dominated by benthic and intermediate larvae in spring and planktonic ones in fall and winter, while benthic ones reappeared in February at Vellerup. Although these genetic differences are suggestive, one has to be cautious when considering if there is a genetic basis of poecilogony of *P. elegans*. Firstly, the switch in developmental mode was observed mainly between breeding seasons (spring 2014 and fall 2015) and not between consecutive broods within a breeding season (fall 2015, spring 2016) since sampling was not conducted in spring 2016. Only a small proportion of benthic and intermediate larvae were detected in February 2015 at Vellerup. Secondly, developmental mode was not inferred directly from females genotyped but from all egg strings found in the sample and the genotypes of their parents are unknown. Unfortunately, as many individuals left their tubes during the sampling and sorting process, there were only a few instances when females were found together with their brood. Furthermore, Kesäniemi *et al.* (2012b) detected isolation by distance for *P. elegans* populations expressing different modes of development, rather than genetic differentiation among populations with different developmental modes, suggesting that different genotypes can produce the same phenotype and that gene flow exists among individuals with different developmental modes. In this study only seven microsatellite loci were used to genotype specimens, yet, a more elaborate study on *S. benedicti* using about 15,000 single nucleotide polymorphisms also revealed recent gene flow between populations with different modes of development (Zakas and Rockman 2015). Lastly, since the third genetic cluster arrived with a new cohort these specimens could have experienced different environmental conditions influencing their developmental mode directly or via epigenetic modifications (Kesäniemi *et al.* 2016).

Also other traits besides reproduction might differ among the three genetic clusters. Average size of specimens belonging to the second genetic cluster is generally small, even if the cluster is dominating the population. Additionally, this cluster shows a low number of gravid females, which in turn have smaller average sizes compared to individuals in the other clusters. Hence, maturity might occur earlier for these individuals and lead to lower total numbers of offspring produced, since number of egg capsules per egg string is related to the number of segments of the mother. Small average sizes and high percentage of asexual reproduction was observed among specimens at Lynæs and Lammefjord where the second genetic cluster predominated, perhaps indicating a prevalence of asexual reproduction in this cluster. Individuals assigned to the third genetic cluster on the other hand always exhibited large average sizes and numbers of gravid females. Specimens of the first genetic cluster showed generally large average sizes similar to those from the third genetic cluster, except at Lynæs and Lammefjord, where the second genetic

cluster dominated. Whether this is due to the abiotic conditions at the two sites or a result of competition between the two genetic clusters is unclear.

To conclude, high seasonal and spatial variation in allele frequencies were found, which are indicative of CGP. The seasonal genetic variation is in line with the turnover of genetically differentiated cohorts and could be a result of the short life span of *P. elegans* and SRS. Several traits such as density, size, reproductive activity and mode of development correlate with the identified genetic clusters. Whether this represents a correlation or causation should be investigated further using reciprocal transplant experiments or manipulative experiments.

3.1.4 Species diversity and macrofauna composition (III)

During the field survey, in total 51 benthic invertebrate taxa were observed. *Hydrobia* spp. was the most common taxon followed by Naididae (Tubifex), and the polychaetes *Hediste diversicolor* and *Pygospio elegans*, which were present in at least 38 out of 48 samples. In contrast, some species were very rare and found in only one sample (with only few individuals): *Sphaeroma serratum* (1), *Malacoceros fuliginosus* (1), *Pectinaria belgica* (1), *Idotea granulosa* (2), *Gibbula cineraria* (3), *Modiolula phaseolina* (4), *Glycera capitata* (4), *Parvicardium pinnulatum* (5), and *Gammarus locusta* (7). Of these rare species one was found at Herslev, one at Lammefjord, two at Lynæs, and six at Vellerup.

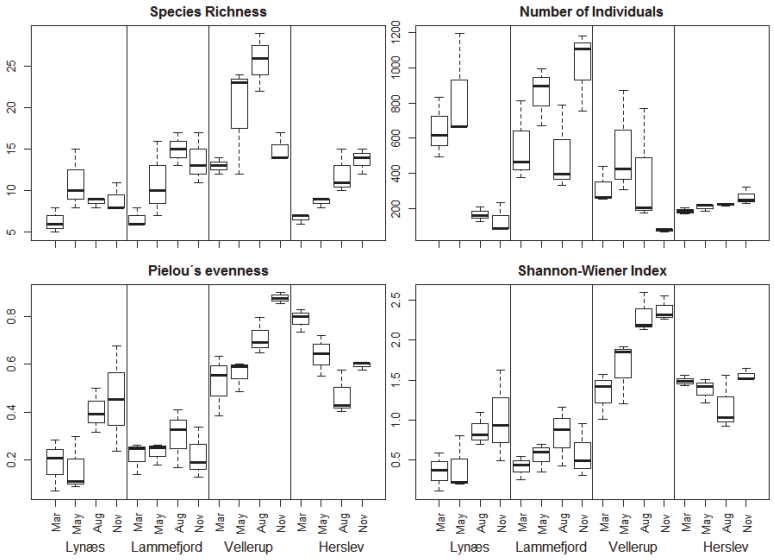

FIGURE 7 Different measures of species diversity averaged over three cores taken at four sites at times during the year 2014: Number of species (species richness), number of individuals, distribution of sampled individuals among the different species (Pielou's evenness), species diversity incorporating species richness and abundance (Shannon-Wiener Index).

Species richness was highest at Vellerup (Fig. 7), however, the functional importance of a species is not only defined by its presence but also by its abundance. The highest total number of individuals was observed at Lammefjord and in March and May at Lynæs (Fig. 7) due to the high abundance of *Hydrobia* spp. in those samples. When *Hydrobia* spp. is removed from the dataset, the highest total abundance was observed at Vellerup, followed by Herslev, Lammefjord and Lynæs. The high abundance of a single species, *Hydrobia* spp., leads to a low evenness and, thus, a low Shannon-Wiener index value at Lammefjord and in March and May at Lynæs (Fig. 7). In contrast, individuals were evenly distributed at Vellerup and Herslev, which resulted in a high Shannon-Wiener index value; higher for Vellerup than for Herslev due to the higher species richness. All diversity indices, species richness, abundance, evenness and Shannon-Wiener index, showed a significant interaction of site and time. Seasonally, the lowest species richness and Shannon-Wiener index was observed in March, while both increased during the year, peaking in August or November. An exception is Herslev, where evenness dropped from March until August and resulted in a decreasing Shannon-Wiener index. Most benthic invertebrates inhabiting mud flats in temperate climates are thought to reproduce in spring, with larvae subsequently settling in spring or summer. Thus, the number of newly arriving individuals would exceed the number of dying ones, leading to an increase in density in spring or summer (Persson 1983, Beukema *et al.* 1999). Such a pattern was observed for *P. elegans* and for the species richness of the benthic community as a whole at Lynæs, Lammefjord and Vellerup. Note, however, that juveniles also can be transported via drift resulting in recruitments later on (Beukema *et al.* 1999).

In the present study species richness was standardized to area, making it a measure of species density (Gotelli and Colwell 2001). Using the sample-based and individual-scaled rarefaction method implemented in EstimateS v. 9.1.0, it was evaluated whether sufficient individuals were sampled per site and time point to determine species richness accurately. Accordingly, enough individuals were collected in half of our samples, since in these samples species richness reached the asymptotic phase. Five of our 16 samples nearly reached the asymptotic phase and three were still in the increasing phase of the rarefaction curves. Overall, sufficient individuals were collected for most of our samples to adequately estimate species richness. The samples in which not enough individuals were collected, leading to a poorer estimate of species richness originated mostly from Lynæs and Herslev.

Species diversity and abundance give information about the community, but to evaluate interactions between members of the community we need to know more about the biology and ecology of specific species and especially their life cycles. Species can have negative interactions due to predation, competition and parasitism but also positive interactions due to facilitation are possible (Gallagher *et al.* 1983, Thrush *et al.* 1992, Bruno *et al.* 2003). *Mytilus edulis*, for example, increases species richness by providing hard substrate and modifies species composition and abundance of the associated community (Norling and Kautsky 2007). In respect to the composition of the total

macrofauna, PERMANOVA revealed an interaction between site and time (p-value 0.001). Based on a NMDS plot, Herslev and Vellerup seemed to differ in macrofauna composition from each other while Lynæs and Lammefjord showed a composition intermediate to the previous sites and similar to each other. Furthermore, March and May look alike, while August differs slightly as does November, except for the sample from Lynæs. These apparent differences were, however, not significant based on pairwise comparisons between samples. SIMPER identified the taxa *Hydrobia* spp., Naididae, *Hediste diversicolor, Scoloplos armiger*, *Polydora* spp., Cardiidae, and *Pygospio elegans* to contribute mainly to the similarity within sites and time points. Lynæs and Lammefjord were thus characterized by high abundance of *Hydrobia* spp., Vellerup by high abundances of *S. armiger*, Naididae, as well as *Mytilus edulis* and Herslev by high abundance of *H. diversicolor*. The abundance of *Hydrobia* spp., Naididae and *P. elegans* was generally high in March and May, the abundance of Cardiidae was high in August, and in November high abundance of Cardiidae and *Polydora* spp. were observed. The focal species *P. elegans* showed highest abundance in May followed by March and lowest abundance in general at Lynæs.

Pygospio elegans lives at the sediment surface and can act as filter- or suspension feeder (Rasmussen 1973) and, as such might interact with bivalves and other polychaetes with similar habits. Bivalves can act as filter feeders, but also facultative surface deposit feeders such as *Macoma balthica* are known (Kube 1996). As such they act as competitors with *P. elegans* for food and space. The effects of filter feeders, including bivalves and epibenthic crustaceans, on larval stages of *P. elegans* are negative in a different way, namely they potentially prey on larvae (Kube and Powilleit 1997). In the present study the abundance of Cardiidae as well as *M. arenaria* and *M. balthica* increase during the year, and one could speculate, that the production of planktonic larvae by *P. elegans* might be a means to escape increased competition pressure, even though these species might feed also on the small larvae of *P. elegans*. *Mytilus edulis,* a suspension feeding bivalve, however, was also described as having a positive effect on *P. elegans* in areas with low suspended food supply by providing faeces that *P. elegans* can use as an extra food source (Kube and Powilleit 1997). In this study, *M. edulis* showed the highest abundance at Vellerup, where the highest species diversity and highest density of *P. elegans* were also observed. The total abundance of bivalves did not differ between sites, but gastropods were more abundant at Lynæs and Lammefjord due to *Hydrobia* spp. in particular. The high abundance of *Hydrobia* spp. is explained by their preference for fine sediment. They also represent deposit feeders that are grazing the sediment surface, and hence, might act as competitor for *P. elegans* at these sites (Newell 1965). Like bivalves, other polychaetes can act as competitors or predators on *P. elegans* adults and larvae. Due to their similar life style, other spionid polychaetes likely act as competitors for food. Furthermore, adult stages also may prey on larval stages (Dauer *et al.* 1981). In this study other small polychaetes or oligochaetes such as *Capitella* sp. and *S. armiger* or Naididae did not seem to have a negative effect on *P. elegans*, since they were

either present to a similar degree at all sites or showed highest abundance at the same site or time point as *P. elegans*. Larger polychaetes, such as Nereididae, and in particular *Alitta virens,* might also prey on *P. elegans* (Rasmussen 1973, Kube and Powilleit 1997). Yet, diversity and abundance of *P. elegans* was high at Herslev where *H. diversicolor* had its highest abundance. Predators of *P. elegans* also include shrimps and fish, which were not monitored during the survey. Muus (1967) described that plaice and flounder mainly feed on the tentacles and prostomia of *P. elegans*. The percentage of individuals regenerating prostomia, exluding the ones clearly performing asexual reproduction, was twice (6 %) as high at Lynæs than at the other sites (3 %). Yet, it remains difficult to draw any conclusions about the predation level at the different sites based on this data. Only large scale temporal and spatial changes were documented during the study while small scale fluctuations in macrofauna were not assessed. As an estimate for small scale spatial heterogeneity, i.e. patchiness, the coefficient of variance between the three replicates of each sample might be useful. Accordingly, no distinct differences in patchiness existed between sites or time points, and unfortunately, the assessment of short-term fluctuations was not possible.

It is unclear how much the dynamics of *P. elegans* are influenced by the species community due to competition and predation and how much the population dynamics of *P. elegans* and the other species in the benthic community respond similarly to common environmental impacts. For example, on the one hand, low density of *P. elegans* and low species diversity at Lynæs and Lammefjord could be a result of competition with *Hydrobia* spp.. On the other hand these two sites could have lower carrying capacities and/or higher predation levels or be more disturbed on a temporal scale, hence supporting lower diversities in general. Species-genetic diversity correlations (SGDC) investigate whether different levels of diversity (species diversity and genetic diversity) are affected by similar ecological and evolutionary processes (Vellend 2003). In the present study a positive correlation between species richness of the benthic community and allelic richness of *P. elegans* was found. Positive correlations are expected when the majority of the species in the community and the focal species exhibit similar life styles and hence probably are affected by the carrying capacity of the habitat and environmental conditions alike. Interactions between the focal species and the species community via facilitation or predation, in contrast, could lead to a negative correlation (Lamy *et al.* 2016). Both species and allelic richness were positively affected by temperature, whereas only species richness increased with coarser sediment structure (environmental parameters are described in detail in 3.1.3.2). The same seasonality of reproduction and larval recruitment common for temperate climates (Beukema *et al.* 1999) thus seems to underlie the diversity of *Pygospio elegans* and the benthic community in the Isefjord-Roskilde-Fjord estuary as well.

In summary, highest diversities were observed at Vellerup and Herslev, although a different composition of macrofauna was present. In contrast, diversities were lower at Lynæs and Lammefjord exhibiting more similar

species composition, most likely due to the presence of *Hydrobia* spp. An increase in diversity could be observed from March to August/November. Referring to the macrofauna composition, the samples in November differed from other time points, except at Lynæs, while the samples at other time points were similar. The biotic interactions between *P. elegans* and the benthic macrofauna are somewhat unclear, although according to the positive SGDC environmental parameters might have a greater impact on *P. elegans* dynamics than the benthic community.

3.1.5 Abiotic parameters and sediment characteristics (I)

Temperature

Temperature did not vary spatially, but showed a strong seasonal pattern. December until February were the coldest months, reaching a weekly minimum of -3 °C in December at Lynæs. Warmest months were July and August with a maximum weekly temperature of 28.6 °C in July at Lammefjord. Rasmussen (1973) described temperatures in Roskilde Fjord being higher than in Isefjord especially in stagnant water, but no such difference was apparent in this study.
Sexual reproduction occurred seasonally and might be induced at low temperatures. Ripe males and gravid females appeared at temperatures below 15 °C and sperm degenerated within males when temperatures rose from 5° to 18 °C (Rasmussen 1973). Likewise, Anger (1984) described higher rates of sexual reproduction at 5° and 12 °C compared to 18 °C. However, sexual reproduction in the field was also reported in summer (Söderström 1920, Gudmundsson 1985, Morgan 1997). According to observations of two peaks of ripe males and gravid females, sexual reproduction probably occurred in two batches at Lynæs, Lammefjord and Vellerup. Gudmundsson (1985) described *P. elegans* as iteroparous, reproducing more than once per lifetime. However, although several cohorts were present at the same time in the present study, usually only one cohort had an average size typical for mature individuals when sexual reproduction occurred. At Lynæs the first cohort that arrived (cohort 1) comprised gravid females only in March and cohort 3 in October, while no gravid females were present in cohort 2 and 4. At Lammefjord cohort 1 included gravid females in March and very few in October, cohort 2 included gravid females in October and cohort 3 in February. At Herslev gravid females were present in cohort 1 in March, in cohort 2 in low numbers in May, August, and November, and in cohort 3 in November and February. Hence, different cohorts might produce gametes at different times indicated by the two peaks of gravid females and ripe males. Yet, individuals were only assigned to cohorts for the months March, May, August, October, November and February, hence, no information about number of females in the different cohorts at other months is given here. Moreover, sexual reproduction might also be possible between cohorts, since females can store sperm in their receptacula seminis already at early ages, before they are sexually mature (Söderström 1920)

Salinity

Salinity showed both spatial and seasonal variation. The mean salinity at Lynæs, Lammefjord and Vellerup was 19–20, while it was distinctly lower at Herslev, about 14, due to its location in the innermost part of Roskilde Fjord. Seasonal fluctuations occurred as previously described by Rasmussen (1973), showing lower salinity in summer at the sites in Isefjord, while higher salinity at Herslev, in Roskilde Fjord. Similar trends were determined from the national monitoring program conducted in Roskilde Bredning and Ydrebredning in Isefjord (National Monitoring and Assessment Programme for the Aquatic and Terrestrial Environment, NOVANA). Additionally, unpredictable short term fluctuations in salinity were observed at all sites, but fluctuations were twice as high at Lammefjord than at the other sites. This might be explained by the location of the data logger close to the entrance of Tuse Å, a larger freshwater input to Isefjord.

Although *P. elegans* represent a very euryhaline species, it was demonstrated that changes in salinity can affect fitness. Accordingly, time to maturity and production of egg strings might be delayed at very low (5) or full strength marine salinities (30) and could hence lead to fewer broods per season (see 3.2). Indeed, gravid females and ripe males occurred one month later at Herslev compared to the other sites (September instead of August), and this was the site with the lowest salinity. Additionally, only one plateau (from September to February) of gravid females and ripe males was present, and only benthic larvae were produced at Herslev. In contrast, two peaks of gravid females and ripe males (August/September and January/February) as well as a seasonal switch from planktonic larvae in winter to benthic ones in spring – although between different breeding seasons – was observed at the other sites. The production of benthic larvae might be more time consuming, since they remain longer in the egg capsules, and could possibly lead to an overlap of the two consecutive broods resulting in the observed plateau at Herslev. Populations only producing benthic larvae were described from low salinity habitats and populations producing only planktonic larvae were described from high salinity habitats, although exceptions also occurred (Gudmundsson 1985, Morgan 1997, Morgan *et al.* 1999, Bolam 2004, Kesäniemi *et al.* 2012b, 2014a, b). The production of benthic larvae that develop in the egg capsules until the size of 14 setigers might represent an adaptation to low salinities, since early life stages are described to be most sensitive to environmental stress in general (Kinne 1966). Similar adaptation to low salinity has been suggested for other polychaetes, for example, *Hediste limnicola*, a viviparous Nereididae that releases its larvae in the freshwater at a stage when larvae of *H. diversicolor* are already capable of osmoregulation (Oglesby 1965). However, it is unclear whether egg capsules prevent larvae from being exposed to low salinities, or rather serve to slow down water inflow, so that abrupt changes in the extra-capsular environment might take several hours to reach and impact the embryo (Pechenik 1983, Richmond and Woodin 1996). Reciprocal transplant experiments would be necessary to confirm whether production of benthic

larvae represents an adaptive response to low salinities. Since benthic larvae were observed during spring at all sampling sites regardless of their salinity, several different factors may act together to influence the developmental mode of *P. elegans*. Anger (1984) did not find a systematic change in developmental mode of *P. elegans* in response to temperature and salinity changes, and observed different types of larvae at only three instances. Dissolved oxygen content of the water could be another abiotic parameter of importance, since Kube and Powilleit (1997) found that *P. elegans* could survive moderate hypoxia but not severe anoxia.

Sediment structure

Spatial variation, but no clear seasonal trend, was observed in sediment structure. In general, Lynæs and Lammefjord had fine sediment, which correlated with high water content and porosity at these sites, and the sediment was moderately well or moderately sorted. At Vellerup and Herslev the sediment was coarse or medium grained, hence, water content and porosity were low, and it was only poor or moderately sorted. The medium grain size increased and water content decreased from May until November in Lynæs, Lammefjord, and Herslev, whereas at Vellerup medium grain size decreased and water content increased. The sediment changed somewhat over the year from March to November, becoming more poorly sorted at Lammefjord and Vellerup, and more moderately well sorted at Lynæs and Herslev. In the present survey, spatial and temporal fluctuations of sediment characteristics were analysed only on a large (site) scale. Sudden disturbances of the sediment that would represent unpredictable temporal changes were not registered. Furthermore, spatial differences within one site (patch scale) also were not analysed, since the different kajak cores were pooled.

Population ecology and genetics of *P. elegans* as well as species richness were affected by sediment structure, including median grain size, sorting, water content, and porosity. The medium to coarse grained sediments at Vellerup and Herslev supported the highest abundances of *P. elegans*, which additionally were largest and had highest numbers of gravid females, ripe males, and egg strings. Hempel (1957) and Armitage (1979) described that *P. elegans* prefers coarse sediment. Furthermore, Kube (1996) observed that high water content hampers the stability of unbranched burrows of the related, but much larger, polychaete *Marenzelleria viridis*, which might also be true for *P. elegans*. The coarser sediment at Vellerup and Herslev also supported higher species richness, species diversity, and when excluding *Hydrobia* spp., also total abundance of individuals. Coarse-grained sediment might provide more microhabitats, and hence allow for higher species diversities and abundance (Kaiser *et al.* 2011). Furthermore, the genetic structure of *P. elegans* correlated to some degree with sediment structure. While the first genetic cluster was more prevalent in coarse and poorly sorted sediment, as in Vellerup and Herslev, the second genetic cluster was more common in fine and well sorted sediment, as found at Lynæs and Lammefjord.

Food supply

In general, the organic content of the sediment was highest at Lynæs, followed by Lammefjord, Vellerup and Herslev. However, the C/N content, which gives information about the bioavailability of carbon, indicated that the most nutritionally valuable material was present at Lynæs, followed by Herslev and Lammefjord, while very refractory material was found at Vellerup. Over the period of the survey, the organic content increased at Lammefjord and Herslev, but decreased at Lynæs and fluctuated at Vellerup. The C/N ratio followed the decreasing trend of water content at Lynæs, Lammefjord and Herslev, while it fluctuated at Vellerup similarly to the organic content.

In general, benthic invertebrates can be predatory, deposit feeders that feed on benthic diatoms and microorganisms in the sediment, or suspension feeders that consume the phytoplankton suspended in the water column. *Pygospio elegans* can thrive as both a deposit and suspension feeder (Fauchald and Jumars 1979). Since the highest densities and largest specimens of *P. elegans* were observed at sites with the lowest organic content and most refractory C/N ratio, it seems that suspension feeding may be preferred over deposit feeding or can support larger populations. A similar pattern was shown for other spionid polychaetes (Dauer *et al*. 1981, Kube 1996). Moreover, Kube and Powilleit (1997) detected a correlation of *P. elegans* abundance with chlorophyll concentration in the water column. According to the Danish national monitoring survey, a peak in chlorophyll content was observed in mid February 2014 in Isefjord and 3–4 weeks later in Roskilde Fjord. Only a small increase in chlorophyll content was observed in August at both locations simultaneously (National Monitoring and Assessment Programme for the Aquatic and Terrestrial Environment, NOVANA). These typical spring and autumn phytoplankton blooms were not reflected in the trends of organic content of the sediment, since in the present study near shore sites were surveyed, which are affected less by sedimentation and more by local processes, including inputs of organic matter from benthic sources (benthic microalgae and macrophytes). Organic matter at deeper stations would likely be more reflective of the patterns in the water column chlorophyll monitored during the national survey. Other species at these sites might provide insights into the organic matter dynamics. Since *M. arenaria* is an obligatory filter feeder and *M. balthica* a facultative deposit feeder (Kamermans 1994), Kube (1996) suggested that the presence of *M. arenaria* might indicate the presence of phytoplankton sources while the presence of *M. balthica* indicates prevalence of benthic diatoms. In the present study *M. arenaria* had high abundance at Lynæs and Herslev, whereas *M. balthica* dominated at Vellerup. Hence, the high abundance and large size of *P. elegans* at Vellerup and Herslev cannot be related to one type of food supply in this study.

In general, the seasonal dynamics of *P. elegans* are largely impacted by temperature. In contrast, organic content and C/N did not affect population dynamics, which might not be surprising since the differences in organic content and C/N were quite modest. Sediment structure correlated strongly with abundance, size and reproductive activity. The heterogeneity in habitat

quality might support source and sink dynamics of a metapopulation. The impact of mean salinity and salinity fluctuations could not be evaluated clearly from the field study and physiological impacts of such will be discussed in more detail in the next section. This study, however, did not investigate small scale disturbances and the effects of, for example, chlorophyll or dissolved oxygen in the water column. These shallow water sites are not likely subjected to low oxygen levels very often, however.

3.2 Salinity tolerance (IV)

The population of *P. elegans* at Herslev reacts to abrupt decrease in salinity from 15 to 5 by increasing body volume and tissue water content due to inflowing water. The maximum body volume was about 2.7 fold larger than initial size and was reached about 30–120 minutes after exposure. Restoration of the initial volume was initiated but could not be fully achieved; within the next week, still a 1.7 fold increase was observed. Tissue water content increased by 9.5 % after 45 min, but decreased subsequently so that after four hours only an increase by 8.2 % was measured. In response to hyperosmotic medium (salinity 30), a decrease in tissue water content could be observed, whereas no change in body volume was apparent. The water content decreased by 11.5 % within 45 minutes and increased after four hours to a reduced tissue water content by 9 % compared to initial water content. The responses were very different depending on the individual, which might be a result of differences in size or condition of the worms. Similar responses with increasing body volume or weight in response to a hyposmotic environment and decreasing body volume or weight in response to a hyperosmotic environment were reported for other polychaetes or cells of polychaetes. The extent of the response differed hereby depending on the osmoregulatory abilities of the species (Oglesby 1965, Fletcher 1974, Costa *et al*. 1980, Dykens and Mangum 1984). The fact that initial body volume could not be restored after a transfer to salinity 5 and that half of the specimens of this treatment died indicates that an abrupt transfer from salinity 15 to 5 is more stressful than a transfer from 15 to 30. These observations suggest that *P. elegans* is a weak cell-volume regulator. This was also supported by the fact that no response in the RNA expression of alanine aminotransferase or tubulin in response to salinity changes could be observed although changes in cell volume affects the cytoskeleton structure (e.g. tubulin) and cell volume can be regulated by adjusting the osmolyte content (e.g. amino acids) in the cell.

Moreover, the RNA expression of genes involved in ionic and osmoregulation, by providing energy (ATP-Synthase), facilitating ion transport (Na^+K^+-ATPase, bicarbonate exchanger, carbonic anhydrase), and cell signalling (IGF) did not change in any clear pattern in response to changing salinities suggesting that *P. elegans* does not regulate ions and osmotic concentration. However, the exposure time in the experiment might have been too short to elicit a strong response at the RNA level. Gene expression changes in response

to salinity change have been described for several molluscs and crustaceans, although those exposures were not necessarily on the same time scale as studied here (Lovett *et al.* 2006, Lockwood and Somero 2011, Towle *et al.* 2011, Zhao *et al.* 2012, Havird *et al.* 2013, Lv *et al.* 2013, Li *et al.* 2014, Hu *et al.* 2015). Immediate response to salinity changes might be achieved via changes in protein concentration or changes in the functionality of existing proteins. To properly evaluate the osmoregulatory capacity of *P. elegans,* however, the osmolality of its body fluids would have to be determined. Measurement of ion concentration as well as osmolality through use of an ion chromatograph and nanoosmometer was pursued in this study, but the extraction of body fluid from these small polychaetes was not reliable and those results were questionable. Thus, they are not presented in this thesis.

Pygospio elegans from the Herslev population does not seem to cope well with sudden large decreases in salinity. At this site, natural fluctuations in salinity down to 5 were observed, however, they were not prolonged, and salinity was low for relatively short periods ranging from a few hours up to one day in duration. Longer periods of low salinity might be achieved at low water levels and during heavy rain storms. Such events could be detrimental for the population if the specimens do not have an avoidance mechanism, such as burrowing deeper in the sediment or closing their tubes.

Also during long-term exposure to different salinities, it became apparent that a transfer to salinity 5 is harsher for *P. elegans* than a transfer to salinity 30. At the control salinity 15 and at salinity 30, asexual reproduction took place, so that the number of individuals actually increased during the experiment, although the mean length of the specimens decreased. In general, the biomass in the two treatments was quite similar. In salinity 5 no asexual reproduction was observed and, in contrast to the other salinities, the number of individuals declined slightly to a minimum of 27. The mean length of these worms increased, however, both after 3 weeks and after 6 weeks. Overall biomass decreased over time at salinity 5. The number of specimens carrying gametes was highest at salinity 15, followed by 30, and 5. Egg strings were only observed at salinity 15 and 30, although in the latter treatment they were only observed after 6 weeks. Anger (1984) described that *P. elegans* had highest reproductive and survival rates in brackish water (salinity 10–16) even when populations originated from full strength marine habitats. Sublethal effects of polychaetes in response to salinity changes include slowed growth and development as well as delayed maturity (Kinne 1966, Qiu and Qian 1997, Pechenik *et al.* 2000). Salinity of 5 seems to be still within the tolerance range of this population of *P. elegans*, however, a decrease in fitness was observed. Populations of *P. elegans* are known to persist in the Northern Baltic Sea at salinities as low as 5 (Kesäniemi *et al.* 2012c). To investigate whether these populations have adapted to the low salinities or whether also *P. elegans* from Herslev could persist at such low salinities, reciprocal transplant experiments would need to be performed. Moreover, experiments would need to last longer and include different life stages to study population persistence, since early life

stages are usually the most vulnerable to salinity stress (Kinne 1966, Qiu and Qian 1997, Pechenik *et al.* 2000).

3.3 Metapopulation dynamics, dispersal and community structure

The population density of *P. elegans* fluctuated at three out of four sites, with the fourth site showing consistently low abundances. Fluctuations like this are characteristic for short-lived opportunistic species that colonize new habitats quickly and then overshoot their carrying capacity (Beukema *et al.* 1999). A high temporal turnover was also visible from the genetic structure of two of the studied populations, indicating CGP. Additionally, there were differences in allele frequencies among sites. Spatial heterogeneity was also apparent in sediment structure and according to abundance, size and reproductive activity of *P. elegans* seemed to affect the quality of the habitat. Kesäniemi and colleagues (2014a) suggested that *P. elegans* in the Isefjord-Roskilde-Fjord estuary complex represents a metapopulation consisting of several subpopulations, however, no real extinctions of any single subpopulation were observed during the present study. Nevertheless, due to the short life span of *P. elegans* with two overlapping size cohorts and low densities, changes in population allele frequencies were observed indicating immigration of individuals from genetically differentiated populations. In two of the study populations not all genotypes persisted, and these were replaced by others, possibly originating from other high quality habitats. Hence, these populations experienced dynamics similar to the metapopulation described for *Pectinaria koreni* in the Baie de Seine (Jolly *et al.* 2014). In addition to seasonal fluctuations, stochastic events such as rainstorms can quickly change the salinity in coastal areas and estuaries and challenge populations of *P. elegans*. As seen in the present study, an abrupt and prolonged decrease in salinity can lead to reduced fitness and ultimately death of *P. elegans*. Kube and Powilleit (1997) described extinctions of populations of three spionids after severe anoxia in the Baltic Sea. Although their numbers increased again quickly afterwards, the same abundances were reached only after about one year.

Considering the population dynamics of *P. elegans* in the Isefjord-Roskilde-Fjord estuary complex, different types of larvae of *P. elegans* might serve different purposes. Planktonic larvae with their high dispersal potential can disperse to or recolonize new habitat patches and thus dampen fluctuations in other subpopulations. Benthic larvae, in contrast, can be retained to maintain the local populations in high quality habitats (Pechenik 1999, Eckert 2003). In this study, local recruitment via benthic larvae resulted in no seasonal variation in allele frequencies at Herslev. Sites with planktonic larvae, namely Lammefjord and Vellerup, in turn exhibited a turnover in genotypes. No clear seasonal genetic variation was observed at Lynæs, although planktonic larvae

were produced in winter. One possible explanation for this could be the high percentage of asexual reproduction at Lynæs, which is another form of local recruitment. Unfortunately, the water circulation in the estuary complex is not known at the appropriate scale to allow an assessment of the impacts water circulation has on larval dispersal and population connectivity. Planktonic larvae could still be locally retained passively via oceanic currents or actively via habitat cues (Strathmann *et al.* 2002, Weersing and Toonen 2009). Likewise, benthic larvae or juvenile stages could disperse via drifting as described e.g. for *S. armiger* and *M. balthica* (Beukema *et al.* 1999). Although different types of larvae are advantageous under certain conditions, these conditions might not be predictable, which could lead to the stochastic production of one type of larvae as a bet-hedging strategy (Chia *et al.* 1996). However, the dispersal polymorphism in *P. elegans* in the Isefjord-Roskilde-Fjord estuary could also be maintained due to asymmetric dispersal between local populations acting as sources or sinks, as has been proposed for *S. benedicti* (Zakas and Hall 2012). Investigating the actual dispersal of larvae and the water circulation in the estuary could help to elucidate the consequences of larval type on population structure.

4 CONCLUSION

The present study confirmed the observations of Rasmussen (1973) that reproduction of *P. elegans* occurs from fall to spring in the Isefjord-Roskilde-Fjord estuary and that multiple types of larvae are present. Yet, in contrast to the results of Gudmundsson (1985), there is evidence that *P. elegans* is semelparous, i.e. produces only one brood per life-time. The two consecutive brood batches are instead produced by different cohorts and lead to the two recruitment events, one in spring and one in fall. Moreover, although *P. elegans* is a euryhaline species with broad distribution and salinity tolerances (Anger 1984), the results of the present study suggest that this species cannot cope with abrupt prolonged salinity changes and might need to adapt to enable persistence at low salinities.

TABLE 1 Summary of the population dynamics of *P. elegans* and the abiotic and biotic environmental conditions at the four sampling sites in the Isefjord-Roskilde-Fjord estuary in 2014/15.

		Lynæs	Lammefjord	Vellerup	Herslev
Environment	Sediment structure	fine	fine	coarse	medium
	Sediment sorting	moderately well	moderately	poorly	moderately
	Mean salinity	19	19	19	14
	Organic content	0.92	1.04	0.84	0.78
	C/N	8.28	8.83	9.53	8.74
	Species diversity	0.66	0.59	1.92	1.4
Pygospio elegans	Density (ind m-2)	0–337, no peak	75–4357	132–7847	189–4791
	Mean length (µm)	1139–1731	1074–1648	1496–1848	1343–1818
	Gravid females	10 %	22 %	32 %	26 %
	Developmental mode	benthic, planktonic	benthic, planktonic	benthic, planktonic	benthic
	Asexual reproduction	8.7 %	3.1 %	1.3 %	1.1 %
	Genetic cluster	2	2, 3	1, 2, 3	1

The main results of the field study relating the population ecology and genetics of the poecilogonous polychaete *P. elegans* in the Isefjord-Roskilde Fjord estuary to environmental parameters are summarized in Tab. 1. According to the size cohort and population genetic data, the populations were very dynamic with high seasonal and spatial turnover. The data supports that this is a result of the short life span of *P. elegans* and sweepstakes reproductive success as suggested by Kesäniemi *et al.* (2014a). Seasonal dynamics influenced timing of reproduction, which was correlated with temperature, while spatial variation was correlated with sediment structure. To properly document the dynamics and identify patterns, a longer field study would be needed. This should ideally include the assessment of larval dispersal via plankton samples, answering questions about the realized dispersal distances, the occurrence of collective dispersal and the water currents.

In respect to developmental mode it is still unclear whether poecilogony in *P. elegans* resembles i) a genetic polymorphism that is maintained via asymmetric dispersal or ii) a plastic response to certain environmental conditions or iii) a bet-hedging strategy in response to environmental unpredictability (Chia *et al.* 1996, Krug 2007, Zakas and Hall 2012). Although genetically differentiated cohorts might have resulted in different types of larvae, an environmental impact cannot be excluded. Reciprocal transplant experiments or mating experiments with populations exhibiting one or the other type of larvae could help to answer this question. Furthermore, manipulative experiments would need to be performed to identify potential selective pressures such as salinity fluctuations or level of predation on the mode of development. In these experiments specimens could be chosen according to genotype, and in addition to mode of development, also other life history traits such as age of maturity and longevity could be documented to get a better picture of their consequences on population dynamics.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Kehitysmuotojen variaatio ja sen vaikutus *Pygospio elegans* -monisukasmadon populaatiodynamiikkaan heterogeenisessä ympäristössä

Meressä elävillä selkärangattomilla pohjaeläinlajeilla on useita erilaisia lisääntymisstrategioita ja toukkamuotoja. Morfologiaerojen lisäksi toukkamuodot eroavat ravinnon lähteiden suhteen. Yleisin muoto on vedessä elävä ja ravintoa etsivä toukka, mutta toukat voivat saada ravintonsa myös maternaalisesti suoraan ravinteikkaasta munasolusta, tai naaraan tuottamista ylimääräisistä ravintomunista. Toukkamuotojen tuottamisessa on suuria energeettisiä eroja: veteen vapautettavia planktisia toukkia voidaan tuottaa pienistä munista suuria määriä, kun taas maternaalista lisäravintoa käyttäviä toukkia tuotetaan yleensä pienempi määrä, sillä isokokoisten munasolujen tai ylimääräisen ravinnon tuottaminen vie enemmän resursseja. Eri lisääntymisstrategioita, eli toukkien kehitysmuotoja suositaan eri ympäristöissä, ja toukkamuoto vaikuttaa myös lajien populaatiorakenteisiin. Esimerkiksi planktisten toukkien tuottamista voidaan suosia aikana jolloin vedessä on runsaasti ravintoa, eli kasviplanktonia. Planktisilla toukilla on korkea levittäytymispotentiaali, jolloin populaatioiden välinen migraatio ja geenivirta voi olla suurta. Planktisia toukkia tuotetaan usein runsaasti, mutta toisaalta niiden kuolleisuus on suuri verrattuna pohjasedimentissä tai suojaavissa rakenteissa eläviin toukkamuotoihin. Jos lajilla ei ole dispersoivaa planktista toukkavaihetta, populaatioiden välinen migraatio voi olla heikkoa ja populaatiot eriytyä geneettisesti. Vaikka eri ympäristöistä peräisin olevia eri toukkamuotoja tuottavia lajeja on vertailtu aikaisemmin, ei toukkamuotojen esiintymiseen liittyviä valintapaineita vielä täysin ymmärretä. Eri lajeja vertailtaessa tuloksiin vaikuttaa myös fylogeneettiset rajoitukset. Tutkimuksiin toukkien kehitysmuotoihin liittyvistä evolutiivisista valintapaineista onkin sopivampaa käyttää lajia, joka pystyy tuottamaan eri toukkamuotoa lajin sisällä (poecilogony).

Vain 14 lajin tiedetään omaavan lajinsisäistä variaatiota toukkamuodoissa. *Pygospio elegans* -hiekkaputkimato on yksi näistä. Nimessä mukaisesti tämä yleinen monisukasmatolaji elää rakentamissaan hiekkaputkissa vuorovesialueilla pohjoisella pallonpuoliskolla. Lajin eri populaatiot voivat tuottaa vain joko yhtä toukkamuotoa, vaihtaa toukkamuotoa vuodenajasta riippuen tai tuottaa erilaisia toukkamuotoja samanaikaisesti. Tässä väitöskirjassa tutkin *P. elegans* -hiekkaputkimatojen lajinsisäistä toukkamuotojen monimuotoisuutta, sen mahdollisia syitä ja seurauksia neljässä tanskalaisessa populaatiossa, joissa on havaittu sekä planktisia että naaraan hiekkaputkessa kasvavia ilman pelagista toukkavaihetta kehittyviä toukkia. Näitä Isefjord-Roskilde Fjord estuaarialueella olevia populaatioita seurattiin vuoden ajan keräten aineistoa populaatioiden rakenteen muutoksista ja populaatiogeneettisistä rakenteista, sekä useista bioottisista ja abioottisista muuttujista.

Tutkimuksessani selvisi, että kyseisissä *P. elegans* populaatioissa suvullista lisääntymistä tapahtui syyskuusta toukokuuhun, elinympäristön lämpötilan ollessa alimmillaan. Joissakin populaatioissa lisääntymisessä oli kaksi selkeää aktiivisuusaikaa vuodessa. Vuoden aikana yksilöiden kokojakaumassa nähtiin populaatiosta riippuen kolmesta neljään erillistä, mutta osittain päällekkäistä kohorttia. Populaatioiden geneettisessä rakenteessa havaittiin paikallista ja vuodenaikojen välistä vaihtelua, joka saattaa johtua *P. elegans* -matojen lyhyestä eliniästä ja suuresta variaatiosta yksilöiden välisessä lisääntymismenestyksessä. Joissakin populaatioissa vuodenaikojen väliset muutokset alleelifrekvensseissä ajoittuvat uusien kokoluokkien ilmestymisen kanssa yhtenäisesti. Lisäksi kolmessa populaatiossa havaittiin vuodenaikaiseroja toukkien lisääntymismuodoissa; vapaana elävät planktiset toukat vallitsivat talvella, kun taas hiekkaputkissa suojatut toukat olivat yleisiä keväällä. Eri toukkamuotoja tuottavat naaraat kuuluvat todennäköisesti geneettisesti erilaisiin kohortteihin. *Streblospio benedicti* monisukasmadon toukkamuotojen monimuotoisuudella on havaittu olevan geneettinen perusta, mutta tämän tutkimuksen tulosten perusteella ei kuitenkaan voida tehdä johtopäätöksiä *P. elegans* -lajin taustasta. Lisäksi myös ympäristön vaikutus ja epigenetiikan rooli olisi tutkittava käyttäen kokeellista näkökulmaa.

Sedimentin ominaisuudet vaikuttivat *P. elegans* -lajin populaatiodynamiikkaan neljässä tutkimuspopulaatiossani. Habitaateissa, joissa sedimentti oli karkeaa ja koostui erikokoisista partikkeleista oli suurin yksilötiheys, isokokoisimmat yksilöt sekä korkein lisääntymisfrekvenssi. Myös muun pohjaeläimistön lajirikkaus oli näillä alueilla suurin. *P. elegans* suosii karkeaa pohjasedimenttiä, ja erikokoisista partikkeleista koostuva sedimentti voi ylläpitävää elinympäristöjä monille eri pohjaeläinlajeille. Lisäksi *P. elegans* -madon geneettinen variaatio (alleelirikkaus) korreloi positiivisesti muun selkärangattomien pohjaeläinyhteisön lajirikkauden kanssa. Tämä viittaa siihen että ympäristömuuttujat, kuten esimerkiksi vuodenaikaisvaihtelut tai habitaatin kantokyky, vaikuttavat *P. elegans* populaatioihin samalla tavalla kuin muuhunkin yhteisöön, kun taas esimerkiksi vuorovaikutussuhteilla muiden pohjaeläinyhteisön lajien kanssa on pienempi vaikutus. Ympäristöolojen muutokset voivat olla ennustettavia, esimerkiksi vuodenajoista riippuvia, tai hyvinkin stokastisia. Vuorovesialueet, erityisesti murtovesialueet ja jokien estuaarit, ovat erittäin dynaamisia habitaatteja, joissa esimerkiksi veden suolapitoisuus saattaa vaihdella ajallisesti merkittävästi. Koska kehitysmuodoiltaan polymorfisia *P. elegans* -populaatioita tavataan erityisesti näillä alueilla, tutkin kokeellisesti näiden matojen fysiologisia ja ekologisia reaktioita muutoksiin veden suolapitoisuudessa. Kokeissani selvisi, että kun veden suolapitoisuutta alennetaan äkillisesti, *P. elegans* -matojen osmoregulaatiokyky heikkenee, joten voimakkaat stokastiset laskut meriveden suolapitoisuuksissa voivat olla lajille vahingollisia. Pitkäkestoisen kokeen mukaan alhainen suolapitoisuus (5 ppt) myös alentaa lajin yksilöiden suvullisen ja suvuttoman lisääntymisen frekvenssiä tai viivästyttää lisääntymistä.

Tutkimukseni antaa lisätietoa *P. elegans* -lajin populaatiodynamiikasta, populaatiogenetiikasta ja fysiologisen toleranssin rajoista. Populaatiotason erot

viittaavat dynaamisen metapopulaation olemassaoloon tällä lajillaTanskan Isefjord-Roskilde Fjordin alueella. Toukkamuodoissa nähtävä polymorfia voi olla metapopulaatiota ylläpitävä tekijä tai sen seurausta. Ympäristön stokastisuus, sekä habitaatin ominaisuudet kuten lämpötila ja sedimentin laatu vaikuttavat lajin populaatiodynamiikkaan. Tutkimukseni tulokset viittaavat *P. elegans* -madon kehitysmuotopolymorfiaa ylläpitävään geneettiseen ja ympäristöstä johtuvaan taustaan, joskin lisätutkimuksia tarvitaan vielä. Koska tällä lajilla eri toukkamuotoja havaitaan usein juuri heterogeenisissä habitaateissa, lajinsisäinen variaatio toukkamuodoissa (poecilogony) saattaa olla strategia ajallisen kelpoisuusvaihtelun vähentämiseksi vaihtelevassa ennalta arvaamattomassa elinympäristössä.

OVERSIGT (RÉSUMÉ IN DANISH)

Effekten af variation I developmental mode på populationsdynamikken af en spionid børsteorm (*Pygospio elegans*) i et heterogent miljø.

Marine bundlevende invertebraters larver udviser stor variabilitet i form og funktion. Udover at variere i morfologi varierer de også i deres fødeoptagelse. De mest almindelige former er planktotrofe, der lever af partikelfiltrering i det pelagiske miljø og de lecitotrofe/ adelphofatiske, der lever i det bentiske miljø og får deres næring fra moderdyret enten i form af blommemasse eller nurse æg og søskendelarver i kuldet. En stor del af variationen kan ligge i at enten produceres der store mængder små æg, der udvikler planktotrofe larver eller færre store æg der udvikler lecitotrofe/ adelphofatiske larver. Forskellige larvestrategier, eller developmental modes favoriseres under forskellige forhold og resulterer i forskellige populationsstrukturer og dynamik; eksempelvis favoriseres planktotrofe larver i situationer med høj planteplankton forekomst og grundet disse larvers store spredningspotentiale resulterer dette i høj populations connectivitet. Omvendt vil høj larvedødelighed i planktonet favorisere udviklingen af lecitrotrofe larver, der udvikler sig i hav bundens miljø. Eftersom lecitotrofe larver oftest bundslår sig i udgangspopulationen bundmiljø fører bentisk developmental mode til populations differentiering. Men det er en kendsgerning, at selektionspresset der fører til den ene eller den anden larvestrategi ikke er fuldstændigt afklaret. Developmental modes mellem forskellige arter og habitater er sammenlignet i tidligere studier for netop at forsøge at afklare selektionspresset. Men disse studier lider under fylogenetiske uklarheder hvorfor anvendelsen af poecilogonous arter, der er kendetegnet ved indenfor en art at producere flere forskellige larvetyper, antageligt er en bedre strategi for at studere selektionspresset på developmental modes.

Til dato er blot 14 arter beskrevet som poecilogonous og en af disse er den lille spionide børsteorm *Pygospio elegans*. Denne er almindelig på lavvandede mudderflader i hele det circumpolare område. Forskellige populationer beskrives som enten fikseret i udviklingen af enten planktotrofe eller lecitotrofe larver eller at udvise sæsonvariation i developmental mode eller endelig at være i stand til at producere flere typer af larver samtidigt. Men meget få populationer er studeret gennem et helt år. I min thesis undersøgte jeg fænomenet poecilogony, dets potentielle årsager og konsekvenser i børsteormen *Pygospio elegans* på fire lokaliteter i det danske Isefjord-Roskilde-Fjord estuarie kompleks, eftersom både planktotorfi og lecitototrofi er beskrevet der. Jeg dokumenterede populationsdynamikken og populationsgenetikken på disse fire lokaliteter ved at indsamle individer gennem et helt år og relatere datamønstret til såvel biotiske som abiotiske variable i økosystemet.

Jeg opdagede at den kønnede formering blev igangsat af relativ lav temperatur og fandt sted fra september til maj samt at på nogle lokaliteter var dyrene reproduktiv aktive i to perioder om året. Jeg identificerede tre til fire forskellige men delvist overlappende størrelses-kohorter gennem året. Hver af

disse størrelses-kohorter varede cirka et halvt år og ny rekruttering fandt sted om foråret samt om efteråret. Den genetiske struktur af populationerne udviste såvel rumlig som tidslig variation som sandsynligvis skal tilskrives den korte generationstid af *P. elegans* samt fænomenet sweepstakes reproduktions succes. Ydermere foreslås at individerne i blot en størrelses-kohorte ad gangen er stor nok til at reproducere sig. Sæsonforskelle i allel frekvenser på nogle af lokaliteterne kunne associeres til fremkomsten af nye størrelses-kohorter. Der blev også fundet at mode of development på tre lokaliteter varierede over sæsonen med planktotrofe larver om vinteren og bundlevende om sommeren. Disse forskellige larvekuld er sandsynligvis produceret af hunner hidrørende fra forskellige størrelses-kohorter der udviser forskellige genetiske karakteristika. Men ud fra disse data kan jeg endnu ikke identificere en basis for poecilogony i *P. elegans* som beskrevet for en anden poecilogonous børsteorm, *Streblospio benedicti*. For at besvare dette spørgsmål skal manipulationsforsøg og/eller mating eksperimenter udføres hvor miljø og epigenetiske effekter kan udelukkes.

Rumlige forskelle i sedimentets grovhed og andre karakteristika mellem de fire lokaliteter blev korreleret med børsteormenes tætheder, kropsstørrelser og reproduktionsaktivitet samt med høj artsdiversitet i bunddyrssamfundet i almindelighed. Det var forventningen ud fra tidligere studier at *P. elegans* foretrækker groft sediment og at ringe sorteret sediment tilbyder forskellige nicher for mange invertebrat arter. Ved kombination af de tidslige og rumlige prøver korrellerede allel richness i *P. elegans* med arts rigdommen i bunddyrssamfundene. Dette indikerer at *P. elegans* udviser samme respons som det øvrige bunddyrssamfund og at alle bunddyrene i højere grad er under indflydelse af miljøvariable såsom sæsonvariation og bærekapaciteten i habitatet end af interaktioner mellem andre bunddyr. Miljøets indflydelse kan være forudsigelige, eks. sæsonvariation, men kan i høj grad også være stokastiske. I denne sammenhæng repræsenterer estuarier et meget udfordrende miljø karakteriseret ved store fluktuationer eksempelvis i saltholdighed forårsaget af kraftige regnvejrsepisoder. Jeg undersøgte fysiologiske og økologiske respons af *P. elegans* af akut og langtids ændringer i saltholdighed eftersom populationer af *P. elegans,* der ikke er fikseret i blot en developmental mode, netop er beskrevet fra estuarier. Jeg fandt at *P. elegans* er en svag volume ion- og osmoregulator ved lave saliniteter som respons på abrupte fald I salinitet. Derfor kan stokastiske salinitetsfald være uhyre skadelige på *P. elegans* populationer. Ifølge langtids eksperimentet var saliniteten 5 indenfor men på grænsen af toleranceområdet eftersom børsteormen voksede men udviste reduceret eller forsinket asexual og sexual reproduktion.

Mine studier tilvejebringer ny viden om *P. elegans* populationsdynamik og populationsgenetik samt fysiologisk salttolerance. Forskellene der blev observeret på *P. elegans* populationsniveau antyder en dynamisk metapopulationsstruktur i Isefjord-Roskilde Fjord estuarie komplekset der kan forklare både årsagen til og konsekvensen af variationen i developmental mode i arten der. Det er tydeligt at såvel temperaturen som sedimentstrukturen

spiller en rolle for populationsdynamikken samt at denne dynamik også er under indflydelse af stokastiske hændelser. Mine resultater antyder såvel genetisk som miljømæssig indflydelse på den observerede variation i developmental mode.i *P. elegans*. Disse opdagelser fortjener yderligere at blive forfulgt i fremtidige studier. Eftersom multible developmental modes oftere er udtrykt I estuarine miljøer kan poecilogony repræsentere en bet-hedging strategi som respons på miljøets ustabilitet.

ZUSAMMENFASSUNG (RÉSUMÉ IN GERMAN)

Der Wurm

von Raphaela Leonhard-Pfleger für Anne Thonig

Er ist nicht oft zulesen,
ein unbeachtet Wesen
und wahrlich auch kein Held,
was ist ein Wurm hier auf der Welt?

Doch ein Wurm hat mehr drauf als man denkt
dazu verschied'ne Wege er vermengt
denn er sich vielfältig selbst multipliziert
die Wissenschaft damit sehr verwirrt.

Zum einen Mal, wenn's ihn langweilt sehr
nimmt er sich selbst zu teilen her.
Schneidet sich entzwei in der Mitte
und wächst komplett nach, so ist's die Sitte.

Ein anderes Phänomen,
bei Würmern schon gar oft geseh'n,
sie folgen dem eingebauten Triebe
und machen heimliche Liebe.

Die Eier - Gott nur weiß warum
zerfallen teils zugrunde stumm.
Die übrige Geschwisterschar
frisst die Zerfall'nen mit Haut und Haar.

Wieviele so sterben ist unbekannt,
die Wissenschaft ist darum sehr gespannt,
wer zuerst steigt hinter all diese Zwänge,
wer als erstes beweist die Zusammenhänge.

Die Auswirkungen einer variablen Larvenentwicklung auf die Populationsdynamik eines Vertreters der Polychätenfamilie Spionidae (*Pygospio elegans*) in einem heterogenen Lebensraum.

Marine benthische Invertebraten besitzen eine groβe Vielfalt an Larven. Neben der Morphologie, unterscheiden sie sich auch anhand der Nahrungsaufnahme. Die häufigsten Formen sind hierbei planktotrophe Larven, die sich im Plankton ernähren, und lecithotrophe/adelphophage Larven, deren Nahrung von der Mutter in Form von Dotter, Nähreiern oder Geschwistern im selben Gelege bereitgestellt wird. Die Bildung von entweder vielen kleinen Eiern (Planktotrophie) oder wenigen groβen Eiern (Lecithotrophie/ Adelphophagie) stellt einen Konflikt zwischen Fertilität und Brutvorsorge dar, und unterliegt zumindest teilweise Schwankungen. Verschiedene Larventypen sind unter verschiedenen Bedingungen von Vorteil und können zu unterschiedlicher Struktur und Dynamik in der Population führen. Beispielsweise wären planktotrophe Larven von Vorteil, wenn der Phytoplanktongehalt im Wasser hoch ist. Diese würden aufgrund ihres hohen Verbreitungspotentials dazu führen, dass verschiedene Populationen im genetischen Austausch miteinander stehen. Im Gegensatz dazu würde hohe Sterblichkeit im Plankton lecithotrophe Larven bevorzugen, die sich im Benthos entwickeln. Da diese Larven typischerweise in ihrer Heimatpopulation siedeln, würden sie dazu führen, dass verschiedene Populationen voneinander isoliert sind. Es ist allerdings noch nicht völlig geklärt, welche Faktoren die eine oder andere Strategie selektieren. Um diese Faktoren zu bestimmen, wurde in vorangegangen Arbeiten untersucht, welcher Larventyp in welchen Lebensräumen vorhanden ist. Hierbei wurden jedoch verschiedene Arten miteinander verglichen, so dass der Einfluss verschiedener Lebensräume auf den Larventyp mit stammesgeschichtlichen Einschränkungen zwischen den Arten vermengt sein könnte. Daher könnten poecilogene Arten, das sind Arten die verschiedene Larventypen produzieren, besser geeignet sein, die Selektion für einen bestimmten Larventyp zu untersuchen.

Bisher sind lediglich 14 Arten bekannt, die tatsächlich poecilogen sind. Eine von ihnen ist *Pygospio elegans*. Dieser kleine röhrenbildende Polychät der Familie der Spioniden ist weit verbreitet im Schlick des Gezeitenbereichs der borealen Breiten. Es ist bekannt, dass einige *P. elegans* Populationen nur adelphophage oder planktotrophe Larven produzieren, während in anderen Populationen ein saisonaler Wechsel zwischen den Larventypen stattfindet oder verschiedene Larventypen gleichzeitig produziert werden. Allerdings beobachteten nur wenige Studien Populationen über einen längeren Zeitraum hinweg. In dieser Arbeit, habe ich das Phänomen der Poecilogonie, sowie seine möglichen Ursachen und Folgen in dem Polychaeten *P. elegans* an vier Standorten im Dänischen Isefjord-Roskilde-Fjord Ästuar untersucht, da hier sowohl plankotrophe als auch adelphophage Larven beschrieben worden sind. Über ein Jahr hinweg habe ich die Populationsdynamik und Populationsgenetik dokumentiert und diese mit den vorherrschenden biotischen und abiotischen Umweltbedingungen verglichen.

Ich habe festgestellt, dass die sexuelle Reproduktion durch niedrige Temperaturen eingeleitet wird und von September bis Mai stattfindet. Hierbei traten an einigen Standorten zwei Maxima sexueller Aktivität auf. Drei bis vier verschiedene, teilweise überlappende Größenkohorten konnten über das Jahr hinweg beobachtet werden; jede überdauerte etwa ein halbes Jahr und neue Kohorten siedelten im Frühling und Herbst. Die genetische Struktur der Populationen zeigte standortbedingte sowie saisonale Unterschiede, welche möglicherweise der kurzen Lebensdauer von *P. elegans* und dem Zufall einer erfolgreichen Fortpflanzung zuzuschreiben sind. Die Ergebnisse deuten darauf hin, dass möglicherweise zu jedem Zeitpunkt Individuen von nur einer Kohorte groß genug waren um sich fortzupflanzen. An einigen Standorten könnten die saisonalen Unterschiede in der genetischen Strukur der Population mit dem Auftreten einer neuen Kohorte übereinstimmen. Darüber hinaus wechselte der Larventyp saisonal an drei Standorten von planktotrophen Larven im Winter zu adelphophagen im Frühling. Diese verschiedenen Gelege werden vermutlich von Weibchen produziert, die verschiedenen Kohorten angehören, welche wiederum genetische Unterschiede aufweisen. Dennoch können wir von diesen Ergebnissen nicht ableiten, dass Poecilogony in *P. elegans* genetisch bedingt ist wie beispielsweise in dem Polychaeten *Streblospio benedicti*. Weitere manipulative Experimente und Paarungsstudien sind nötig um Umwelt- oder epigenetische Effekte auszuschließen.

Die unterschiedliche Sedimentstruktur an den verschiedenen Standorten beeinflusste die Populationen dahingehend, dass in grob körnigem Sediment, welches zusätzlich unterschiedliche Korngrößen aufwies, die Populationsdichte von *P. elegans* und der Anteil an reproduzierenden Individuen höher war und außerdem die Individuen größer waren. Dies war zu erwarten, da aus früheren Studien bekannt ist, dass *P. elegans* grob körniges Sediment bevorzugt. Weiterhin weiste die benthische Invertebratengemeinschaft in diesem Sediment eine höhere Artenvielfalt auf, was darauf beruhen könnte, dass verschiedene Nischen durch das heterogene Sediment vorhanden sind. Wenn man die Proben aller Standorte und Zeitpunkte zusammennimmt, korrelierte der Alleelreichtum von *P. elegans* mit dem Artenreichtum der benthischen Invertebratengemeinschaft. Dies weist daraufhin, dass *P. elegans* einen ähnlichen Lebensstil besitzt wie ein Großteil der Invertebratengemeinschaft und dass beide stärker durch Umweltbedingungen, wie saisonale Zyklen oder der Kapazität des Lebensraumes beeinflusst werden als durch die Interaktionen miteinander. Umweltbedingungen können vorhersehbar, beispielsweise saisonal sein, aber sie können auch unvorhersehbar sein. Dahingehend stellen Ästuare einen sehr anspruchsvollen Lebensraum dar, der starken Schwankungen, zum Beispiel im Salzgehalt aufgrund von plötzlichem Starkregen unterliegt. Da *P. elegans* nicht nur einen Larventyp, sondern meist verschiedene Larventypen in Ästuaren aufweist, habe ich die Reaktion von *P. elegans* sowohl auf akute als auch auf langwierige Veränderungen des Salzgehaltes auf physiologischer und ökologischer Ebene untersucht. Es stellte sich heraus, dass *P. elegans* das Zellvolumen sowie die Ionen- und osmotische Konzentration seiner Hämolymphe in niedrigen Salzgehalten vermutlich kaum

regulieren kann. Daher könnte ein plötzlicher Abfall im Salzgehalt verheerende Auswirkungen auf Populationen von *P. elegans* haben. Einem Langzeitexperiment zufolge befindet sich ein Salzgehalt von 5 zwar noch innerhalb - allerdings am Rande - des Toleranzbereiches von *P. elegans*, da es Individuen noch möglich ist zu wachsen jedoch die asexuelle und sexuelle Fortpflanzung verringert oder verzögert ist.

Im Allgemeinen liefert meine Arbeit neue Informationen über die Populationsdynamik und Populationsgenetik sowie die physiologische Toleranz von *P. elegans*. Unterschiede in der Dynamik der verschiedenen Populationen weisen darauf hin, dass eine Metapopulationsstruktur im Isefjord-Roskilde-Fjord vorherrschen könnte, welche sowohl Ursache für das Vorhandensein verschiedener Larventypen, als auch eine Konsequenz davon sein könnte. Temperatur und Sedimentstruktur spielen eine klare Rolle in der Populationsdynamik; zusätzlich sind beispielsweise auch unvorhersehbare Wetterereignissen von Bedeutung. Meine Ergebnisse lassen sowohl einen genetischen als auch umweltbedingten Einfluss auf den Larventyp in *P. elegans* vermuten, welcher allerdings weiter untersucht werden sollte. Da unterschiedliche Larventypen in *P. elegans* vorzugsweise in Ästuaren auftreten, könnte Poecilogonie eine Strategie darstellen um Fitnessschwankungen in unvorhersehbaren Lebensräumen auf längere Sicht zu verringern.

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ORIGINAL PUBLICATIONS

I

POPULATION AND REPRODUCTIVE DYNAMICS OF THE POLYCHAETE *PYGOSPIO ELEGANS* IN A BOREAL ESTUARY COMPLEX

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Population and reproductive dynamics of the polychaete *Pygospio elegans* in a boreal estuary complex

Anne Thonig,[1,2] K. Emily Knott,[2] Jenni Emilia Kesäniemi,[2,3] Benni Winding Hansen,[1] and Gary Thomas Banta[1]

[1]Department of Science and Environment, Roskilde University, Roskilde 4000, Denmark
[2]Department of Biological and Environmental Science, University of Jyvaskyla, Jyvaskyla 40014, Finland
[3]Department of Ecology and Genetics, University of Oulu, Oulu 90014, Finland

Abstract. *Pygospio elegans* is an opportunistic, wide-spread spionid polychaete that reproduces asexually via fragmentation and can produce benthic and pelagic larvae, hence combining different developmental modes in one species. We documented the density, size distribution, and reproductive activity of *P. elegans* at four sites in the Danish Isefjord-Roskilde Fjord estuary complex, where all modes of reproduction were reported. We compared population dynamics of this species to environmental parameters such as salinity, temperature, and sediment characteristics (grain size, sorting, porosity, water content, organic content, C/N). We observed that new cohorts—resulting either from sexual or asexual reproduction—appeared in spring and fall, and old ones disappeared in late summer and winter. Sexual reproduction occurred from September until May, and although their timing was variable, there were two reproductive peaks at three sites. At those sites, we also observed a switch in larval developmental mode. Asexual reproduction peaked in April. While the seasonal dynamics can be related to temperature to a large extent, the differences in population dynamics among sites also correlated with sediment structure and salinity. Populations from sites with coarse and heterogeneous sediment had high levels of sexual reproduction. At the site with lower salinity, intermediate and benthic larvae were present during winter in contrast to pelagic larvae found at the other sites. However, we could not identify one clear environmental factor determining the mode of development. At present, it remains unclear to what degree genetic background contributes to mode of development. Hence, whether the differences in developmental mode are the result of genetically different cohorts will be further investigated.

Additional key words: life history, poecilogony, development, spatiotemporal variation environmental impact

A variety of types of larvae have evolved independently among marine taxa (Strathmann 1993). Larvae are an integral part of the different life histories of invertebrates, which affect population dynamics and how populations respond to environmental conditions (Marshall et al. 2012). Pelagic larvae that have high dispersal potential might dampen population fluctuations (Eckert 2003). Thus, they would be advantageous for species living in seasonal environments (Thorson 1950, Marshall & Burgess 2015) but also for opportunistic species that rapidly colonize disturbed areas (McEdward 2000). However, the dispersal potential of pelagic larvae does not always translate into higher connectivity among populations (Weersing & Toonen 2009). Also, the quality of the new colonizers determines their establishment and reproductive success (Marshall et al. 2010; Burgess & Marshall 2011). Benthic larvae, with their predominantly local recruitment, could be favored in temporally constant but spatially variable environments and when predation in the plankton is high (Pechenik 1999).

The effect of developmental mode on population structure and dynamics can be investigated best in species that express different developmental modes,

[a]Author for correspondence. E-mail: anne.a.thonig@student.jyu.fi

as speciation effects cannot be excluded between sibling species with different modes of development (Knott & McHugh 2012). Variation in developmental mode, also called poecilogony, was described for sacoglossan sea slugs (Krug 2007, 2009; Vendetti et al. 2012) and several spionid polychaetes (Blake & Kudenov 1981; Duchêne 1984; Levin et al. 1991; MacKay & Gibson 1999), including *Pygospio elegans* (Claparède 1863) (Morgan et al. 1999; Kesäniemi et al. 2012b). It is a common, small (10–15 mm), tube-dwelling estuarine species with a circumboreal distribution, and found primarily on intertidal mud and sand flats. Members of the species can form high density patches, or tube-beds, with densities up to 600,000 individuals m^{-2} (Morgan 1997). Populations of *P. elegans* thrive in a wide range of habitats, temperatures, and salinities (Hempel 1957; Armitage 1979; Anger 1984; Morgan 1997). The average life span of individuals is ~9 months (Anger et al. 1986). The time from hatching (as pelagic larvae) to first reproduction takes ~15–17 weeks (Anger et al. 1986). Reproduction was reported to be seasonal, with sexual reproduction that may consist of two broods occurring in winter and asexual reproduction peaking afterward in spring (Rasmussen 1973; Gudmundsson 1985). The species' versatile reproductive biology, consisting of asexual and sexual reproduction and polymorphism in larval developmental mode with both benthic and pelagic larvae (Rasmussen 1973), allows for different life histories. Within gravid females, two different kinds of eggs can be distinguished: nurse eggs containing yolk and fertile eggs (true or genuine eggs *sensu* Rasmussen 1973) with a distinct nucleus. The fertile eggs develop into embryos that consume the nurse eggs while in egg capsules. The ratio of nurse eggs to fertile eggs indicates the mode of development (Rasmussen 1973). Pelagic larvae are expected from capsules containing a large number of fertile eggs (>10) and few nurse eggs. These larvae emerge from the capsules at the three-chaetiger stage, possess swimming chaetae, and feed and develop in the plankton for ~4–5 weeks until they are 12–16 chaetigers in size, when they settle as juveniles (Hannerz 1956; Rasmussen 1973; Anger et al. 1986). By contrast, benthic larvae are expected from capsules with few (1–3) fertile eggs and a large number of nurse eggs. They hatch when they are ~14–20 chaetigers in size and immediately settle (Hannerz 1956; Hempel 1957; Anger et al. 1986). Intermediate types of larvae that hatch at about ten chaetigers and spend a short time in the plankton can also be found (Hannerz 1956; Kesäniemi 2012). Mature individuals of *P. elegans* are usually larger than 35 chaetigers, in most cases ~45 chaetigers in size (Gudmundsson 1985). Asexual reproduction occurs via fragmentation of the worm into three to four pieces that subsequently remain in the tube and regenerate heads, tails, or both (Rasmussen 1953).

It is not unusual for life history traits to differ among populations of the same species, particularly for poecilogonous species (Levin 1984; Lam & Calow 1989; Blanck & Lamouroux 2007; Marshall & Keough 2008). This is the case for *Pygospio elegans*, in which some populations rely solely or predominantly on asexual reproduction (e.g., Kiel Bight [Germany], Anger 1977), while others show no signs of asexual reproduction (e.g., Drum Sands [North Sea], Bolam 2004). Furthermore, the mode of development can differ even among spatially close populations (Isefjord-Roskilde Fjord complex [Kattegat], Kesäniemi et al. 2014; English Channel, Morgan et al. 1999). For some populations, the mode of development is expected to be fixed to either pelagic (e.g., Drum Sands [Bolam 2004] and Somme Bay, English Channel [Morgan et al. 1999]) or benthic larvae (e.g., Cullercoats [Gudmundsson 1985] and Ängsö, Finland [Kesäniemi et al. 2012a]). However, there may also be seasonal switches in mode of development from pelagic larvae in winter and benthic larvae in spring (Blyth estuary [Gudmundsson 1985] and Horsens Fjord [Rasmussen 1973]) and simultaneous occurrence of multiple types of larvae have been observed (Isefjord [Rasmussen 1973] and Schiermonnikoog, Netherlands [Kesäniemi et al. 2012a]). The basis for variation in developmental mode could be a genetically based polymorphism (Levin et al. 1991), epigenetic regulation of gene expression (Kesäniemi et al. 2016), or a plastic response to environmental cues (Krug 2009). Low genetic divergence among populations, however, indicates that poecilogony in *P. elegans* is probably not solely a genetically based polymorphism, but also influenced by the environment. So far, variation in developmental mode of *P. elegans* has been observed in estuarine environments (Rasmussen 1973; Gudmundsson 1985). Hence, poecilogony in *P. elegans* might represent a bet-hedging strategy that is favored in unpredictable, highly dynamic habitats (Chia et al. 1996; Collin 2012), while at more constant sites the mode of reproduction might be fixed.

Because developmental mode can have an impact on population persistence and connectivity (Jeffery & Emlet 2003), we wanted to document how populations and their developmental modes change over time, and how those changes are affected by environmental parameters. For this reason, we surveyed

population and reproductive dynamics of the poecilogonous polychaete *Pygospio elegans* at four sites in the Isefjord-Roskilde Fjord estuary in Denmark, where members of the species reproduce via multiple types of larvae, both seasonally and simultaneously (Rasmussen 1973; Kesäniemi et al. 2014).

Methods

We monitored *Pygospio elegans* and several environmental parameters in the Danish Isefjord-Roskilde Fjord complex from March 2014 until February 2015. Four sites, Lynæs, Lammefjord and Vellerup in Isefjord, and Herslev in Roskilde Fjord (Fig. 1), were sampled monthly at shallow areas along the shore (each ~10 m^2 with 0.5–1 m water depth) (see Supporting information, Table S1 for coordinates and exact sampling dates). These sites were chosen to cover genetically different populations of *P. elegans* and different habitats, as described by Kesäniemi et al. (2014). The Isefjord-Roskilde Fjord complex is the second largest estuary in Denmark, located on the north of Zealand with an opening to the Kattegat. Isefjord has a surface area of 280 km^2 with mean depth of 7 m and salinities ranging from 18 to 30 ppt. Roskilde Fjord is connected to the Kattegat via the Isefjord, has a surface area of 117 km^2, and lower salinities, ranging between 5 and 18 ppt. It is divided into a long and narrow outer region and a shallow interior, which is not deeper than 6 m. The two estuaries are similar in temperature, but not in salinity patterns (Rasmussen 1973).

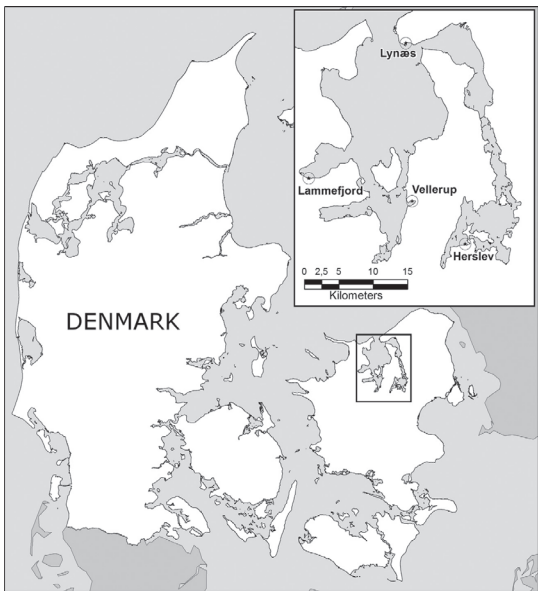

Fig. 1. Location of our four sampling sites in the Isefjord-Roskilde Fjord estuary complex, Denmark.

Population dynamics of *Pygospio elegans*

Pygospio elegans were sampled monthly, excluding December, to determine size, gender, reproductive activity, and mode of development. Surface sediment was randomly sampled (using a shovel) and sieved on site with a 1-mm mesh. Sand tubes of *P. elegans* were collected and transferred to the lab. In the lab, sand tubes were spread evenly on a white photo tray marked with equal quadrants, and worms were sampled as they were leaving their sand tubes. By sampling all individuals from a certain quadrant we avoided biased sampling (e.g., sampling only the largest worms) and hence obtained a quantitative and representative subsample to determine size and population structure.

At least 30 individuals were used to measure length in order to analyze the cohort structure of each population. It is important to note that in this study the term cohort refers only to size classes and not to generations because asexual reproduction disrupts the relation between size and age. Hence, individuals of the same size or assigned to the same cohort could be of different ages. However, individuals clearly resulting from asexual reproduction (those with small regenerated heads or tails, Supporting information, Fig. S1F) constituted on average 3% or less of the samples in all populations except Lynæs (ca. 9%). The worms were first narcotized in seawater containing 10% sparkling water and then photographed with a Nikon camera mounted on a dissecting microscope. Measurements were made using NIS BR software v. 4.2 (Nikon, RAMCON A/S Birkerød, DK). The coefficient of variation for our size measurement was maximally 8% (obtained from measuring ten individuals each ten times). Since many worms were damaged or regenerating, we decided to measure the length from the eyespot to the start of the gills (see Fig. S1A). Length frequency plots were created using SPSS Statistics 22 (IBM, Armonk, NY, USA) with automated binning to identify the best grouping of the data. Cohort analysis was performed in FiSat II (FAO-ICLARM Stock Assessment Tool) using Bhattacharya's method to identify the cohorts and NORMSEP to optimize the fit of a normal distribution. The mean of the normal distribution is used as the mean size of the respective cohort. We aimed

for the identification of a maximal number of cohorts with minimum overlap (separation index SI >2) (Bhattacharya 1967). Since we could not fit a von Bertalanffy growth curve through our data using the method implemented in FiSat II, we followed a procedure similar to that of Bolam (1999, 2004). We followed the progression of each cohort and obtained a growth rate via a regression analysis of the weighted mean size of the cohorts using Systat 13 (Systat Software, Inc., San Jose, CA, USA).

A subsample of at least 50 live specimens, including the 30 individuals used to measure length, was characterized according to Table 1 and Fig. S1. The assessment of asexual reproduction was noted beginning in April. In addition to the live specimens, all sand tubes were checked for the presence of egg strings and, if found, the mode of development was determined (see Table 1 and Fig. S1). Due to seasonal variation in the number of worms collected, the absolute number of egg strings was normalized to the total sample size (egg strings per number of worms collected).

For determining density of *P. elegans,* benthic macrofauna were quantitatively sampled in March, May, August, and November using a hand-held corer (15 cm diameter, 30 cm length). Three samples were taken randomly at each sampling site, and each was sieved through a 1-mm mesh and fixed with 5% formaldehyde on site. In the lab, formaldehyde was removed in several washing steps, and samples were stored in 95% ethanol. To better visualize the macrofauna, the samples were stained overnight by adding 5 mL of saturated Rose Bengal. Afterward, the Rose Bengal/ethanol solution was discarded and *P. elegans* retained on a 1-mm sieve were identified and counted.

Table 1. Characterization of *Pygospio elegans* and its developmental modes. For explanations, see Introduction.

Developmental mode	Description
Non-reproductive	Individuals without gametes
Male	Individuals with soft appendages at second chaetiger and sperm in coelom
Female	Individuals with eggs in coelom
Asexual reproduction	One individual fragmented architomically, hence more than one individual is occupying a given sand tube; specimens are regenerating
Larvae	
Benthic	1–3 larvae per egg capsule
Intermediate	4–10 larvae per egg capsule
Pelagic	>10 larvae per egg capsule

Environmental parameters

At each site, a data logger (HOBO U24-002-C salinity logger, 100–55,000 μS cm^{-1}, Onset Computer Corporation, Bourne, MA, USA) was deployed, which documented conductivity and temperature every 10 min during the survey period. Salinity was calculated according to the PSS-78 using the conductivity and temperature measurements of the logger (UNESCO 1981). The salinity of reference samples taken monthly were measured with a salinometer (MS-310e Micro-salinometer, RBR-global, Kanata, Ontario, Canada) and used to correct the logger for drift. Due to biofouling and frost, salinity data are not available for Lammefjord from June until August, for Vellerup in August, and for Lynæs in January. Temperature and salinity data were excluded when salinity dropped below 2 ppt as these indicated exposure of the logger due to low water levels.

Sediment characteristics were determined in March, May, August, and November. For sediment characteristics, three kajak cores (5 cm diameter, at least 15 cm length) were taken randomly at each sampling site. These were sectioned into four layers (0–1 cm, 1–2 cm, 2–6 cm, 6–15 cm) and the respective layers of each core were pooled and mixed. Wet weight and dry weight (24 h at 105°C) of 5 cm^3 sediment from each layer was determined for calculating porosity and water content.

Particle size was determined from 50–150 g of remaining wet sediment using a set of sieves corresponding to the Wentworth size scale (8 mm, 4 mm, 2 mm, 1 mm, 0.5 mm, 0.25 mm, 0.125 mm, 0.063 mm). The weight percent of each size fraction was determined after 24 h at 105°C. Median grain size ($\Phi_{50\%}$) and sorting (inclusive graphic standard deviation coefficient [IGSD], $(\Phi_{84\%}-\Phi_{16\%})/4+(\Phi_{95\%}-\Phi_{5\%})/6.6$) were calculated according to Gray & Elliott (2009). For that purpose, the Wentworth scale (mm) was converted into the arithmetic Phi (Φ) scale, which is defined as the $-\log_2$ of the size in mm. About 500 mg of the dried sediment from the samples was reserved for C/N analysis, and the rest was used to determine organic content (%) via loss on ignition (LOI, 2 h at 550°C).

Carbon and nitrogen content of 30–50 mg ground sediment from the top layer (0–1 cm) were analyzed in three analytical replicates using an element analyzer (Flash 2000 NCS- Analyzer, and FlashEA® 1112 CHNO Analyzer, Thermo Scientific). Due to a

high quantity of shells in some samples, the difference in LOI between dried and pre-combusted (2 h at 500°C) samples was used to calculate the carbonate free organic C content.

Relation of population and environmental dynamics

Temporal and spatial differences in the population dynamics of *P. elegans* were determined using distance-based permutational multivariate analysis of variance (PERMANOVA) in PRIMER-E v.6 (Clarke & Gorley 2006). The monthly data collected for *P. elegans* (size, proportion of males, females and non-reproductive individuals, number and developmental mode of larvae, occurrence of asexual reproduction) at each location were normalized and a resemblance matrix based on Euclidian distance was calculated comparing all samples. A two-way (time, location) PERMANOVA design without interaction (due to lack of replication) was performed using 9999 permutations and default settings. Subsequently, pair-wise comparisons among locations or among times were performed. The assumption of identical, independent residuals was fulfilled. Residuals were distributed homogenously according to PERMDISP using distances to median (location, p=0.170; time, p=0.098) and variances between different time points across sites were equal according to Levene's test (p=0.989).

Furthermore, a distance-based linear model routine (DistLM) was used to analyze and model the relationship between the population parameters of *P. elegans* (as was done for PERMANOVA, but also including worm density) and the environmental data (mean temperature and SD; mean salinity and SD; and sediment characteristics as median grain size, sorting, porosity and water content, organic content, and C/N). For this purpose we summarized the data into quartiles to account for the different sampling schemes: March (consists of the data from January and February 2015 and March 2014), May (April–June 2014), August (July–September 2014), and November (October–December 2014). For the DistLM procedure, we used two Euclidian resemblance matrices of the normalized data (*P. elegans* data and environmental data), 9999 permutations, and best selection procedure. The model (a subset of the environmental parameters) that best explained the variation among the *P. elegans* and environmental data was determined according to the selection criteria BIC and AICc. Subsequently, this best-fit model was entered in a distance-based redundancy analysis (dbRDA) to visualize the variation in the *P. elegans* data that is explained by the selected model.

Results

Population dynamics of *Pygospio elegans*

In general, worms were smallest at Lynæs (monthly means ranged from 1139–1731 μm) and Lammefjord (1074–1648 μm), followed by Herslev (1343–1818 μm), with the largest worms at Vellerup (1496–1848 μm) (Fig. 2). The differences among populations were most noticeable during fall, when worms at Vellerup remained a constant size while the average worm size at the other sites decreased. Worms were similar in size across all populations at other times of the year.

Using our length measurements, we determined the number of cohorts present each month during the survey. We distinguished one to four overlapping cohorts present at any one time (see Fig. S2). The pattern at each site is summarized and simplified in Fig. 3, which shows the mean worm size of each identified cohort and the fraction of the total population in that cohort. At Lynæs, two to three cohorts were present at any one time and we observed four to five cohorts over the entire period that had growth rates ranging 3.31–6.41 μm d^{-1}. Small worms appeared in April, June, September, and November. At Lammefjord, mostly two cohorts

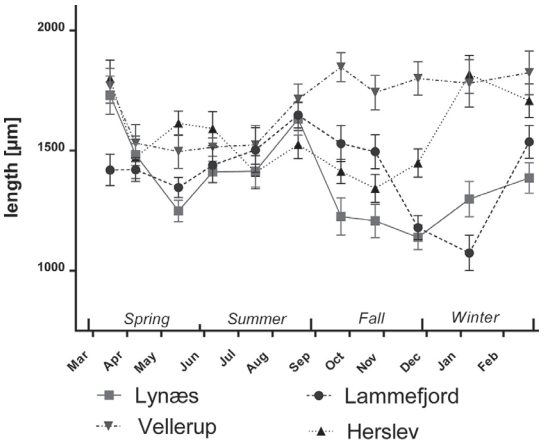

Fig. 2. Mean length±standard error from head to gills of at least 30 individuals of *Pygospio elegans* per month and site. There are no data available for December. Based on a regression (see details in Discussion) between number of segments and length measurements, young individuals, with 14 body segments and ready to settle, were expected to have a mean length from head to gills of 1085 μm; mature individuals, with 40 segments, were expected to have a mean length of 1489 μm.

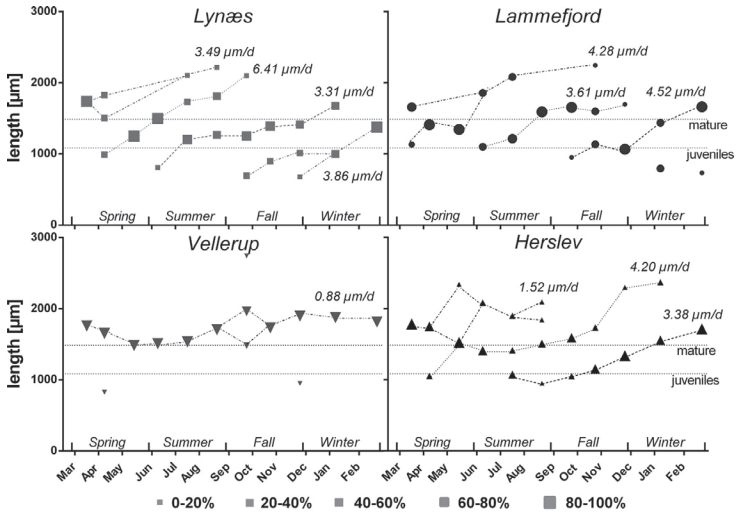

Fig. 3. Cohorts identified with FiSatII: mean of each size class as length from head to gills (µm) is illustrated per month and site. The size of each symbol represents the percentage of the total population in that cohort (<20%, <40%, <60%, <80%, <100%). Growth rates for each cohort were calculated via linear regression, with normality being fulfilled in most cases. The size of small individuals of ~14 chaetigers (1085 µm) and minimum size of mature individuals (1489 µm) as described in the legend of Fig. 4 are indicated. Detailed length frequency histograms can be found in Supporting information, Fig. S2.

were present at the same time, and we could determine four to five distinguishable cohorts during the whole period with growth rates ranging 3.61–4.52 µm d^{-1}. Small worms appeared in March, June, September, and January. Likewise, mostly two cohorts were present at Herslev at any one time, although three (to four) cohorts could be observed during summer, with growth rates ranging 1.52–4.20 µm d^{-1}. Small worms appeared in April and July. For the most part, only one cohort was present at Vellerup during the whole period, with a low overall growth rate of 0.88 µm d^{-1}, and thus, almost stable worm size. Small worms appeared at Vellerup in April and November.

Sexual reproduction by *P. elegans* at our study sites was most prevalent during winter and spring (Fig 4A). The percentage of gravid females and males carrying sperm was lowest at all sites during the summer (from May to August). Two peaks of gravid females and males with sperm were observed in October and February at Lynæs, Lammefjord, and Vellerup, whereas only one broad peak (November to March) was observed at Herslev. The percentage of males carrying sperm was similar to or slightly higher than the percentage of gravid females, and males either preceded gravid females or occurred simultaneously. The percentage of gravid females was much lower at Lynæs (max. 10%) than in Lammefjord (max. 22%), Vellerup (max. 26%), and Herslev (max. 32%).

We observed egg strings in the tubes of *P. elegans* in winter and spring (Fig. 4B), which coincides for the most part with the presence of gravid females. Gravid females were observed in October at Lynæs, Lammefjord, and Vellerup, but egg strings were not observed at these sites until November. Two peaks in the number of egg strings, in accordance with the two peaks in gravid females, were noted only in Vellerup. At Herslev, one major peak in number of egg strings resembles the single broad peak of gravid females. Likewise, the lower normalized number of egg strings observed at Lynæs (max. 0.09) and Lammefjord (0.12), compared to Vellerup (0.28) and Herslev (0.44), is in accordance with the observed lower number of gravid females.

We observed a difference in the larval developmental mode between spring and winter, as well as between sites in winter (Fig. 4B). In spring, multiple types of larvae (pelagic, benthic, and intermediate) were found at all sites, whereas in winter, pelagic larvae were predominant at Lynæs, Lammefjord, and Vellerup, and benthic and intermediate larvae were predominant at Herslev. At Vellerup, the co-occurrence of the second peak in gravid females and number of egg strings in February also coincides with a switch from only pelagic larvae to a mixture

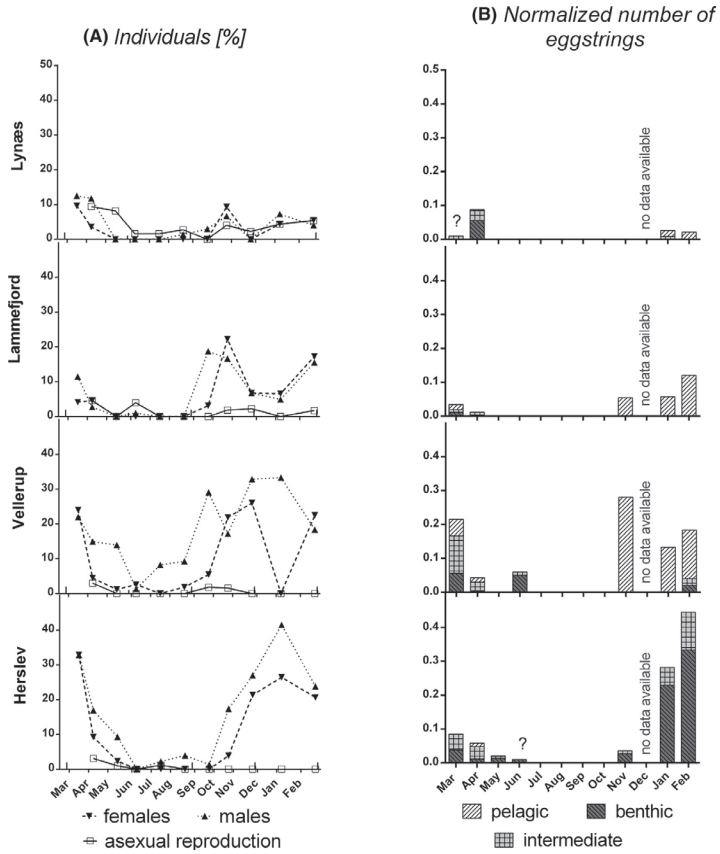

Fig. 4. Reproductive activity (**A**) Percentage of males (sperm and soft appendages at second chaetiger present), females (eggs or egg strings present), and individuals performing asexual reproduction (several worms sharing one tube and regenerating) per month and site. (**B**) Number of egg strings normalized to the total number of individuals captured. The mode of development of the resulting larvae is indicated. Due to missing data, the number of egg strings in March at Lynæs and number of individuals sampled in total in June at Herslev was estimated by interpolation (indicated by "?"). No sampling took place in December.

of benthic, intermediate, and pelagic larvae. At all sites, mainly in January and February, we found females brooding egg capsules while also developing the next batch of eggs in their coelom. At Herslev, the developmental mode of the brood in the egg capsules was benthic, and the developing eggs in the brooding mother were also likely to have a benthic developmental mode, since only a few of the developing eggs were fertile eggs. At the other sites developmental mode of the brood was pelagic, but the stage of the developing eggs in the mothers was too early to allow determination of their developmental mode. Asexual reproduction occurs throughout the year but peaks in April when the frequency of sexual reproduction is in decline (Fig. 4A). The highest prevalence of asexual reproduction was observed in Lynæs (up to 26%).

The mean density of *P. elegans* was lowest at Lynæs (means between sampling times ranged 0–377 individuals m^{-2}), distinctly higher at Lammefjord (75–4357 individuals m^{-2}) and Herslev (189–4791 individuals m^{-2}) and highest at Vellerup (132–7847 individuals m^{-2}) (Fig. 5). While at three sites, the population density was highest in May, with a maximum of 7847±6051 individuals m^{-2} in Vellerup, it was generally low and constant at Lynæs. Furthermore, the distribution of *P. elegans* was patchy, most noticeably during April and May at Herslev and in October at Lynæs when the worms were associated with the presence of diatom mats (unpubl. data).

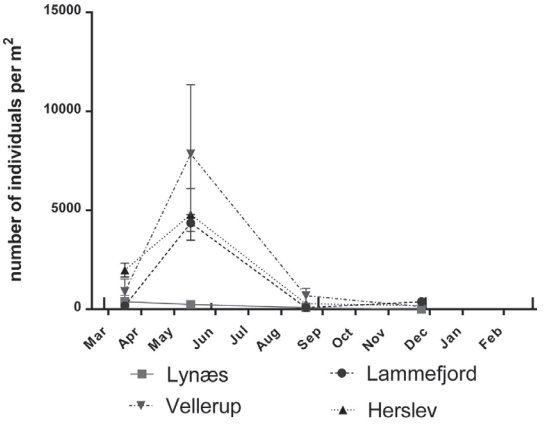

Fig. 5. Population density (mean number of individuals m^{-2}±standard error) in *Pygospio elegans* at four sites in March, May, August, and November.

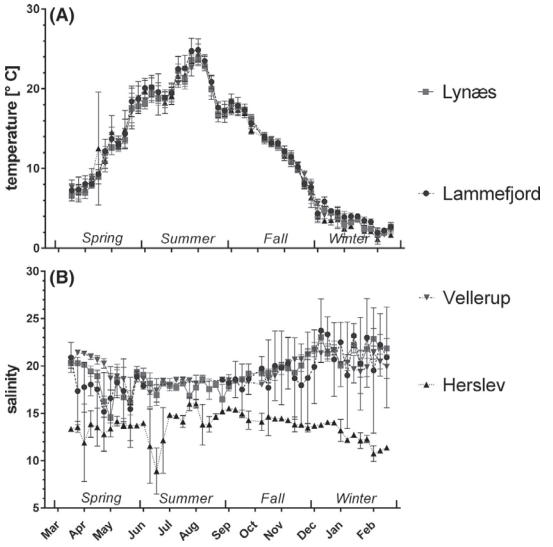

Fig. 6. Temperature (**A**) and salinity (**B**) patterns at our study sites: weekly mean and standard deviation obtained from continuous logger data. Data are missing for 1 week in October and 1 week in January, when the loggers were taken in for maintenance. The logger at Lammefjord was deployed in the mouth of Lammefjords Søkanal, which likely contributed to the large salinity fluctuations observed there.

Environmental dynamics

The temperature and salinity data are illustrated in Fig. 6 and summarized in Table 2. Temperature patterns at the sites were similar. Lowest weekly temperatures were observed from December through February, with the minimum (−2.97°C) in December at Lynæs. Highest weekly temperatures were observed in July and August with the maximum (28.61°C) in July at Lammefjord. There was more variation in temperature during spring than in fall. In contrast to temperature, salinity patterns differed notably between the sites. In Lammefjord there was more variation in salinity (SD=4.0) in comparison to the other sites, and in Herslev mean salinity was low (13.5).

Characteristics of the surface sediments (0–1 cm), which represents the habitat of *P. elegans,* are illustrated in Figs. S3 and S4, and summarized in Table. 2. Median particle size was negatively correlated with water content (Pearson correlation coefficient, r=0.775, p=0.003, *n*=16, df=6), porosity (r=0.725, p=0.009) and sorting (r=−0.818, p=0.001). Hence, sediments at Lynæs and Lammefjord were fine grained, had highest water content and porosity, and were moderately to moderately well sorted. Vellerup had poorly sorted coarse sediment with lowest water content and porosity, while sediment at Herslev was medium in particle size, water content, porosity, and sorting. There were no major seasonal changes in sediment characteristics. Sediment characteristics, except particle size, showed similar patterns with depth at the different sites (data not shown).

Organic content of the sediments was generally higher in Lynæs and Lammefjord than in Vellerup and Herslev (Table 2, Fig. S4A). There was no difference between the sites when comparing organic content depth profiles (data not shown). Seasonally, the percentage of organic content was variable in Lammefjord and Vellerup, whereas it was stable in Lynæs and Herslev. The amount of organic matter in Lammefjord and Herslev increased slightly during the year, while it decreased in Lynæs and Vellerup. Moreover, the C/N ratio was lower in Lynæs, indicating more labile organic matter, compared to Lammefjord and Herslev. The most refractory material was present in Vellerup, except for May (Fig. S4B). The C/N ratio was nearly constant at Lammefjord, decreased during the year at Lynæs and Herslev, and was quite variable at Vellerup.

Relation of population and environmental dynamics

We found significant temporal (p=0.0006) and spatial (p=0.0001) patterns in the population dynamics of *P. elegans*. Pair-wise comparisons revealed significant changes in the population dynamics (for all locations) mostly between late spring until summer

Table 2. Annual mean and standard deviation of environmental parameters. Sediment characteristics refer to the top layer (0–1 cm) of sediment only. IGSD, inclusive graphic standard deviation coefficient (see Methods). The scale for median grain size and sorting is the arithmetic Phi (Φ) which is defined as the $-\log_2$ of the size in mm.

	Lynæs		Lammefjord		Vellerup		Herslev	
	mean	SD	mean	SD	mean	SD	mean	SD
Temperature (°C)	12.39	6.86	13.14	6.97	12.18	6.85	12.50	7.07
Salinity (ppt)	19.07	2.07	19.27	4.00	19.55	1.63	13.53	2.00
Median grain size,$\Phi_{50\%}$ (mm)	2.38 Fine	0.25	2.18 Fine	0.30	0.95 Coarse	0.24	1.68 Medium	0.22
Sorting, IGSD (mm)	0.54 Moderately well	0.06	0.96 Moderately	0.30	1.66 Poorly	0.38	0.82 Moderately	0.20
Water content (%)	19.91	1.43	19.94	0.67	16.68	1.42	18.50	1.38
Porosity (%)	0.40	0.06	0.39	0.01	0.32	0.02	0.35	0.02
Organic matter (%)	0.92	0.09	1.04	0.18	0.84	0.20	0.78	0.06
C/N (mol %)	8.28	1.43	8.83	0.27	9.53	1.40	8.74	0.80

(May until August) and fall until beginning of spring (October until April) (Table S2). Significant site differences (averaging over sampling times) were found between pairs of sites including Lynæs and all other sites (Lammefjord p=0.033, Vellerup p=0.001, Herslev p=0.011), and between Lammefjord and Vellerup (p=0.003) (Table S2). The environmental parameters best correlating with the variation in population dynamics, predicting 59% of the total population variation, were mean temperature, sorting, and mean salinity. Ordination of the *P. elegans* samples fitted to the model showed that population dynamics differed according to it being warmer during May and August, due to the fact that Lynæs had generally finer sediments, and that Herslev had lower salinities (Fig. 7).

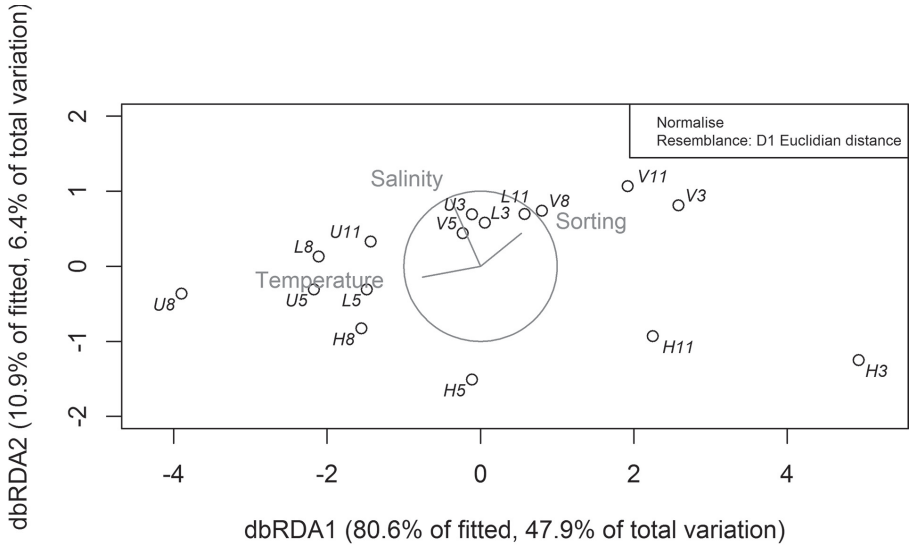

Fig. 7. Distance-based redundancy analysis (dbRDA): Ordination of the population dynamics data for *Pygospio elegans* (U, Lynæs; L, Lammefjord; V, Vellerup; H, Herslev; 3, March; 5, May; 8, August; 11, November) fitted to the significant predictor environmental parameters temperature, sorting, and salinity. The parameters explain 59% of the total variation in the population dynamics, with 54% explained by the first two axes as shown. Overlaid vectors indicate the loadings (importance) of the predictor parameters temperature, sorting, and salinity on the two axes. The circle illustrates a loading of 1.0.

Discussion

We performed a field survey of four populations of *Pygospio elegans* in the Danish Isefjord-Roskilde Fjord estuary complex to gain further insight into the population dynamics of this poecilogenous polychaete. Our specific focus was on its reproductive modes and whether its life history variation is related to environmental conditions in the studied populations.

Seasonal dynamics

We observed a clear seasonality in the population and reproductive dynamics of *P. elegans*. New cohorts appeared in spring and fall. Similar seasonal cohort structures have been observed in surveys of *P. elegans* at other sites. For example, Gudmundsson (1985), Rasmussen (1973), and Bolam (2004) all observed a continuous arrival of juveniles of *P. elegans* with one to two peaks in spring and/or fall. Larvae of *P. elegans* settle when 14–20 chaetigers in size (Hannerz 1956; Hempel 1957; Anger et al. 1986) and reach sexual maturity within a few months (Smidt 1951; Gudmundsson 1985; Anger et al. 1986; Bolam 2004). Accordingly, the spring and fall cohorts at our sites corresponded to a mean size of 30 chaetigers and reached maturity after 5–6 months (spring cohort in September/October, fall cohort in February/March), with an estimated growth rate of ~1.5 chaetigers per month. Bolam (2004) observed slightly higher growth rates of four chaetigers per month for specimens of similar size.

Sexual reproduction occurred from winter until spring, indicated by the presence of gravid females, males carrying sperm, and egg strings. Similar patterns of seasonal sexual reproduction by *P. elegans* were observed at other sites (e.g., Rasmussen 1973; Gudmundsson 1985; Bolam 2004), although there are exceptions. For example, Morgan (1997) found that the number of gravid females peaked during spring/winter in 1990/91 and during summer in 1992, and found egg strings almost year round, but mostly during summer, at Somme Bay. We observed two peaks in the numbers of gravid females and males with sperm at most sites. The two reproductive peaks most likely reflect the maturity of different cohorts at different times. However, we also observed that some individuals within a single cohort were able to produce two consecutive broods, making the peaks of reproduction broad and the cohorts less distinct. Mainly during January and February, we observed females bearing eggs and brooding egg strings simultaneously. A similar finding was made by Gudmundsson (1985) for the population at Cullercoats.

Given that planktonic larvae of *P. elegans* are expected to spend 4–5 weeks in the plankton before settlement, we expected to see new cohorts appearing with an approximate 1-month delay after the disappearance of egg capsules. Although the planktonic larval development mode was prevalent at many of our study sites, we only observed the expected 1-month delay between appearance of new cohorts and disappearance of egg capsules at Vellerup. By contrast, when there is benthic development, juveniles are expected to settle immediately after emerging from the capsules. Therefore, at Herslev, where we observed predominantly the benthic developmental mode, we expected to see new cohorts coinciding with the disappearance of egg capsules. Yet, this was not the case. The general lack of synchronization of reproduction and the combination of different developmental modes in populations of *P. elegans* are possible reasons for the appearance of new cohorts at different times. In addition, as mentioned in the introduction, the occurrence of asexual reproduction disrupts clear definition of cohorts in this species. When sexual reproduction declined in April, we observed an increase in asexual reproduction similar to that observed by Rasmussen (1953), Gudmundsson (1985), and Wilson (1985). Rasmussen (1953) proposed that asexual reproduction after periods of low temperatures might help *P. elegans* populations recover from declines due to severe winter conditions.

At the end of summer and during winter some cohorts disappeared. Accordingly, we observed many pale, inactive, and even degenerating individuals in July at Lynæs and Lammefjord and in January at Lammefjord. Considering the short life span of *P. elegans* (Anger et al. 1986), the appearance of new cohorts combined with the disappearance of old ones slightly afterward might have led to the drop in mean size we observed after summer and spring, indicating that the population was partly substituted by smaller individuals. If so, the highest densities might be present after new cohorts arrived but before old ones disappeared, at the end of spring and beginning of winter. Indeed, we observed highest densities in May with ~4000–8000 individuals m^{-2}, but we did not measure density in December/January. In a previous study at Blyth estuary, the highest densities were reached after the reproductive phase in May/June (Gudmundsson 1985), and at Drum Sands, highest densities (~13,000 individuals m^{-2}) were reached in December and

February (Bolam 2004). By contrast, the populations at Somme Bay had almost stable density levels of ~2500 and 15,000 individuals m^{-2} (Morgan 1997). In general, the densities we observed were in the range of 200–8000 individuals m^{-2}, similar to what has been described for several locations in Denmark (Muus 1967) and in the English Channel (4000 individuals m^{-2}, Morgan et al. 1999). Although our measurements exceed the densities of *P. elegans* observed by Gudmundsson (1985) and Blomqvist & Bonsdorff (1986), they are far below the maximum densities of up to 50,000–500,000 individuals m^{-2} described at other sites (Linke 1939; Hempel 1957; Anger 1977; Armitage 1979; Wilson 1985; Bolam 1999; Morgan et al. 1999).

To summarize, the population and reproductive dynamics of *P. elegans* were distinguished seasonally into a non-reproductive phase lasting from May until August and a reproductive phase, characterized by the presence of gravid females, egg strings, and asexual reproduction that lasted from September until April. The dbRDA plot shows that the seasonal dynamics of *P. elegans* population were correlated with temperature. These observations support the previous work by Rasmussen (1973), who reported that sexually mature individuals appeared when temperature dropped below 15°C, and Anger et al. (1986), who detected a higher rate of sexual reproduction at 5°C and 12°C compared to 18°C. Moreover, male *P. elegans* exposed to a temperature increase from 5 to 18°C lost their soft appendages, and their sperm degenerated (Rasmussen 1973). The influence of temperature on asexual reproduction is less clear. Rasmussen (1953) induced asexual reproduction by exposing *P. elegans* to temperatures of 4–5°C. However, we observed asexual reproduction throughout the year (as did Rasmussen 1953). Furthermore, asexual reproduction was prevalent at Lynæs and less common at Vellerup and Herslev despite nearly identical water temperatures at all sites. Hence, in addition to a strong seasonality in reproduction, there might be additional influences from other factors, such as food availability and worm density (Branch 1975; Wilson 1985) that affect reproductive patterns.

In addition, there are some uncertainties in our cohort estimates of *P. elegans* due to the following issues. First, since we were interested in development mode, we focused on sexually mature individuals and we used a 1-mm mesh for sampling, which might not have been sufficient for sampling juveniles. Using a 500-μm or 212-μm mesh would have been more appropriate for sampling and quantifying the smallest specimens accurately (Gudmundsson 1985; Morgan 1997; Bolam 2004). Although we identified new cohorts in spring and fall with timing matching the results of previous studies (Gudmundsson 1985; Morgan 1997; Bolam 2004), we likely underestimated the number of small individuals, especially at Vellerup and Herslev, where coarse and poorly sorted sediment hindered the sampling. This might have led us to conclude that small individuals appeared later than they actually did. In order to estimate the maximum delay in detection of small individuals due to our sampling methods, we assumed a minimum juvenile growth rate similar to adult growth rate (since growth rates seem to decrease with age [Anger et al. 1986: 18 chaetigers a month for planktonic larvae; Bolam 2004: 5 chaetigers a month for settled individuals]) and calculated that newly settling *P. elegans* of 14 chaetigers would likely need a month to grow to a size large enough (>20 chaetigers) for our detection. The coarse heterogeneous sediment might have also contributed to a sampling artifact that can explain the unrealistic high growth rates of 36 μm d^{-1} and 22.5 μm d^{-1} estimated for Vellerup. Here, new cohorts appeared in April and November and seemed to merge instantaneously with the one cohort present during the survey period. It is likely that we did not observe the true growth rate of cohorts at Vellerup given our limitations for sampling small individuals.

Second, there could have been some inaccuracy in our size measurements. Instead of counting the total number of chaetigers (Gudmundsson 1985; Morgan 1997) or measuring width of the fifth chaetiger (Bolam 2004), we chose to assess worm size by measuring the length from the eyespot to the gills so that we could include broken and regenerating individuals in the sample. In addition, because we wanted to save the specimens for additional genetic analysis (to be reported in a future contribution), we measured live animals that might have moved slightly, despite being narcotized. To test the accuracy of our method, we measured the length from eyespot to the gills and the total number of segments for 62 individuals collected from all sites from July to October, and found only a moderate positive correlation between length and segment number ($r=0.435$, $p<0.001$), which suggests that the two methods do not precisely agree. However, we believe that our measurements are adequate for comparisons among times and sites presented in this study given that the same method is used for all samples.

Site differences

Besides a seasonal difference, we also observed consistent differences in the population dynamics of

P. elegans between the different sampling locations. Lynæs was unique due to its high fraction of asexual reproduction and low worm density. Asexual reproduction might have led to the small mean size of worms and the presence of many separate cohorts. Vellerup and Herslev differed from Lynæs and Lammefjord because of their high number of egg strings, gravid females, and males with sperm. Furthermore, Vellerup and Herslev had the highest population densities and largest mean sizes. Herslev was characterized by a high number of benthic larvae in winter.

DistLM and dbRDA indicated that sorting and mean salinity were the parameters that best explained the observed site differences in population dynamics. In many ways, sorting describes the general sediment characteristics well, as it correlated significantly with median grain size, porosity, and water content. In general, sites with medium to coarse sediment (i.e., Herslev and Vellerup) had highest numbers of egg strings but also highest densities, largest mean sizes, and highest percentages of gravid females and males carrying sperm. By these criteria, populations of *Pygospio elegans* performed better in sandy and heterogeneous sediments in our study, as has been described previously (Smidt 1951; Armitage 1979), despite the lower organic content and higher refractory fraction.

Although the fraction of asexual reproduction was higher at sites with low numbers of egg strings, no correlation between output from sexual reproduction and asexual reproduction was found. However, Lynæs, which had the highest amount of asexual reproduction, was distinguished by the most labile organic matter, lowest densities, and best sorted sediment. Wilson (1985) observed that the asexual fission rate of *P. elegans* is proportional to food availability and inversely proportional to density. In comparison to the study of Wilson (1985), which tested densities of 12,000 to 50,000 individuals m^{-2}, all of our locations would be considered to have low density populations and thus should have high levels of asexual reproduction; however, this was not the case. Therefore, low population density and high sediment organic content might not be the causes of high percentage of asexual reproduction at Lynæs. Instead, the well-sorted sediment might facilitate predation or other disturbances that increase fission rates.

The lowest mean salinity was present at Herslev, which in turn was also the only site where no pelagic larvae, but benthic and intermediate ones, were found during winter. *Pygospio elegans* is a euryhaline species that occurs in salinities down to 5 ppt (Hempel 1957), and all our sites are well within the species' tolerance range. Anger (1984) showed that *P. elegans* has a higher reproductive rate at brackish sites compared to full marine sites, however. Generally, benthic larvae have been found in brackish habitats such as Blyth estuary (Gudmundsson 1985) or the Baltic Sea (Finland, Denmark [Kesäniemi et al. 2014; Rasmussen 1973]), whereas pelagic larvae are mostly known from full marine habitats (Drum Sands [Bolam 2004] and Somme Bay [Morgan 1997]). In addition, a previous study in the Isefjord-Roskilde Fjord estuaries from April 2010 found predominantly benthic and intermediate larvae in Roskilde Fjord and mainly pelagic larvae or all three kinds of larvae in Isefjord (Kesäniemi et al. 2014). Although we could not test it statistically, the fact that mode of development differs among sites only in winter suggest there may be an interaction between temperature and salinity in determining the mode of development, as described for other species (Schlieper 1929; Krug 2007). However, no combined effect of temperature and salinity on the mode of reproduction of *P. elegans* was found in previous lab experiments (Anger 1984). We combined data from different years (March 2014 and January, February 2015) in the March sample for the DistLM analyses in order to summarize the seasonal patterns, but in doing so neglected any interannual changes. Moreover, considering that we monitored only four different sites, and that only one had lower mean salinity, it is difficult to draw final conclusions from our results. Further manipulative lab experiments are needed to fully investigate the effect of sediment and salinity on the degree and mode of reproduction. Furthermore, additional parameters not monitored here, such as predation and disturbance, might play a role in the population and reproductive dynamics.

Although the mode of development of *P. elegans* was not fixed at our sites, we could not clearly relate the presence of different developmental modes with the studied environmental parameters. The co-occurrence of benthic and pelagic larvae might indicate that both exhibit a similar fitness, as otherwise one mode would have been preferred via selection already (Levin & Huggett 1990). Indeed, Levin & Bridges (1995) detected similar population dynamics between benthic and pelagic populations of the spionid polychaete *Streblospio benedicti*. Likewise, we observed similar population dynamics at Herslev compared to Lammefjord and Vellerup, despite a different larval development mode in winter. Furthermore, heterogeneity of the environment might promote the coexistence of different modes of

reproduction as a bet-hedging strategy (Eckert 2003). Thus, the variance in fitness and risk of failure is reduced in the long run (Collin 2012). Members of *P. elegans* are common in shallow and estuarine habitats which are exposed to unpredictable environmental fluctuations. Poecilogony might support persistence in these heterogeneous environments. Given that the genetic background of the populations may also affect the mode of development (Levin et al. 1991), we will further investigate whether the different broods and larvae observed in this study are produced by genetically different cohorts. At this point of time, we have not found a single clear factor determining the variable patterns of reproduction and population dynamics for *P. elegans* at our study sites. It is likely that a combination of environmental, genetic, and stochastic factors interact to produce the dynamic and somewhat unpredictable population dynamics that we have observed.

Conclusion

The population dynamics of *Pygospio elegans* in the Isefjord-Roskilde Fjord estuary complex showed similar seasonal dynamics to those observed previously by Rasmussen (1973), Gudmundsson (1985), and Bolam (2004) in other populations. Seasonality in sexual and asexual reproduction might be temperature induced. The populations at the four study sites, however, also differed in some characteristics, such as proportion of asexual reproduction and proportions of gravid females and males carrying sperm, as well as density and mean sizes. These differences were correlated with differences in environmental conditions at the sites, such as sediment characteristics and salinity. We observed two reproductive peaks at three of the sites. At the same sites, we also found a switch in mode of development from spring to fall 2014, whereas at one site developmental mode remained constant. Consequently, we intend to use molecular tools to further investigate whether the shift in larval developmental mode reflects reproduction of genetically differentiated cohorts.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Fig. S1. Example for size measurement from head until beginning of gills in *Pygospio elegans* (**A**). In *P. elegans* the sex is only distinguishable during the reproductive season: males develop sperm in the coelom and soft appendages at the second chaetiger to transfer the spermatophores to the female (**B**); females develop eggs in their coelom (**C**). The eggs of each segment will be exerted via the nephridioductus forming one egg capsule of the egg string. Depending on the ratio of fertile eggs to nurse eggs small pelagic larvae bearing swimming chaetae will hatch early (**D**) or large benthic larvae containing a lot of yolk (**E**) will develop. After asexual reproduction the specimens regenerate head, tail, or both (**F**).
Fig. S2. Histograms (**a–d**) of length frequency data for each month and location including the fitted normal distribution by FiSatII to identify cohorts. Dotted lines were inserted by eye to follow the development of each cohort during the year.
Fig. S3. Sediment characteristics: median grain size (**A**), sorting (**B**), water content (**C**), and porosity (**D**) in March, May, August, and November. Categories for grain size and sorting are applied from Gray & Elliott (2009). The standard deviation of porosity (<6.6%) and water content (<5.5%) was calculated from three analytical replicates of one sample per site.
Fig. S4. Characteristics of organic content: organic content (**A**) and mean and standard deviation of C/N (**B**) in March, May, August, and November. The standard deviation of organic content (<5%) was calculated from three analytical replicates of one sample per site.
Table S1. GPS coordinates of the sampling sites and sampling dates.
Table S2. Results of pair-wise comparisons using PERMANOVA.

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLE S1 GPS coordinates of the sampling sites and sampling dates.

	Location of sampling	
	Latitude	Longitude
Lammefjord	N 55°46´ 20.61´´	E 11° 37´ 51.00´´
Lynaes	N 55°56´ 38.16´´	E 11° 52´ 7.44´´
Herslev	N 55°40´ 41.29´´	E 11° 59´ 13.07´´
Vellerup	N 55°44´ 14.28´´	E 11° 52´ 4.80´´

	Lynaes	Lammefjord	Vellerup	Herslev
March 2014	18/03/2014	17/03/2014	20/03/2014	20/03/2014
April 2014	10/04/2014	10/04/2014	24/04/2014	11/04/2014
May 2014	13/05/2014	14/05/2014	12/05/2014	13/05/2014
June 2014	12/06/2014	11/06/2014	11/06/2014	12/06/2014
July 2014	15/07/2014	16/07/2014	16/07/2014	15/07/2014
August 2014	20/08/2014	19/08/2014	19/08/2014	20/08/2014
September 2014	23/09/2014	24/09/2014	24/09/2014	23/09/2014
October 2014	21/10/2014	22/10/2014	21/10/2014	22/10/2014
November 2014	26/11/2014	27/11/2014	26/11/2014	27/11/2014
January 2015	06/01/2015	07/01/2015	06/01/2015	07/01/2015
February 2015	25/02/2015	26/02/2015	25/02/2015	26/02/2015

SUPPLEMENTARY TABLE 2 Results of pair-wise comparisons using PERMANOVA

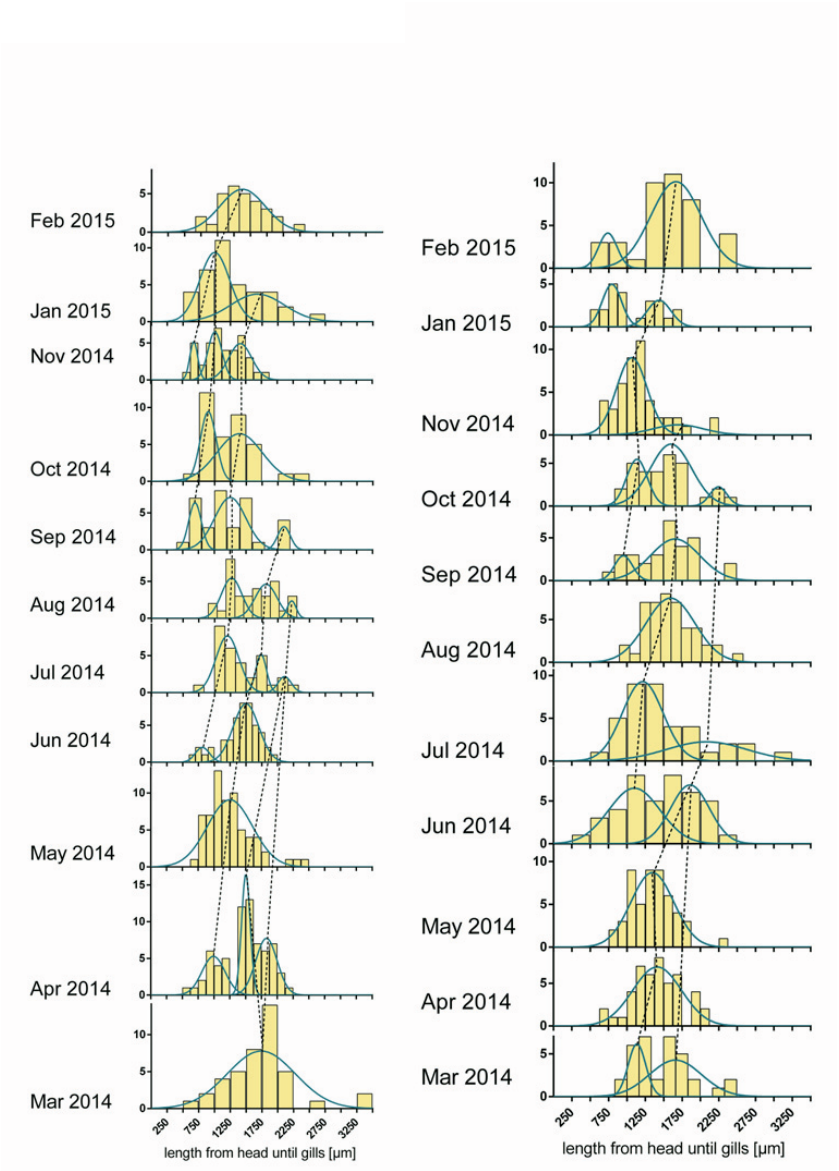
Groups	p-value	unique permutations
Mar, Apr	0.067	425
Mar, May	0.065	425
Mar, Jun	0.066	425
Mar, Jul	0.079	425
Mar, Aug	0.083	425
Mar, Sep	0.164	424
Mar, Oct	0.239	425
Mar, Nov	0.192	425
Mar, Jan	0.471	425
Mar, Feb	0.513	425
Apr, May	**0.031**	**425**
Apr, Jun	0.100	425
Apr, Jul	**0.046**	**425**
Apr, Aug	**0.026**	**425**
Apr, Sep	0.163	425
Apr, Oct	0.065	425
Apr, Nov	0.257	425
Apr, Jan	0.199	425
Apr, Feb	0.201	425
May, Jun	0.736	425
May, Jul	0.510	425
May, Aug	0.154	425
May, Sep	0.640	425
May, Oct	0.102	425
May, Nov	0.228	425
May, Jan	0.130	425
May, Feb	0.101	423
Jun, Jul	0.640	425
Jun, Aug	0.327	425
Jun, Sep	0.263	425
Jun, Oct	**0.048**	**425**
Jun, Nov	0.171	425
Jun, Jan	0.180	425
Jun, Feb	0.120	425
Jul, Aug	**0.032**	**425**
Jul, Sep	0.245	425
Jul, Oct	**0.045**	**425**
Jul, Nov	0.178	425
Jul, Jan	0.175	425

Jul, Feb	0.098	425
Aug, Sep	0.272	425
Aug, Oct	**0.043**	**425**
Aug, Nov	0.139	425
Aug, Jan	0.167	425
Aug, Feb	0.109	425
Sep, Oct	0.069	425
Sep, Nov	0.288	425
Sep, Jan	0.480	425
Sep, Feb	0.188	425
Oct, Nov	0.502	425
Oct, Jan	0.475	425
Oct, Feb	0.241	425
Nov, Jan	0.701	425
Nov, Feb	0.473	425
Jan, Feb	0.324	424

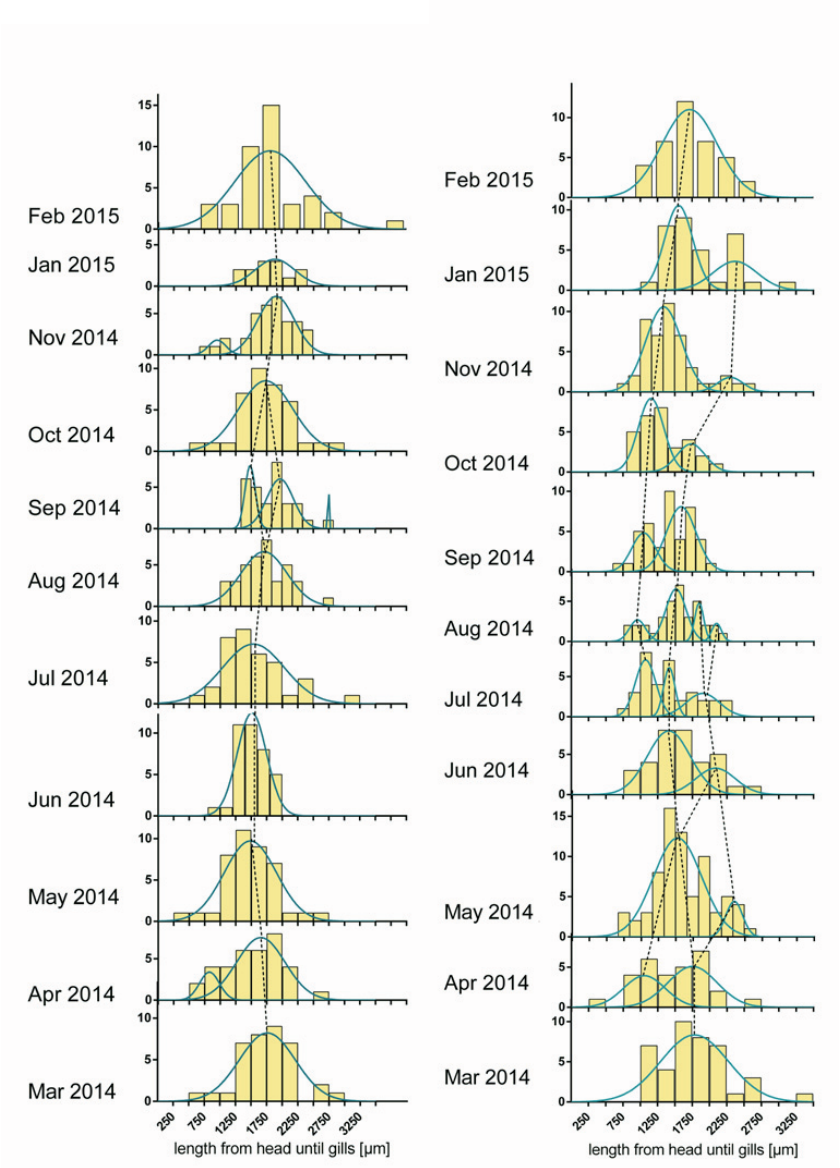
Groups	p-value	unique permutations
Lynæs, Lammefjord	**0.033**	**9947**
Lynæs, Vellerup	**0.001**	**9950**
Lynæs, Herslev	**0.011**	**9946**
Lammefjord, Vellerup	**0.003**	**9957**
Lammefjord, Herslev	0.060	9949
Vellerup, Herslev	0.169	9956

SUPPLEMENTARY FIGURE S1 Example for size measurement from head until beginning of gills in *P. elegans* (A). In *P. elegans* the sex is only distinguishable during the reproductive season: males develop sperm in the coelom and soft appendages at the second setiger to transfer the spermatophores to the female (B); females develop eggs in their coloem (C). The eggs of each segment will be exerted via the nephridioductus forming one egg capsule of the egg string. Depending on the ratio of true eggs to nurse eggs small pelagic larvae bearing swimming setae will hatch early (D) or large benthic larvae containing a lot of yolk (E) will develop. After asexual reproduction the specimens regenerate head, tail or both (F).

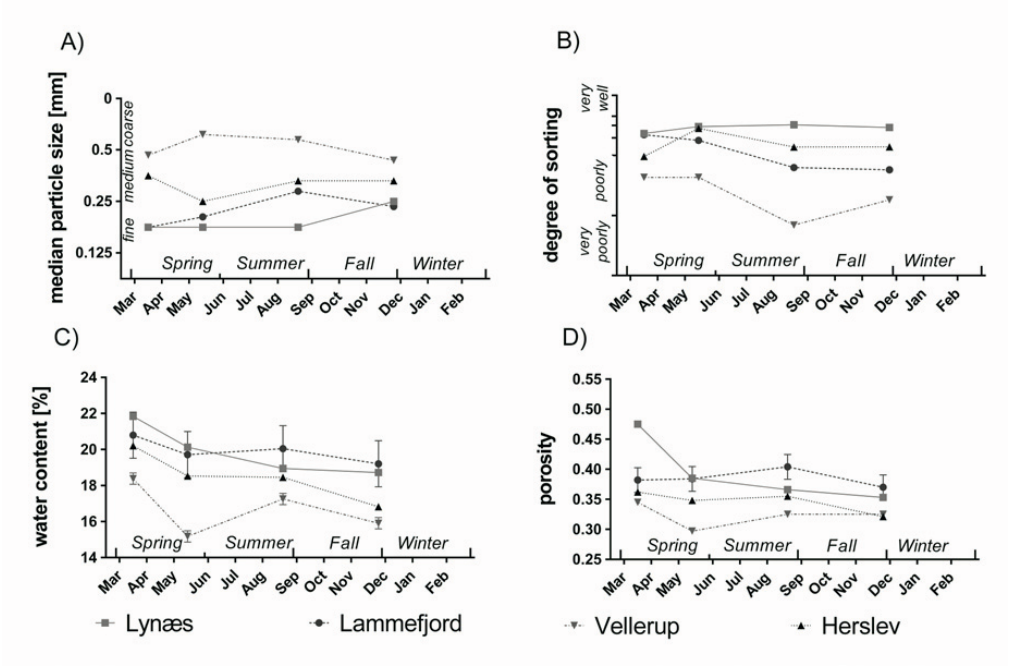
a) Lynaes b) Lammefjord



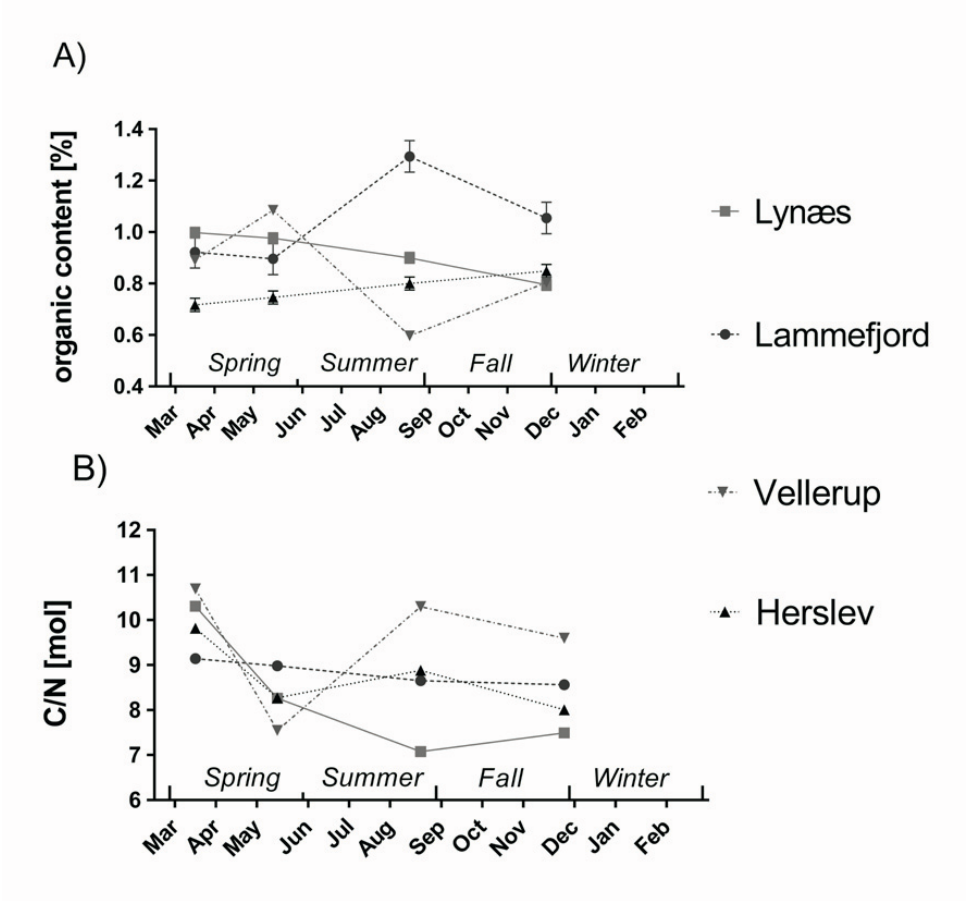
c) Vellerup d) Herslev



SUPPLEMENTARY FIGURE 2a-d Histograms of length frequency data for each month and location including the fitted normal distribution by FiSatII to identify cohorts. Dotted lines were inserted by eye to follow the development of each cohort during the year.



SUPPLEMENTARY FIGURE S3 Sediment characteristics: median grain size A), sorting B), water content C) and porosity D) in March, May, August and November. Categories for grain size and sorting are applied from Gray and Elliot (2003). The standard deviation of porosity (<6.6%) and water content (<5.5%) was calculated from three analytical replicates of one sample per site.



SUPPLEMENTARY FIGURE 4 Characteristics of organic content: organic content A) and mean and standard deviation of C/N, B) in March, May, August and November. The standard deviation of organic content (<5%) was calculated from three analytical replicates of one sample per site.

II

SEASONAL GENETIC VARIATION ASSOCIATED WITH POPULATION DYNAMICS OF A POECILOGONOUS POLYCHAETE WORM

Thonig, A., Banta, G.T., Winding Hansen, B. & Knott, K.E. 2017.

Ecology and Evolution, in press

SEASONAL GENETIC VARIATION ASSOCIATED WITH POPULATION DYNAMICS OF A POECILOGONOUS POLYCHAETE WORM

Thonig, A.[1,2], Banta, G. T.[1],Winding Hansen, B.[1], Knott, K. E.[2]

[1]Department of Science and Environment, Roskilde University, DK-4000 Roskilde, Denmark
[2]Department of Biological and Environmental Science, University of Jyvaskyla, FI-40014 University of Jyvaskyla, Finland

ABSTRACT

Poecilogonous species show variation in developmental mode, with larvae that differ both morphologically and ecologically. The spionid polychaete *Pygospio elegans* shows variation in developmental mode not only between populations, but also seasonally within populations. We investigated the consequences of this developmental polymorphism on the spatial and seasonal genetic structure of *P. elegans* at four sites in the Danish Isefjord-Roskilde-Fjord estuary at six time points, from March 2014 until February 2015. We found genetic differentiation between our sampling sites as well as seasonal differentiation at two of the sites. The seasonal genetic shift correlated with the appearance of new size cohorts in the populations. Additionally, we found that the genetic composition of reproductive individuals did not always reflect the genetic composition of the entire sample, indicating that variance in reproductive success among individuals is a likely explanation for the patterns of chaotic genetic patchiness observed during this and previous studies. The heterogeneous, unpredictable character of the estuary might maintain poecilogony in *P. elegans* as a bet-hedging strategy in the Isefjord-Roskilde-Fjord complex in comparison to other sites where *P. elegans* are expected to be fixed to a certain mode of development.

INTRODUCTION

Phenotypic variation within a single population or species is a classical focus of ecology and evolution, and both the causes and consequences of polymorphism are actively investigated (e.g. Schwander & Leimar 2011, Wennersten & Forsman 2012). Even though phenotypic variation can arise via different mechanisms, e.g. genetic polymorphism, developmental plasticity or randomized switching, the consequences for populations can be very similar. Populations with high phenotypic variation are expected to have larger niche breadths and increased colonization potential, as well as decreased intra-specific competition, decreased vulnerability to environmental changes and decreased fluctuations in population size (Wennersten & Forsman 2012). However, the consequences of phenotypic variation are also influenced by what kind of traits show variation. For example, variation in life history traits are likely to have strong effects on colonization potential and fluctuations in population size, whereas traits affecting nutrient acquisition might have a stronger effect on intra-specific competition (Wennersten & Forsman 2012).

Variation in developmental mode, when a single species produces different types of larvae, is called poecilogony. Poecilogony is known only in some marine invertebrates (notably among spionid polychaete worms and sacoglossan sea slugs), and the degree of variation in developmental mode can differ between poecilogonous species (see Collin 2012, Knott & McHugh 2012, McDonald *et al.* 2014). For example, variation in developmental mode can occur between populations, between females within the same population, between broods of the same female, or even within broods. Likewise, there are different possible mechanisms allowing for poecilogony, including fixed genetic polymorphisms, plasticity in response to environmental cues, or maternal effects (Collin 2012, Knott & McHugh 2012). Because the larval stage of benthic marine invertebrates has a significant impact on their dispersal ability, variation in the mode of development has consequences for spatial and temporal population genetic structure (Collin 2001, Eckert 2003, Cowen & Sponaugle 2009, Lee & Boulding 2009). High population connectivity and low spatial genetic structure is expected for species with planktonic larvae due to their higher dispersal potential in comparison to species with non-planktonic larvae (Bohonak 1999, Hellberg 2009). However, dispersal potential does not always translate into realized dispersal and connectivity, and might not predict population genetic structure (e.g. Weersing & Toonen 2009). Moreover, temporal fluctuations in genetic structure can occur in species with planktonic larvae due to sweepstakes reproductive success, particularly in highly fecund species, and/or due to selection during the planktonic phase (Lee & Boulding 2009, Hedgecock & Pudovkin 2011).

One species exhibiting poecilogony is *Pygospio elegans*, a small (max. 20mm), tube-dwelling spionid polychaete with an average life span of 9 months, which exhibits a broad range of habitat tolerances, population densities

and a variety of feeding modes (Anger 1984, Anger *et al.* 1986, Hempel 1957). It can reproduce asexually via fragmentation (Rasmussen 1953, Anger 1984), whereas embryos resulting from sexual reproduction are laid in egg capsules within the mother's sand tube. Larvae spend part of their development within the egg capsules feeding on unfertilized nurse eggs provided by the mother (oophagy). When there are few embryos and many nurse eggs (> 10), the larvae are classified as benthic larvae: these hatch from the egg capsules at a large size and do not have a large potential for dispersal. In contrast, when there are many embryos and few (< 3) or no nurse eggs laid in the capsules, the larvae are classified planktonic larvae: these hatch at a small size and complete their development in the plankton and have a greater potential for dispersal. However, the difference between benthic and planktonic larvae is not always discrete and intermediate larvae also exist (Rasmussen 1973, Thonig *et al.* 2016). The association of developmental mode and nurse egg production suggests a possible maternal effect (as noted for other poecilogonous polychaetes, e.g. Oyarzun & Brante 2014). However, the underlying mechanism of poecilogony in *P. elegans* is still not known, and multiple mechanisms might work in concert. For example, different developmental modes in *P. elegans* are found both between populations and within populations, at times showing seasonal switches (Rasmussen 1973, Gudmundsson 1985, Thonig *et al.* 2016), suggesting a possible environmental influence. Also, the possibility of genetic polymorphism has been suggested because some populations are presumed to have a fixed developmental mode (Morgan *et al.* 1999, Bolam 2004), and this possibility has not been ruled out.

In the first part of this study we investigated the population dynamics of the poecilogonous spionid *Pygospio elegans* at four sites in the Danish Isefjord-Roskilde Fjord estuary and how environmental parameters might affect the population dynamics (Thonig *et al.* 2016). We identified two main recruitments, seen as the appearance of new size cohorts, one in spring and one in fall. Previous cohorts seemed to disappear during summer and winter, thus resulting in a turnover of the population. Sexual reproduction occurred predominantly from September until May. These results confirmed observations of Rasmussen (1973), Gudmundsson (1985) and Bolam (2004). Two separate peaks of gravid females were observed at three out of four sites, and these showed a switch in type of larvae from planktonic larvae in winter to intermediate and benthic larvae in spring. One peak of gravid females and only intermediate and benthic larvae were observed at the innermost site, Herslev. The seasonal population dynamics were related to temperature, with reproduction occurring at low temperature. Median grain size and sorting of the sediment correlated with the spatial differences, where higher densities of *P. elegans* and larger specimens were observed at sites with coarse and poorly sorted sediment (Herslev and Vellerup).

In this part of the study we analysed the population genetic structure of *P. elegans* using seven microsatellite loci to genotype individuals sampled from the same four locations at six different time points over one year. Our aim was to determine whether genetic differences among individuals and cohorts are

associated with the population dynamics we described in Thonig *et al.* (2016). Previous studies of population genetic structure in *P. elegans* have not been able to adequately follow individual worms with known developmental modes, but rather, examined populations with different larval types, or populations categorized based on the developmental mode observed in a sample (e.g. Kesäniemi *et al.* 2012a, 2012b). Here we compare genotypes and phenotypes of individuals sampled both spatially and temporally to describe consequences of poecilogony on the population genetic structure of *P. elegans*.

MATERIALS AND METHODS

Sampling

We conducted a field survey from March 2014 until February 2015 to document the population dynamics of *Pygospio elegans* at four sampling sites in the Danish Isefjord-Roskilde-Fjord estuary complex: Lynæs, Lammefjord, Vellerup and Herslev (described in detail in Thonig *et al.* 2016). In this study, we examine population genetic structure at the four sites from samples collected at six time points (in March, May, August, October, November and February) in order to determine if genetic differences can be detected between size cohorts and how variation in developmental mode is related to the spatial and temporal population genetic structure. *Pygospio elegans* were sampled from the top layer of sediment and sieved on site with a 1 mm mesh. In the laboratory, subsamples of 27 to 44 individuals were sized (Thonig *et al.* 2016) and afterwards stored in 99% ethanol for DNA extraction.

DNA extraction and Microsatellite Genotyping

DNA was extracted from whole individuals using the Qiagen DNeasy Blood & Tissue Kit following the manufacturer´s protocol for animal tissue. We developed two multiplex reactions to amplify ten microsatellite loci in *P. elegans.* Seven of the microsatellite loci were identified from a draft transcriptome of *P. elegans* (Heikkinen *et al.* 2017) and primers were designed to amplify these loci using WebSat software (Martins *et al.* 2009). Three of the loci (Pe6, Pe7, and Pe19) were described previously (Kesäniemi *et al.* 2012a) (see Table 1). Multiplex PCR reactions of 10 µl were performed containing 1x Qiagen Multiplex PCR Master Mix, 0.2 µM of each primer and 1 µl DNA template (diluted 1:20). The PCR had an initial activation step of 15 min at 95 °C followed by 30 cycles of 30 sec at 94 °C, 90 sec at 60 °C and 60 sec at 72 °C, and a final extension for 30 min at 60 °C. Fragments were separated using an ABI PRISM 3130xl Genetic analyzer with Gene Scan™ 500 LIZ™ size standard (Applied Biosystems) in our own lab. The results were analysed with GeneMapper® v.5 Software (Applied Biosystems).

Quality of loci

To ensure the quality of the data, every allele that occurred only once in the data set was double-checked and confirmed in the raw data. Individuals missing information for more than two loci were discarded. Three loci had more than 5 % missing data (Pe7- 5.7 %; Pe159- 6.1 %; and Pe309- 8.1 %) and were suspected to have null alleles. We used Micro-Checker (Van Oosterhout *et al.* 2004) to estimate null allele frequencies for all loci and found that loci Pe7, Pe159 and Pe309 had a significant proportion of null alleles (Oosterhout calculation: up to 2.2 % in Pe7, 2.9 % in Pe309 and 3.3 % in Pe159) in many of the samples. Locus Pe385 also showed possible null alleles, but these were always less than 2 %, which is not expected to affect downstream analyses significantly (Putman & Carbone 2014). Gametic disequilibrium and Hardy-Weinberg-Equilibrium (HWE) were checked per locus and sample using Fstat v.2.9.3.2 (Goudet 1995). Loci Pe7 and Pe159 were not in HWE in the majority of the samples. Consequently, we decided to eliminate three loci from the data set: Pe7, Pe159 and Pe309. Therefore, further statistics are calculated based on data from the remaining seven polymorphic loci (see Table 1). An outlier test was performed in LOSITAN (Beaumont & Nichols 1996, Antao *et al.* 2008) for all loci except Pe7 and Pe159 using "Neutral mean Fst", "Force mean Fst" and "100.000" simulations. This test indicated that Pe385 might be subject to positive selection and Pe294 might be subject to balancing selection.

Genetic diversity

For each sample, observed and expected heterozygosity (H_o and H_e), gene diversity and F_{IS} averaged over all loci were calculated using Arlequin v.3.5.2 (Excoffier & Lischer 2010). F_{IS} was calculated for each sample separately, assuming no temporal or spatial groups, from a distance matrix based on the number of different alleles and 20,000 permutations were performed to calculate the p-values. We calculated allelic richness and number of private alleles using the rarefaction method implemented in HP-Rare v1.1 (Kalinowski 2005). These values are calculated based on the same number of individuals per sample to enable comparisons between samples. Relatedness within each sample was calculated using the triadic likelihood estimator implemented in Coancestry v.1 (Wang 2007, Wang 2011), which infers allele frequencies from the genotypic data and accounts for inbreeding. Hereby, 100 individuals are used as a reference sample and 100 bootstrapping samples were used to calculate the 95 % confidence intervals.

Population structure

Population structure was analysed using three different approaches. Firstly, analysis of molecular variance (AMOVA) was performed in Arlequin v.3.5.2 (Excoffier & Lischer 2010). For AMOVA, samples were grouped either in temporal or spatial groups. The distance matrix used in the analysis was based

on the number of different alleles and the p-values were calculated based on 20,000 permutations. Secondly, population differentiation was estimated using G'_{ST} (Hedrick 2005) and Jost´s D (Jost 2008) statistics implemented in the R package diveRsity (Keenan *et al.* 2013) for each pair of samples. The correlation between different statistics (F_{ST}, G_{ST}, G'_{ST}, Jost´s D) and the mean number of alleles for each locus showed a similar trend (data not shown). Thirdly, the model-based clustering method implemented in Structure v.2.3.4 (Pritchard *et al.* 2000) was used to assign individuals to distinct clusters. We used the admixture model, correlated allele frequencies, a burn-in of 100,000 iterations and subsequently 500,000 iterations to calculate the likelihood of the different models. We performed five replicate runs for each K ranging from k=1 to k=5. The number of clusters was determined according to the MedMeaK and MaxMeaK method (Puechmaille 2016) with a threshold of 60 % for the mean membership coefficient. The results were illustrated using DISTRUCT (Rosenberg 2004). In addition to the structure analysis, clustering methods implemented in InStruct (Gao *et al.* 2007) and Flock (Duchesne & Turgeon 2012) were investigated. The Bayesian clustering method of InStruct inferred the number of subpopulations only with admixture by comparing the log likelihoods and DIC (deviance information criterion) for the number of subpopulations ranging from K=2 to K=5. For that purpose, samples were taken every 100 iterations from 3 independent chains with 1,000,000 iterations and 500,000 iterations as burn-in. Convergence was checked with Gelman-Rudin statistics. In Flock, the plateau lengths were determined for 2 to 9 reference groups using 30 iterations and 50 runs with a random choice of samples as the initial separation mode. Furthermore, identical multi-locus genotypes were identified using GenClone2 (Arnaud-Haond & Belkhir 2007). Accordingly, we removed 107 individuals so that only one copy of each genotype is present per sample. The analyses of population structure were repeated with the purged samples. However, since the results were similar to the analyses including all individuals and purging might reduce precision of the fixation index (Waples & Anderson 2017) we only show results of the analyses including all individuals.

Comparing genotype with cohort, sex and environmental data

Individuals were assigned to a distinct genetic cluster defined by Structure when membership to that cluster was higher than 60 %. Choosing higher membership thresholds resulted in an increase of unassigned individuals, but did not change the trends. Additionally, when possible, individuals used in this study were assigned to distinct cohorts based on their size (Thonig *et al.* 2016). We analysed whether the different size cohorts are composed of individuals assigned to distinct genetic clusters. Similarly, since some individuals were identified as bearing gametes, we analysed whether these females and males were assigned to different genetic clusters. We estimated genetic differentiation (1) between different cohorts within each site, (2) of females/males between every sampling within each site, and (3) between males, females and all individuals within each sample, using the fixation indices G'_{ST} (Hedrick 2005)

and Jost´s D (Jost 2008) as implemented in the R package diveRsity (Keenan *et al.* 2013). This was only applicable when more than one specimen was present per group and more than two groups were present for comparison.

We compared the observed genetic structure at four time points (March, May, August, November) with the environmental parameters described previously (Thonig *et al.* 2016) using Primer-E v.6 (Clarke & Gorley 2006). For that purpose, genetic differentiation G'_{ST} calculated with the R package diveRsity (Keenan *et al.* 2013) was input in Primer-E as a dissimilarity matrix. The following environmental parameters were normalized and used to calculate a resemblance matrix based on Euclidian distance: median particle size (correlating significantly with sorting r = -0.818, porosity r = 0.725 and water content r = 0.775), organic content, C/N, mean temperature (correlating significantly with standard deviation of temperature r = 0.905), mean salinity and standard deviation of salinity. The Spearman rank correlation between the two matrices was calculated using RELATE and the environmental parameters best explaining the observed genetic differentiation were determined via DistLM based on the Bayesian information criterion (BIC) using 9999 permutations.

RESULTS

Genetic diversity

The genetic diversity of the metapopulation is described in Table 2. Allelic richness and expected heterozygosity are similar among sites throughout the year, being highest in August and October. However, this increase in diversity is less distinct in Lynæs and Herslev than at the other sites. Depending on the location, the percentage of private alleles increases from May to November and is highest at Vellerup and lowest at Lynæs. Gene diversity fluctuates, but also seems to peak in August and October. Accordingly, relatedness is lowest in August and October, most drastically at Lammefjord and Vellerup. Gene diversity and relatedness are otherwise similar among the sites. At Vellerup and Herslev the observed heterozygosity fluctuates through the year more than it does at the other sites. In almost all of the samples a deficiency of heterozygotes was observed, with significant differences from HWE in the majority of samples from Lammefjord and Vellerup.

Population structure

The AMOVA results of the temporal and spatial differences between the samples are shown in Table 3. When samples are grouped according to location (across time), a similar percentage of the variation is explained by location (1.99 %) and time point within location (2.05 %). When samples are grouped according to time point (across locations) a greater percentage of variation is

explained by location within time points (3.17 %) than among time points (0.51 %). These results suggest that the four locations are genetically differentiated. Moreover, the results suggest that there is no general seasonal pattern in the population structure common to all locations; instead, temporal genetic changes differ among the locations.

The two summary statistics for population differentiation, G'_{ST} and Jost´s D (Fig. 1), show similar patterns, but Jost´s D shows less pronounced differentiation between the samples. Except in August, Herslev is more similar to Vellerup, whereas Lammefjord and Lynæs are more alike in allele frequencies, but these two groups differ from each other. There are no seasonal differences in samples from Lynæs, and only weak differences among the samples from Herslev. However, strong seasonality occurs at Lammefjord and Vellerup. Allele frequencies in August, and, to some degree also in October, differ from those in the other months at these sites and also differ from allele frequencies at other locations. However, allele frequencies in August and October at Lammefjord and Vellerup are similar. The confidence intervals of G'_{ST} and Jost´s D can be found in Supplementary Table S1.

The cluster analysis in Structure revealed three genetic clusters when analysing the whole metapopulation (Fig. 2). The first cluster (light-grey) was composed of all samples from Herslev as well as samples from March, November and February from Vellerup. The second cluster (grey) included all samples from Lynæs, all samples from Lammefjord except for August and October, and the sample from May from Vellerup. The third cluster (dark-grey) contained the samples from August and October from both Lammefjord and Vellerup. For k=3 the allele frequency divergence among clusters computed by Structure using point estimates is lower between the first and second cluster (0.0339) compared to the divergence of the third cluster from the other two (first to third 0.0509, second to third 0.0749). Analysing every location separately resulted in a single cluster for both Herslev and Lynæs, whereas two clusters were the best solution for the samples at Lammefjord and Vellerup (graphs not shown). In both cases, the first cluster included the samples from March, May, November and February, while the samples from August and October belonged to the second cluster.

Genetic clusters were also estimated with the program InStruct, which accounts for inbreeding and might be more suitable for *P. elegans*, since we observed high and significant FIS values and since *P. elegans* also is able to reproduce asexually. This program recommended two clusters as a best explanation for the data according to deviance information criterion (DIC). The program Flock determines genetic clusters by partitioning the sample and reallocating genotypes. Several runs starting with a different initial partitioning are performed for a different number of clusters. The number of genetic clusters k is reached when an identical final partitioning is obtained for more than six runs. For k = 2 to 9 cluster, no more than three identical partitions were obtained for our sample, indicating that either no population structure is present or that our data do not contain enough information, i.e. too few microsatellites, to infer the number of genetic clusters. Of the three methods

used to estimate genetic clusters, the three-cluster solution from Structure reflects the G'_{ST} values best.

Comparing genotype with cohort, sex and environmental data

Cohorts based on size of the worms were distinguished previously (Thonig *et al.* 2016) and the genetic composition of these cohorts is shown in Fig. 3 and Supplementary Table S2. Vellerup is not included, since we could not distinguish cohorts based on size at this location (see Thonig *et al.* 2016 for more details). At the other sites, about 50 individuals per site could not be assigned to a distinct size cohort due to overlapping size ranges of the cohorts (cohort not characterized– n.c.). About 25 individuals per site that could be assigned to a cohort, however, could not be assigned to a distinct genetic cluster as their membership coefficient was below 60 % (cluster 0). At Herslev, the fixation index G_{ST} suggests a genetic difference between cohort 2 and 3 (G_{ST} = 0.0138). Most individuals in the cohorts at Herslev were assigned to genetic cluster 1 (68 %), but some individuals in the size cohorts were assigned to genetic cluster 2 (23 %) and genetic cluster 3 (9%). Likewise, at Lynæs, all four size cohorts are primarily composed of individuals assigned to a single genetic cluster: cluster 2 (67%). Nonetheless, individuals assigned to the other two genetic clusters also exist and vary in frequency. Note that 31 % of the individuals of the third size cohort belong to genetic cluster 3, whereas individuals assigned to genetic cluster 3 make up only 7-14 % of the other size cohorts. A significant G_{ST} value indicates that cohort 3 differs from all other cohorts (G_{ST} ranging from 0.0124 to 0.0243). In contrast to relatively stable genetic composition of size cohorts in Herslev and Lynæs, Lammefjord shows a different pattern: the first and third size cohorts are dominated by individuals assigned to genetic cluster 2 (~70 %), while the second size cohort is dominated by individuals assigned to genetic cluster 3 (80 %). The genetic difference between size cohorts is evidenced by a significant G_{ST} values between cohorts (G_{ST} ranging from 0.0694 to 0.0826). These patterns reflect the seasonal variation noted in the initial structure analysis (Fig 2). The third size cohort at Lynæs and the second size cohort at Lammefjord, which show higher frequencies of individuals assigned to genetic cluster 3 are both present from June to October/November. The second cohort at Herslev that differed slightly from the other two cohorts due to less individuals assigned to cluster 2 and more individuals assigned to cluster 1 was present during the whole study period, but dominated from June to September.

In Fig. 4 and Supplementary Tables S3 and S4 the genetic composition of the individuals bearing gametes are shown (panel A) in comparison to the genetic composition of the whole sample (panel B). Individuals with membership coefficients below 60 % are excluded (cluster 0). At Herslev, we did not observe a significant genetic change among individuals with eggs or sperm between samplings: these individuals mostly belong to genetic cluster 1 throughout the study period. At the other sites, individuals reproducing in winter/spring are primarily assigned to genetic clusters 1 and 2, whereas individuals reproducing in fall/winter are primarily assigned to genetic cluster

3. However, at Vellerup individuals assigned to genetic cluster 1 are also reproductive in fall. While too few sexually mature individuals were captured at Lynæs to test for genetic differences, at Lammefjord and Vellerup, gravid females and ripe males sampled in March showed genetic differences from those sampled in October and November, and at Lammefjord, gravid females and ripe males sampled in October also differed genetically from those sampled in February.

Comparing the genetic composition of reproductive individuals and that of all individuals in each sample, we can see whether individuals contributing to the next generation represent a subsample of the available genetic variation (see Fig. 4 and Table 5). At Herslev, the percentages of individuals in the different genetic clusters among reproductive females and males are similar to those of the whole population, with genetic cluster 1 dominating both. This is further supported by the fact that we observed no significant G'_{ST} value between reproductive individuals and the whole sample. At the other sites, discrepancies are seen from October to February, when genetic cluster 3 is more common among reproductive females and males even though in the total population a different genetic cluster is more prevalent: cluster 2 at Lynæs in October and at Lammefjord in February, or cluster 1 at Vellerup in November. At Vellerup, no individuals with gametes belong to cluster 2 in fall even though individuals in cluster 2 are relatively common in the population. Likewise, significant genetic differentiation was observed between females/males and the whole sample at Lynæs in October and at Lammefjord in October and November. No differentiation was observed at Vellerup, however, probably because of the genetic similarity of clusters 1 and 2.

We observed a significant moderate correlation (Rho = 0.4, p = 0.001) between genetic and environmental differences between samples. The environmental parameters best correlating with the genetic differentiation and explaining 74.02 % of the genetic variation are median grain size, mean temperature and mean salinity. These results are displayed in a distance-based redundancy analysis (dbRDA) in Supplementary Figure S1.

DISCUSSION

We investigated the genetic structure of the poecilogonous polychaete *Pygospio elegans* from four sites in the Isefjord-Roskilde-Fjord estuary complex using six temporal samples collected over one year. We aimed to evaluate the relationship between the genotype of sampled individuals and previously described differences in population dynamics in these populations (see Thonig *et al.* 2016). We observed genetic differences between the sites as well as changes during the year at two of the sites. Similar population genetic structure was evident from summary statistics and fixation indices, cluster analysis and AMOVA. Overall, differentiation is low, which we expected given that the sites

are geographically close and that the time between sampling is short. Nevertheless, significant genetic differentiation was found between cohorts as well as between reproductive individuals and the total population. Previously, Kesäniemi *et al.* (2014a) also detected three genetically different clusters among 16 sampling sites within Isefjord-Roskilde-Fjord estuary complex at a single time point in 2010. Furthermore, Kesäniemi *et al.* (2014b) found either temporal stability or differences in allele frequencies, depending on the population, when sampling different populations in Baltic Sea to North Sea over 1-2 years. However, neither of these previous studies included sufficient sampling and phenotypic data to allow the assessment of genetic composition of size cohorts or reproducing individuals.

Seasonal dynamics

The genetic data collected in this study suggest the arrival of genetically distinct recruits of *P. elegans* after May and after October at Lammefjord and Vellerup, indicated by a temporal change of the predominant genetic cluster. The timing of the genetic shift at these sites correlates with the appearance of new cohorts defined by size (Thonig *et al.* 2016). Accordingly, small individuals (< 30 setigers) appeared at the four study sites in spring (April to June) and in autumn (September to November), and individuals died in summer (July) and winter (January) (Thonig *et al.* 2016). Similar recruitment times in spring and fall have been reported for other populations of *P. elegans* (Gudmundsson 1985, Morgan 1997, Bolam 2004). Moreover, the second size cohort at Lammefjord and Herslev as well as the third size cohort at Lynæs was composed of genetically different individuals compared to the other cohorts at this site (Fig. 3). Since we were unable to distinguish size cohorts at Vellerup, we cannot say whether the observed seasonal genetic switch represents different size cohorts, but we assume this to be the case.

The occurrence of genetically distinct clusters in Lammefjord and Vellerup, correlated with arrival of new recruits and expected seasonal reproductive periods, suggests that these individuals immigrated from a genetically differentiated, but unknown, source population. Considering the structure of the estuary, the source of the recruits might be located within Isefjord, in close proximity to Lammefjord and Vellerup. It is also possible that larvae immigrated from the Kattegat, outside the estuary. Although the recruiting individuals were assigned to the same genetic cluster, it is important to keep in mind that they might not have originated from the same source population, since population structure in this estuarine system is known to be patchy (Kesaniemi *et al.* 2014a). Kesäniemi and colleagues (2014a) also included samples from Lammefjord and Vellerup in their broader spatial study of samples collected at a single time point (April 2010), but in that study, the two populations were assigned to different genetic clusters. Our analysis indicates some differentiation between the populations in spring as well, despite their genetic similarities in the fall. At least in March, the sample from Vellerup was assigned to cluster 1 while the sample from Lammefjord was assigned to cluster

2, but in May, individuals from both populations were primarily grouped in cluster 2. In contrast to what was observed at Lammefjord and Vellerup, we could not detect any seasonal genetic change at Lynæs and Herslev in the structure analysis, indicating that the majority of new recruits at these locations did not originate from differentiated populations or are the result of self-recruitment. However, the presence of some immigrants belonging to the third genetic cluster at these two sites might also reflect pre-settlement selection due to dispersal limitation or missing habitat cues.

Along with the seasonal genetic change noted for populations at Lammefjord and Vellerup, we observed that the reproductive individuals also show a genetic change at these sites, and surprisingly, also at Lynæs, where population-level seasonal variation was not detected. Gamete-bearing individuals were assigned primarily to genetic clusters 1 and 2 in winter to spring, but assigned primarily to cluster 3 in fall, and persisting partly in winter. This pattern correlates with the two peaks of gravid females and ripe males, in September/October and in January/February that we observed at these sites (Thonig *et al.* 2016). In contrast, only a single peak of individuals with gametes was noted at Herslev, and here individuals were primarily assigned to cluster 1 during the whole period. Hereby, the genetic change in females is particularly of interest since they can store sperm in *receptacula seminis* and so, the contribution of ripe males to the next generation is not clear.

In our previous study (Thonig *et al.* 2016), we examined egg strings produced in these populations in order to determine larval developmental mode, and found that at Lammefjord, Lynæs, and Vellerup, planktonic larvae were produced primarily from November to February, but benthic and intermediate larvae were produced primarily from February to June. At Herslev, only benthic and intermediate larvae were predominant throughout the reproductive period (November to May). Seasonal switches in developmental mode that we observed (Thonig *et al.* 2016) have also been noted by others (Rasmussen 1973 and Gudmundsson 1985) and might indicate asynchronous local population dynamics (isolation by time) where gene flow is restricted due to reproductive season (Hendry & Day 2005, Eldon *et al.* 2016). Such dynamics were noted for the polychaete *Pectinaria koreni* in Baie de Seine (Jolly *et al.* 2014). With asynchronous population dynamics we would expect to observe genetic changes between seasons, but not years (Hendry & Day 2005). The results of this study show seasonal genetic change at some sites, but not others. Although our sampling did not cover multiple years, Kesäniemi and colleagues (2014b) observed temporal genetic change at Vellerup between spring 2009 and spring 2010, but no genetic change between fall 2008 and spring 2009/2010, in other words, annual but not seasonal genetic change. Together, these results highlight temporal variation with no clear pattern that even can differ among geographically close populations.

Chaotic genetic patchiness

Although we analysed populations at only four sites located in close proximity in the same estuary, we observed different genetic clusters and different seasonal dynamics among them, confirming chaotic genetic patchiness (CGP) among *P. elegans* in the Isefjord-Roskilde-Fjord estuary complex as reported by Kesäniemi *et al.* (2014a). Chaotic genetic patchiness describes spatial genetic structure with high temporal turnover at a scale where dispersal should be able to efficiently homogenize genetic variation (Eldon **et al.** 2016). One likely mechanism of CGP is sweepstakes reproductive success (SRS), the variance in reproductive success of highly fecund marine organisms and unequal contributions to the future reproductive population due to the high degree of stochasticity of oceanographic processes, spawning success and fates of planktonic larvae (Hedgecock 1994, Hedgecock & Pudovkin 2011, Broquet *et al.* 2013, Cornwell *et al.* 2016). Although the reproductive biology of *P. elegans* does not match that of species for which SRS is described originally, our analysis indicates that reproductive individuals are not necessarily a random subset of the population: genetic cluster 2 is present at all sites, but these individuals do not contribute to reproduction in respective proportions. Moreover, at Vellerup and Herslev max. 40-60 % of the individuals carry gametes while only 20-40 % of the population are reproductive at Lammefjord and Lynæs (Thonig *et al.* 2016). Hence, SRS might be more likely at the latter sites, in particular at Lynæs, where reproducing individuals do not belong to the dominant genetic cluster. The effect of SRS can be counter-balanced via larval dispersal that redistributes genetic variation between locations (Eldon *et al.* 2016). However, if larvae from different populations are not well-mixed, but instead disperse together with others from the same cohort, termed collective dispersal, the effect of genetic drift due to small effective population size will be maintained (Broquet *et al.* 2013, Eldon *et al.* 2016). Our observations of a change in the predominate genetic cluster with the appearance of a new size cohort suggest that collective dispersal could occur, but additional study of larval cohorts and their genetics is needed to support this hypothesis. The short life span of *P. elegans* and its seasonal reproduction also likely enhance the consequences of SRS.

Diversifying selection is another mechanism that can cause CGP in unstable and patchy environments (Eldon *et al.* 2016). Therefore, poecilogony of *P. elegans* alone (without SRS or collective dispersal) might explain the patterns of chaotic genetic patchiness we observed in the Isefjord-Roskilde-Fjord estuary complex. Heterogeneous environments can promote the evolution of phenotypic polymorphisms, depending on the accuracy of both genetic and environmental cues that influence development of the phenotype (Leimar 2009) and are expected to favour the evolution of poecilogony (Chia *et al.* 1996). For example, when environmental heterogeneity is unpredictable, diversifying bet-hedging within cohorts might explain observed variation in developmental mode (Krug 2009). In this and our previous study, the environmental parameters explaining best the population genetic structure and population dynamics, respectively, were temperature, sediment grain size correlating with

sorting, and mean salinity (Thonig *et al*. 2016) and these variables likely describe different aspects of the environmental heterogeneity. Temperature reflects the seasonal changes in reproductive activity and population genetic structure observed in Lammefjord and Vellerup. However, the populations that did not show seasonal genetic changes also experienced seasonal fluctuations in temperature. The coarse and poorly sorted sediment at Vellerup and Herslev, was inhabited by large specimen of *P. elegans* and populations showed high densities as well as a high percentage of gamete-bearing individuals. These populations were additionally the ones where most individuals were assigned to the first genetic cluster (38 % at Vellerup, 55 % at Herslev). In contrast, at Lynæs and Lammefjord the sediment was fine and well-sorted, specimens were smaller, occurred in lower densities and had higher percentage of asexual reproduction. These populations were dominated by the second genetic cluster (57 % at Lynæs, 47 % at Lammefjord). Salinity was only lower at Herslev (~15 PSU) compared to the other sites (~ 20 PSU). Herslev was also the only site where no switch in developmental mode and genetic composition of reproductive individuals was observed. Other environmental variables not measured here might be more effective in explaining the patterns of genetic differentiation we observed here.

Zakas & Hall (2012) proposed dispersal polymorphism in *S. benedicti* between similar sized patches is maintained due to asymmetric dispersal, as in typical source-sink metapopulations. Source-sink metapopulations are composed of several subpopulations with heterogeneous habitat quality. Demographic excess in high quality habitats (sources) can lead to emigration, while low quality habitats (sinks) with a demographic deficit might not persist without immigration, can go extinct and be re-colonized (Dias 1996). If such is the case also for *P. elegans,* the different population dynamics we observed suggest that Herslev and Vellerup would be characterized as source subpopulations, while Lynæs and Lammefjord would be sinks, according to criteria described by Jolly *et al.* (2014). However, DeMeester *et al.* (2002) and Jolly *et al.* (2014) proposed that genetic heterogeneity and temporal change is high and allelic richness low in sink populations and vice versa in sources. This is only partly the case for populations in our study. Hence, the heterogeneous, unpredictable character of the estuary and metapopulation dynamics might maintain poecilogony in *P. elegans* as a bet-hedging strategy in the Isefjord-Roskilde-Fjord estuary complex in comparison to other sites where *P. elegans* are expected to be fixed to a certain mode of development (Morgan *et al* 1999, Bolam 2004).

CONCLUSION

We found spatial and seasonal population genetic structure of *Pygospio elegans* in the Danish Isefjord-Roskilde-Fjord estuary complex, but seasonal genetic

structure varied among the four study sites. When present, the seasonal genetic switch correlated with the arrival of new size cohorts. Phenotypic variation in larval developmental mode of *P. elegans* contributes to patterns of chaotic genetic patchiness observed in the estuary metapopulation. We found that the genotypes of individuals bearing gametes did not resemble the genotypes of the whole sample, indicating a possibility for variance in reproductive success. However, the genetics of larval cohorts and the effects of pre- and post-larval settlement on the population genetics are yet to be determined. Diversifying selection could lead to poecilogony in *P. elegans* as a bet-hedging strategy to allow persistence in the unpredictable estuarine environment resulting in chaotic genetic patchiness among populations.

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DATA ACCESSIBILITY

Microsatellite loci described in this study are available in GenBank under accession numbers MG021816-MG021822, GU321899, GU321900 and GU321906. Genotype and phenotype data for Pygospio elegans analysed in this study are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.9c5s0

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TABLES

TABLE 1 Microsatellite loci, repeat found in reference sequence, the primers used for amplification, and GenBank Accession number. Loci marked with an asterisk were discarded from the study because they showed a high estimated null allele frequency. The number of alleles and size-range observed in this study are shown. Loci Pe6, Pe7 and Pe19 were described in Kesäniemi *et al.* (2012a). The loci were grouped into two multiplex panels: Multiplex 1 contained Loci Pe307, Pe309, Pe385, Pe6 and Pe7; Multiplex 2 contained Loci Pe19, Pe159, Pe234, Pe294 and Pe369.

Locus Name	Repeat sequence	Primer sequences	GenBank Accession Number	No. of Alleles	Size Range (bp)
Pe307	(TG)6	F: AGCTAAATCTTGACACTGGCCT R: GAAGTCAGCCATCTTGGATTCT	MG021816	12	181-202
Pe309*	(ATG)8	F: CCAGAGGAAATGATGTAGGCTC R: ATTCACACTTGACCATGACCAC	MG021817	11	377-402
Pe385	(GGT)8	F: TCAATAGGAGAAGCACAACGAA R: CGCTGGTTATTTTAGGGATGAG	MG021818	13	392-430
Pe6	(CA)28	F: ACTACGGAAACTGCCTGCAC R: ATATGGCCACCGAAACCTCT	GU321899	6	265-287
Pe7*	(CATA)13	F: CTCACCCTTTACACCCAAGG R: AGCGTCTGTTATGGGGTACAG	GU321900	38	124-255
Pe19	(GA)23	F: TATCCAACGCACACCTACCA R: TTGAGTGATGGTGCGAGGTA	GU321906	13	214-285
Pe159*	(GT)10	F: TTGGTTTGAGCAATGTGGAA R: GCCCTTTGCACTCATTGTTT	MG021819	35	184-255
Pe234	(AG)6AA (AG)4	F: AGCAGTAAAAGCGGATCACAAC R: TGTCTCTGGCGTAATTTTCTCA	MG021820	5	374-384
Pe294	(AG)5	F: AGTGGGTGTGTGAGAAGAGC R: AGTTGAGCCGTGATACAAAATC	MG021821	5	231-239
Pe369	(GT)8	F: CTTTCTTCCCCAAGGCTTCT R: TTTCTCACCCTCCTGACCTG	MG021829	17	190-227

TABLE 2 Genetic diversity for each sample. Expected and observed heterozygosity (He and Ho), gene diversity, and inbreeding coefficient (F_{IS}) were calculated using Arlequin v.3.5.2. F_{IS} values with a p-value smaller than 0.05 are indicated with *. Allelic richness and number of private alleles were determined with HP-Rare v1.1. Relatedness was calculated using Coancestry v.1. N – number of individuals per sample.

Sample	N	H_e	H_o	Gene diversity	F_{IS}	Allelic richness (N=26)	Private alleles (N=26)	Mean relatedness
Lynæs								
Mar	35	0.313	0.281	0.222	0.099	2.54	0	0.393
May	36	0.322	0.268	0.263	0.143*	3.29	0.1	0.226
Aug	29	0.315	0.283	0.239	0.077	4.02	0.01	0.246
Oct	35	0.364	0.310	0.364	0.150*	4.16	0.11	0.199
Nov	38	0.337	0.320	0.289	0.051	2.86	0.1	0.311
Feb	29	0.339	0.345	0.338	-0.021	3.34	0.0	0.231
Lammefjord								
Mar	31	0.330	0.347	0.276	-0.068	2.78	0	0.287
May	44	0.319	0.320	0.264	-0.022	3.07	0	0.344
Aug	41	0.416	0.348	0.416	0.165*	5.71	0.21	0.131
Oct	30	0.414	0.346	0.351	0.142*	5.2	0.13	0.119
Nov	40	0.335	0.291	0.321	0.112*	4.33	0	0.184
Feb	40	0.371	0.301	0.366	0.183*	4.42	0.11	0.194
Vellerup								
Mar	32	0.355	0.372	0.300	-0.056	2.52		0.297
May	37	0.330	0.251	0.299	0.226*	3.75	0.33	0.231
Aug	27	0.400	0.349	0.388	0.115*	5.51	0.37	0.147
Oct	37	0.427	0.344	0.415	0.182*	5.65	0.58	0.140
Nov	33	0.333	0.262	0.276	0.201*	3.64	0	0.295
Feb	39	0.321	0.313	0.316	0.014	3.04	0	0.245
Herslev								
Mar	41	0.318	0.280	0.314	0.112*	2.85	0.04	0.287
May	37	0.369	0.330	0.192	0.085	2.5	0.07	0.320
Aug	34	0.429	0.421	0.358	0.003	3.41	0.03	0.216
Oct	30	0.400	0.341	0.393	0.141*	3.89	0.06	0.189
Nov	43	0.336	0.309	0.283	0.072	3.09	0.3	0.272
Feb	37	0.350	0.352	0.284	-0.025	3.22	0	0.202

TABLE 3 Analysis of molecular variance (AMOVA) of temporal and spatial groups of samples performed with Arlequin v.3.5.2.

Source of variation	df	Sum of squares	Variance components	Percentage of variation	p-value
Spatial groups (across time)					
Among locations	3	39.073	0.02349	1.99	< 0.0001
Among time points within locations	20	59.26	0.02425	2.05	< 0.0001
Among individuals within sample	831	1030.743	0.10527	8.9	< 0.0001
Among loci within individuals	855	880.5	1.02982	87.06	< 0.0001
Temporal groups (across locations)					
Among time points	5	28.308	0.006	0.51	0.11517
Among locations within time points	18	70.025	0.03735	3.17	< 0.0001
Among individuals within sample	831	1030.743	0.10527	8.93	< 0.0001
Among loci within individuals	855	880.5	1.02982	87.38	< 0.0001

FIGURES

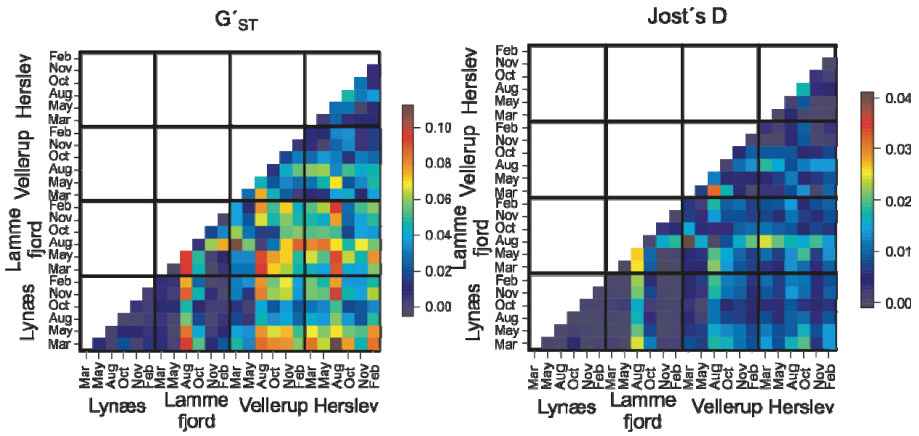


FIGURE 1 Heatmap illustrating the differentiation between the sampled populations using the fixation index G'_{ST} (panel A) and Jost´s D (panel B) calculated with diversity, ranging from genetically similar populations in blue (0.00) to genetically differentiated populations in red (up to 0.1). The respective confidence intervals can be found in Supplementary Table S1.

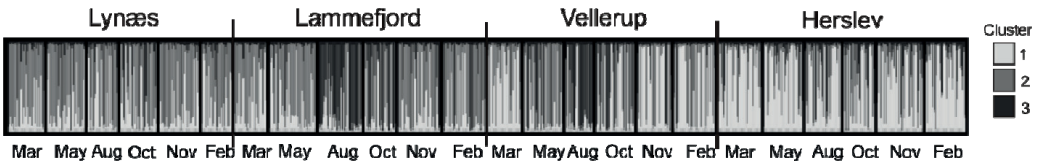


FIGURE 2 Assignment of sampled individuals to different genetic clusters as determined with the program Structure for K=3 clusters. Each line represents one individual sampled from four locations at six different months in chronological order from left to right. The colour of the line describes the membership of that individual to the three respective clusters. Cluster 1 (light grey) was composed of samples from Herslev in Mar, May, *Aug*, *Oct*, Nov, *Feb;* and from Vellerup in Mar, Nov, *Feb*. Cluster 2 (grey) was composed of samples from Lynæs in Mar, *May, Aug, Oct,* Nov, *Feb*; from Lammefjord in *Mar*, May, *Nov*, Feb; and from Vellerup in *May*. Cluster 3 (dark grey) was composed of samples from Lammefjord in Aug, *Oct*; and from Vellerup in Aug, *Oct*. If the average membership in one cluster for a sample was less than 60 %, the sample is listed in italic.

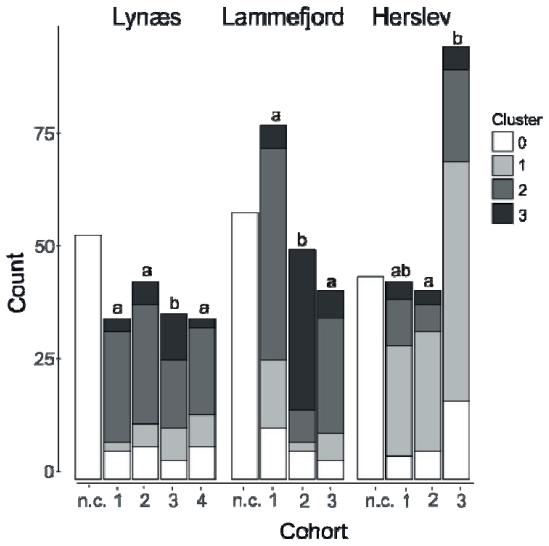


FIGURE 3 Assignment of individuals to different size cohorts (according to Thonig *et al.* 2016) and genetic clusters (1, 2, or 3) as determined with Structure when membership coefficient was larger than 60 %. Cluster 0 indicates individuals that could not be assigned to a distinct genetic cluster (membership coefficient was less than 60 %). Size cohort is listed in the x axis, n.c. groups those individuals that could not be assigned to a size cohort because of overlapping size ranges and for that reason were not assigned to a genetic cluster. No size cohorts could be distinguished at Vellerup, hence it was excluded from this graph. Genetic differentiation between cohorts within site was tested using G'_{ST} and are indicated with lower case letters. The respective values and confidence intervals can be found in Supplementary Table S2.

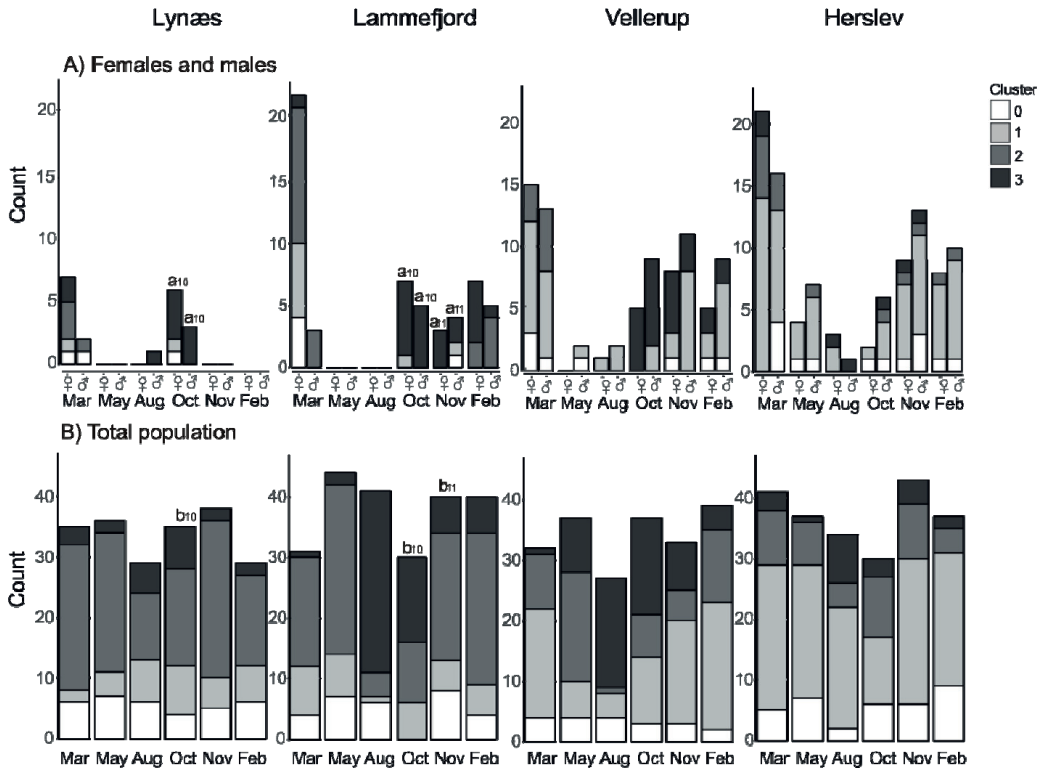


FIGURE 4 Assignment of females and males (A) as well as the total population (B) to the three genetic clusters (1, 2, or 3) determined with Structure when membership coefficient was larger than 60 %, at each time point and site. Females and males were identified by the presence of eggs or sperm in their coelom, respectively. Cluster 0 indicates individuals that could not be assigned to a distinct genetic cluster (membership coefficient was less than 60 %). Genetic differentiation among females, males and the total sample was tested using G'_{ST} and significant differences are indicated with lower case letters. No statistics could be calculated when less than two individuals were in one sample or when there were less than three samples per comparison. Hence, no results are available for within sample comparisons for Lynæs except in October, for Lammefjord and Vellerup in May and August and for Herslev in August. The respective values and confidence intervals can be found in Supplementary Tables S3 and S4.

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLE S1 Population differentiation estimated with G'_{ST} and Jost´s D and their respective confidence intervals (CI) calculated with the R package diveRsity. Values are significant when 0 is not included in the CI and are displayed in bold.

	G'_{ST}			Jost´s D		
Populations	value	lower CI	upper CI	value	lower CI	upper CI
H03 vs H05	0.0026	-0.0175	0.0297	0	-0.0019	0.0035
H03 vs H08	**0.0262**	0.0081	0.0489	0.0037	-0.0035	0.0104
H03 vs H10	0.0076	-0.0047	0.0218	0.0007	-0.0035	0.0064
H03 vs H11	0.0108	-0.001	0.0286	0.0001	-0.0025	0.0027
H03 vs H02	0.0102	-0.0035	0.0386	0	-0.0033	0.0046
H03 vs L03	**0.0437**	0.0189	0.0719	**0.0062**	0.0026	0.0113
H03 vs L05	**0.0663**	0.0418	0.0944	**0.0107**	0.0068	0.0162
H03 vs L08	**0.0802**	0.0496	0.0979	**0.0242**	0.014	0.0337
H03 vs L10	**0.0529**	0.0258	0.0727	**0.011**	0.0041	0.0151
H03 vs L11	**0.0474**	0.024	0.0663	**0.0074**	0.0034	0.0131
H03 vs L02	**0.0522**	0.0316	0.0719	**0.0076**	0.0041	0.0104
H03 vs U03	**0.0832**	0.0558	0.1134	**0.0141**	0.0093	0.0217
H03 vs U05	**0.067**	0.0477	0.0882	**0.0116**	0.0067	0.018
H03 vs U08	**0.0425**	0.017	0.0904	**0.0081**	0.0033	0.0133
H03 vs U10	**0.0283**	0.0118	0.0553	**0.0054**	0.0009	0.0105
H03 vs U11	**0.0573**	0.0385	0.0959	**0.0088**	0.0058	0.0116
H03 vs U02	**0.0571**	0.0255	0.0745	**0.0105**	0.0037	0.0173
H03 vs U02	0.0134	-0.001	0.0346	0.0026	-0.0021	0.0093
H03 vs V05	**0.0295**	0.0052	0.0717	0.0049	0	0.0155
H03 vs V08	**0.059**	0.0263	0.0822	**0.0185**	0.0011	0.0335
H03 vs V10	0.0275	-0.0029	0.044	0.0088	-0.0034	0.0175
H03 vs V11	0.0068	-0.0042	0.0215	0.0001	-0.0026	0.0039
H03 vs V02	0.0128	-0.0061	0.0357	0.0024	-0.0012	0.0067
H05 vs H08	**0.0264**	0.0134	0.0494	0.0016	-0.0036	0.0083
H05 vs H10	0.0297	-0.0005	0.0824	**0.0089**	0.0004	0.0205
H05 vs H11	-0.0012	-0.0155	0.0062	0	-0.0024	0.0017
H05 vs H02	0.0124	-0.0002	0.0367	0.0002	-0.0035	0.0081
H05 vs L03	**0.0382**	0.0149	0.0724	**0.0046**	0.0008	0.0098
H05 vs L05	**0.0527**	0.0363	0.0761	**0.0064**	0.0037	0.0114
H05 vs L08	**0.0844**	0.0569	0.1232	**0.0206**	0.0115	0.0383
H05 vs L10	**0.0551**	0.0238	0.0852	**0.0091**	0.0031	0.0167
H05 vs L11	**0.0416**	0.0278	0.0679	**0.0052**	0.0022	0.0121
H05 vs L02	**0.0617**	0.0425	0.0777	**0.0084**	0.0052	0.0108
H05 vs U03	**0.064**	0.047	0.0969	**0.0077**	0.0054	0.0136
H05 vs U05	**0.058**	0.0223	0.0903	**0.0077**	0.0037	0.0112

H05 vs U08	**0.0364**	0.0043	0.0738	**0.0051**	0.0006	0.0111
H05 vs U10	**0.0326**	0.0103	0.0473	**0.0051**	0.001	0.0101
H05 vs U11	**0.0545**	0.0394	0.0698	**0.0065**	0.0049	0.0079
H05 vs U02	**0.0478**	0.0285	0.0771	**0.0066**	0.0015	0.0126
H05 vs U02	0.0149	-0.0006	0.0357	0.003	-0.0011	0.0106
H05 vs V05	**0.0451**	0.0219	0.0747	**0.0068**	0.0033	0.0116
H05 vs V08	**0.0608**	0.02	0.1064	**0.0153**	0.0012	0.0357
H05 vs V10	**0.0371**	0.0169	0.053	**0.0089**	0.0029	0.0154
H05 vs V11	0.0002	-0.0142	0.0174	0	-0.0018	0.0032
H05 vs V02	**0.0113**	0.0014	0.0356	0.0013	-0.0008	0.0045
H08 vs H10	**0.0467**	0.0243	0.0808	**0.0168**	0.0062	0.0331
H08 vs H11	**0.0308**	0.0107	0.0478	0.0031	-0.0037	0.0116
H08 vs H02	**0.0367**	0.011	0.0539	0.0043	-0.0086	0.0141
H08 vs L03	**0.0888**	0.0522	0.1199	**0.0132**	0.0062	0.02
H08 vs L05	**0.0921**	0.0712	0.1184	**0.0132**	0.0086	0.0225
H08 vs L08	**0.0687**	0.0382	0.1066	**0.0172**	0.0102	0.0247
H08 vs L10	**0.0502**	0.0208	0.0837	**0.0084**	0.0037	0.0124
H08 vs L11	**0.0761**	0.0432	0.1075	**0.0112**	0.0062	0.0168
H08 vs L02	**0.094**	0.0597	0.1268	**0.0139**	0.009	0.0194
H08 vs U03	**0.1116**	0.0897	0.1386	**0.0165**	0.0125	0.0244
H08 vs U05	**0.0701**	0.0366	0.1039	**0.009**	0.0047	0.0129
H08 vs U08	**0.059**	0.0252	0.0896	**0.0093**	0.0038	0.0161
H08 vs U10	**0.0519**	0.0284	0.0728	**0.0087**	0.0041	0.0151
H08 vs U11	**0.0985**	0.0629	0.1334	**0.0141**	0.0086	0.0202
H08 vs U02	**0.0771**	0.05	0.1053	**0.0119**	0.0059	0.0189
H08 vs U02	**0.0373**	0.0114	0.067	0.0077	-0.0011	0.0208
H08 vs V05	**0.0685**	0.0488	0.0861	**0.0116**	0.0092	0.0141
H08 vs V08	**0.0331**	0.0056	0.0568	**0.008**	0.0002	0.0162
H08 vs V10	**0.031**	0.0114	0.0505	**0.0089**	0.0023	0.0145
H08 vs V11	**0.0261**	0.0094	0.0465	0.0039	-0.0034	0.0119
H08 vs V02	**0.0329**	0.0058	0.0638	0.0052	-0.0003	0.0128
H10 vs H11	**0.0292**	0.0013	0.0632	0.0027	-0.0008	0.0089
H10 vs H02	0.0077	-0.0128	0.0267	0.0011	-0.0031	0.0072
H10 vs L03	0.0395	-0.003	0.0805	0.0104	-0.0013	0.0191
H10 vs L05	**0.0637**	0.0098	0.0966	**0.0191**	0.003	0.0284
H10 vs L08	**0.0479**	0.0104	0.116	**0.0151**	0.0035	0.0418
H10 vs L10	0.0182	-0.0302	0.0663	0.0046	-0.0086	0.0191
H10 vs L11	0.026	-0.008	0.0556	0.0063	-0.0029	0.0189
H10 vs L02	0.027	-0.0077	0.0583	0.0047	-0.0047	0.0127
H10 vs U03	**0.0769**	0.0224	0.112	**0.02**	0.0057	0.0306
H10 vs U05	**0.0567**	0.0033	0.1016	**0.0165**	0.0001	0.0332
H10 vs U08	**0.0358**	0.0025	0.0855	**0.0107**	0.0016	0.0234
H10 vs U10	0.0174	-0.0119	0.0519	0.006	-0.0021	0.0147
H10 vs U11	**0.0451**	0.0098	0.0843	**0.0106**	0.0014	0.0197
H10 vs U02	0.037	-0.0104	0.0759	0.0111	-0.0008	0.022

H10 vs U02	**0.0416**	0.0172	0.0663	**0.0129**	0.0024	0.0241
H10 vs V05	0.0222	-0.0166	0.063	0.0043	-0.0053	0.0164
H10 vs V08	**0.0507**	0.0118	0.0917	0.0117	0	0.0204
H10 vs V10	0.0143	-0.0123	0.0406	0.0048	-0.0054	0.0118
H10 vs V11	**0.0323**	0.0018	0.0683	**0.0089**	0.0006	0.0187
H10 vs V02	**0.0353**	0.0069	0.0704	**0.0095**	0.0037	0.0174
H11 vs H02	0.0069	-0.0107	0.0186	0	-0.0032	0.0046
H11 vs L03	**0.0378**	0.0102	0.0715	**0.0055**	0.001	0.0124
H11 vs L05	**0.0453**	0.021	0.0879	**0.0063**	0.0026	0.0139
H11 vs L08	**0.0668**	0.0542	0.0812	**0.0188**	0.013	0.0235
H11 vs L10	**0.0384**	0.012	0.054	**0.007**	0.0011	0.0115
H11 vs L11	**0.0378**	0.0191	0.0607	**0.0056**	0.0027	0.0093
H11 vs L02	**0.0505**	0.0268	0.0709	**0.0081**	0.0041	0.0139
H11 vs U03	**0.0448**	0.0211	0.0998	**0.0059**	0.0019	0.0181
H11 vs U05	**0.0522**	0.0177	0.0867	**0.0077**	0.0019	0.016
H11 vs U08	**0.0216**	0.0034	0.0383	**0.0035**	0.0005	0.0063
H11 vs U10	**0.0213**	0.0044	0.0328	0.0037	0	0.0072
H11 vs U11	**0.0387**	0.0125	0.0638	**0.0053**	0.0022	0.0098
H11 vs U02	**0.0358**	0.0137	0.0736	**0.0055**	0.0012	0.0133
H11 vs U02	0.0108	-0.0088	0.0301	0.001	-0.0046	0.0079
H11 vs V05	**0.0327**	0.0172	0.0564	**0.0059**	0.0029	0.0099
H11 vs V08	**0.0425**	0.0033	0.0701	0.0135	-0.0005	0.0235
H11 vs V10	**0.0301**	0.0177	0.0442	0.0051	-0.0022	0.0101
H11 vs V11	0.0058	-0.0008	0.0131	0.0008	-0.001	0.0027
H11 vs V02	0.0055	-0.0087	0.0217	0.0008	-0.0017	0.0032
H02 vs L03	**0.0575**	0.0158	0.093	**0.0091**	0.002	0.0201
H02 vs L05	**0.0784**	0.0524	0.101	**0.0131**	0.0092	0.0169
H02 vs L08	**0.0574**	0.0297	0.0873	**0.0138**	0.0055	0.0211
H02 vs L10	**0.0362**	0.0195	0.0666	**0.0062**	0.0025	0.0122
H02 vs L11	**0.0501**	0.0313	0.0683	**0.0078**	0.004	0.0128
H02 vs L02	**0.0584**	0.0322	0.0794	**0.0091**	0.0037	0.0135
H02 vs U03	**0.0832**	0.063	0.1038	**0.0136**	0.0104	0.0206
H02 vs U05	**0.0784**	0.0547	0.1001	**0.0138**	0.0081	0.0184
H02 vs U08	**0.0485**	0.0111	0.0967	**0.0087**	0.0016	0.0151
H02 vs U10	**0.0319**	0.0142	0.0562	**0.0061**	0.0029	0.0103
H02 vs U11	**0.0617**	0.0472	0.0852	**0.0093**	0.0066	0.0138
H02 vs U02	**0.0482**	0.0298	0.0689	**0.0082**	0.0047	0.0149
H02 vs U02	**0.0376**	0.0216	0.0612	**0.01**	0.0028	0.0178
H02 vs V05	**0.0466**	0.0247	0.0696	**0.0083**	0.0039	0.0164
H02 vs V08	**0.0464**	0.0248	0.0747	**0.0138**	0.0071	0.0228
H02 vs V10	**0.0245**	0.0132	0.039	**0.0071**	0.0015	0.0158
H02 vs V11	**0.016**	0.0075	0.0342	**0.0014**	-0.0022	0.0065
H02 vs V02	**0.0164**	0.0005	0.0433	**0.0029**	-0.0008	0.0075
L03 vs L05	-0.004	-0.0121	0.0107	-0.0006	-0.0013	0.0014
L03 vs L08	**0.0924**	0.0539	0.1206	**0.0258**	0.0142	0.0392

L03 vs L10	**0.0459**	0.0114	0.0742	**0.0092**	0.0022	0.023
L03 vs L11	-0.0036	-0.015	0.0125	-0.0006	-0.0022	0.0029
L03 vs L02	0.005	-0.0134	0.0194	**0.0003**	-0.0038	0.0044
L03 vs U03	0.0119	-0.0103	0.037	**0.0013**	-0.0022	0.0068
L03 vs U05	0.0063	-0.0038	0.0185	0	-0.0022	0.0029
L03 vs U08	0.006	-0.016	0.0502	**0.0007**	-0.003	0.0074
L03 vs U10	0.0102	-0.0066	0.0373	**0.0013**	-0.0023	0.0071
L03 vs U11	0.0072	-0.0116	0.0437	**0.0008**	-0.0025	0.0077
L03 vs U02	0.0065	-0.0062	0.0283	0	-0.0025	0.0054
L03 vs U02	**0.0294**	0.0132	0.046	**0.0043**	0.0008	0.0086
L03 vs V05	**0.0294**	0.0157	0.051	**0.0043**	0.0013	0.0085
L03 vs V08	**0.094**	0.0346	0.1324	**0.0216**	0.0027	0.0395
L03 vs V10	**0.0709**	0.0412	0.0919	**0.0136**	0.008	0.0169
L03 vs V11	**0.0625**	0.0255	0.1094	**0.0089**	0.0043	0.018
L03 vs V02	**0.0459**	0.0217	0.0725	**0.0063**	0.0033	0.0103
L05 vs L08	**0.0978**	0.0608	0.1299	**0.0261**	0.0133	0.0395
L05 vs L10	**0.0491**	0.0244	0.0784	**0.0101**	0.0038	0.0162
L05 vs L11	0.0018	-0.0052	0.0091	0	-0.0018	0.0015
L05 vs L02	0.0098	-0.0106	0.0262	0.0006	-0.0039	0.0039
L05 vs U03	0.0007	-0.0111	0.0152	0	-0.0016	0.0046
L05 vs U05	-0.0013	-0.0111	0.0198	-0.0001	-0.0018	0.0038
L05 vs U08	-0.0009	-0.017	0.0255	0	-0.0022	0.0034
L05 vs U10	0.0133	-0.0016	0.0332	0.0021	-0.0009	0.0072
L05 vs U11	0.0037	-0.013	0.028	0.0003	-0.0021	0.0048
L05 vs U02	0.0055	-0.0076	0.0172	0	-0.0021	0.0032
L05 vs U02	**0.0319**	0.0175	0.0588	**0.0047**	0.002	0.0087
L05 vs V05	**0.0329**	0.0082	0.0543	**0.0057**	0.0021	0.0096
L05 vs V08	**0.0921**	0.0524	0.1244	**0.02**	0.0062	0.0351
L05 vs V10	**0.0765**	0.0465	0.1052	**0.0151**	0.0087	0.0216
L05 vs V11	**0.0719**	0.0304	0.1054	**0.0101**	0.005	0.0139
L05 vs V02	**0.0493**	0.0246	0.0673	**0.0067**	0.0043	0.0086
L08 vs L10	0.0043	-0.0182	0.0294	0	-0.0048	0.0059
L08 vs L11	**0.0597**	0.0429	0.0757	**0.0157**	0.0087	0.0269
L08 vs L02	**0.0747**	0.0447	0.1071	**0.0184**	0.012	0.0268
L08 vs U03	**0.0932**	0.0622	0.1235	**0.0245**	0.0111	0.0363
L08 vs U05	**0.084**	0.0396	0.1384	**0.0207**	0.0127	0.041
L08 vs U08	**0.0568**	0.0177	0.1	**0.0166**	0.0085	0.0283
L08 vs U10	**0.0465**	0.0168	0.0892	**0.0155**	0.0061	0.0303
L08 vs U11	**0.0878**	0.0587	0.1082	**0.0212**	0.0114	0.0314
L08 vs U02	**0.0756**	0.0499	0.0986	**0.0223**	0.01	0.0342
L08 vs U02	**0.1077**	0.0634	0.1406	**0.0407**	0.0212	0.0597
L08 vs V05	**0.0559**	0.03	0.106	**0.016**	0.0085	0.0277
L08 vs V08	0.0022	-0.0086	0.0271	0.0003	-0.0037	0.0083
L08 vs V10	**0.0293**	0.0109	0.0537	**0.0082**	0.0002	0.0203
L08 vs V11	**0.0705**	0.0525	0.0965	**0.0157**	0.0102	0.0212

L08 vs V02	**0.0879**	0.0612	0.1328	**0.0206**	0.0124	0.0323
L10 vs L11	**0.0175**	0.0004	0.035	0.0026	-0.0016	0.0095
L10 vs L02	**0.0321**	0.0081	0.07	**0.0052**	0.0009	0.0121
L10 vs U03	**0.0531**	0.012	0.0847	**0.0116**	0.0021	0.0194
L10 vs U05	0.0393	-0.0041	0.0962	0.007	-0.0018	0.0151
L10 vs U08	0.0181	-0.0097	0.0501	0.0035	-0.0018	0.0075
L10 vs U10	0.0098	-0.01	0.0346	0.0017	-0.0019	0.0071
L10 vs U11	**0.0447**	0.0175	0.0775	**0.0074**	0.0033	0.0127
L10 vs U02	0.0256	-0.0014	0.052	0.0052	-0.0014	0.0136
L10 vs U02	**0.0604**	0.0316	0.0822	**0.0154**	0.0051	0.0231
L10 vs V05	**0.0289**	0.0054	0.0541	**0.0051**	0.0012	0.0085
L10 vs V08	0.0164	-0.0065	0.0383	0.0033	-0.0033	0.0106
L10 vs V10	0.0167	-0.0015	0.0322	0.0031	0	0.0078
L10 vs V11	**0.0547**	0.0421	0.071	**0.0091**	0.0077	0.0105
L10 vs V02	**0.0591**	0.0307	0.0901	**0.0104**	0.0057	0.0153
L11 vs L02	0.0001	-0.0159	0.0139	0.0001	-0.0023	0.0022
L11 vs U03	0.013	-0.0051	0.036	0.0016	-0.0022	0.0064
L11 vs U05	0.0036	-0.01	0.0344	0	-0.0033	0.0111
L11 vs U08	-0.0006	-0.0319	0.0428	0	-0.0049	0.0061
L11 vs U10	0.0033	-0.0122	0.018	0.0005	-0.0021	0.0033
L11 vs U11	0.0066	-0.0108	0.0401	0.0007	-0.0015	0.0042
L11 vs U02	-0.0016	-0.0148	0.0158	-0.0002	-0.0025	0.0058
L11 vs U02	**0.0385**	0.0088	0.062	0.0065	-0.0002	0.0172
L11 vs V05	0.0219	-0.0021	0.0619	0.0031	-0.0003	0.01
L11 vs V08	**0.0663**	0.027	0.0908	**0.0141**	0.0037	0.0219
L11 vs V10	**0.0467**	0.0119	0.0802	**0.0079**	0.0018	0.0148
L11 vs V11	**0.0582**	0.0369	0.0755	**0.0082**	0.0062	0.0108
L11 vs V02	**0.0478**	0.0205	0.0724	**0.0068**	0.0039	0.0102
L02 vs U03	**0.0213**	0.0028	0.0448	0.0012	-0.0029	0.0062
L02 vs U05	**0.0096**	0.001	0.0146	0.0007	-0.002	0.0034
L02 vs U08	0.0046	-0.0143	0.0336	0.0006	-0.0037	0.009
L02 vs U10	0.003	-0.0071	0.0171	0.0005	-0.002	0.0035
L02 vs U11	-0.0016	-0.0164	0.0156	-0.0001	-0.0025	0.0029
L02 vs U02	0.0024	-0.0098	0.0185	0.0002	-0.0023	0.0039
L02 vs U02	**0.0376**	0.0109	0.0582	0.006	-0.0003	0.0127
L02 vs V05	0.013	-0.0092	0.044	0.0017	-0.0027	0.0078
L02 vs V08	**0.0793**	0.0463	0.1035	**0.0176**	0.0092	0.0248
L02 vs V10	**0.0518**	0.0165	0.0805	**0.0093**	0.0021	0.0175
L02 vs V11	**0.0704**	0.0429	0.1003	**0.0108**	0.0067	0.0151
L02 vs V02	**0.0432**	0.0203	0.0606	**0.0065**	0.0027	0.0108
U03 vs U05	0.0132	-0.0017	0.0459	0.0008	-0.0027	0.0095
U03 vs U08	0.0011	-0.0152	0.0211	0	-0.0029	0.0056
U03 vs U10	**0.0193**	0.0023	0.0359	0.0039	-0.001	0.0094
U03 vs U11	0.0017	-0.0145	0.0272	0	-0.0025	0.0043
U03 vs U02	0.0149	-0.0056	0.0355	0.001	-0.0041	0.009

U03 vs U02	**0.0471**	0.0213	0.0702	**0.0079**	0.0024	0.0169
U03 vs V05	**0.0336**	0.0111	0.0631	**0.0066**	0.0017	0.0134
U03 vs V08	**0.0852**	0.0424	0.1303	**0.0178**	0.004	0.0304
U03 vs V10	**0.0809**	0.0476	0.1272	**0.0166**	0.0104	0.0309
U03 vs V11	**0.0788**	0.0415	0.1129	**0.0109**	0.0066	0.0144
U03 vs V02	**0.0579**	0.0306	0.0861	**0.0079**	0.0041	0.0141
U05 vs U08	0.002	-0.0144	0.0204	0.0002	-0.003	0.006
U05 vs U10	0.0102	-0.0099	0.0344	0.0014	-0.0021	0.0057
U05 vs U11	0.0099	-0.0061	0.0285	0.0008	-0.0021	0.0044
U05 vs U02	0.0044	-0.011	0.0233	0	-0.0023	0.0039
U05 vs U02	**0.0375**	0.0242	0.0617	**0.0058**	0.003	0.0129
U05 vs V05	**0.0353**	0.0068	0.0593	**0.0063**	0.0009	0.0108
U05 vs V08	**0.0778**	0.0214	0.1289	**0.0159**	0.0006	0.0366
U05 vs V10	**0.0664**	0.0483	0.0939	**0.0124**	0.009	0.0171
U05 vs V11	**0.0734**	0.0315	0.1065	**0.011**	0.0053	0.0169
U05 vs V02	**0.0503**	0.0222	0.08	**0.0073**	0.0029	0.0113
U08 vs U10	-0.002	-0.017	0.0312	-0.0004	-0.0035	0.0061
U08 vs U11	-0.0001	-0.0225	0.0271	0	-0.0037	0.0042
U08 vs U02	0.0037	-0.0207	0.0494	0	-0.0055	0.0087
U08 vs U02	0.0182	-0.0019	0.0537	0.0038	-0.0013	0.012
U08 vs V05	0.0059	-0.0046	0.0141	0.0011	-0.0011	0.0026
U08 vs V08	**0.0452**	0.0297	0.0663	**0.011**	0.0035	0.0227
U08 vs V10	**0.035**	0.0107	0.0649	**0.0075**	0.0011	0.0157
U08 vs V11	**0.0401**	0.0136	0.0799	**0.0065**	0.0029	0.0097
U08 vs V02	**0.025**	0.0099	0.039	**0.0041**	0.0019	0.0056
U10 vs U11	0.0052	-0.0085	0.0191	0.0006	-0.0015	0.0028
U10 vs U02	0.004	-0.0132	0.0181	0.0004	-0.0027	0.0052
U10 vs U02	0.0145	-0.0049	0.0302	0.0026	-0.0031	0.0099
U10 vs V05	0.0021	-0.0101	0.0151	0.0003	-0.002	0.0033
U10 vs V08	**0.04**	0.0053	0.0632	0.0115	-0.0008	0.0276
U10 vs V10	0.0208	-0.003	0.0537	0.0048	-0.0017	0.0147
U10 vs V11	**0.0356**	0.0173	0.0625	**0.0064**	0.0037	0.01
U10 vs V02	**0.0217**	0.0054	0.0448	**0.0038**	0.0013	0.0081
U11 vs U02	0.0017	-0.0184	0.0197	0.0001	-0.0028	0.0029
U11 vs U02	**0.0309**	0.0167	0.0608	**0.0046**	0.002	0.012
U11 vs V05	**0.0145**	0.0009	0.0362	0.0025	-0.0003	0.0066
U11 vs V08	**0.0831**	0.0453	0.1126	**0.0177**	0.0055	0.0327
U11 vs V10	**0.0601**	0.0268	0.0925	**0.0115**	0.007	0.0173
U11 vs V11	**0.0642**	0.0452	0.0816	**0.009**	0.0059	0.011
U11 vs V02	**0.0359**	0.0119	0.0716	**0.0047**	0.0015	0.0079
U02 vs U02	**0.0317**	0.0036	0.0546	0.0049	-0.0011	0.0147
U02 vs V05	**0.0351**	0.0111	0.0583	**0.0066**	0.002	0.0123
U02 vs V08	**0.0787**	0.0405	0.105	**0.0188**	0.0052	0.0344
U02 vs V10	**0.0572**	0.0354	0.0896	**0.0118**	0.0065	0.0199
U02 vs V11	**0.0673**	0.0356	0.0996	**0.0107**	0.0056	0.0182

U02 vs V02	**0.0386**	0.0178	0.0605	**0.0057**	0.0028	0.0088
U02 vs V05	**0.0274**	0.0037	0.0529	0.0065	-0.0001	0.014
U02 vs V08	**0.0745**	0.021	0.1067	**0.0317**	0.0049	0.0502
U02 vs V10	**0.0437**	0.012	0.0842	**0.0173**	0.0019	0.0368
U02 vs V11	0.0244	-0.0017	0.0521	0.004	-0.0039	0.0134
U02 vs V02	0.0041	-0.0102	0.0181	0.0001	-0.0029	0.0041
V05 vs V08	**0.0434**	0.0005	0.0767	0.0121	-0.0007	0.0228
V05 vs V10	**0.0221**	0.0001	0.0387	0.0048	-0.0009	0.0106
V05 vs V11	**0.0333**	0.0081	0.062	**0.0056**	0.002	0.0101
V05 vs V02	**0.0269**	0.0096	0.0545	**0.0046**	0.0014	0.0096
V08 vs V10	0.0148	-0.0008	0.0333	0.0013	-0.0035	0.0119
V08 vs V11	**0.0422**	0.0151	0.0767	**0.0108**	0.0017	0.0215
V08 vs V02	**0.0543**	0.0077	0.0826	**0.0134**	0.0003	0.0204
V10 vs V11	**0.0181**	0.0047	0.0414	**0.0049**	0.0013	0.0136
V10 vs V02	**0.0317**	0.0113	0.0635	**0.0092**	0.0033	0.018
V11 vs V02	0.0053	-0.0119	0.038	0.0006	-0.0028	0.005

SUPPLEMENTARY TABLE S2 Genetic differentiation between cohorts within sites estimated via G'_{ST} and the respective confidence intervals (CI). Values are significant when 0 is not included in the CI and are displayed in bold.

		G'_{ST}		
	Comparison	actual	lower CI	upper CI
Lynæs	cohort1 - cohort2	0.012	-0.0038	0.0217
	cohort1- cohort3	**0.0243**	**0.0016**	**0.0818**
	cohort1-cohort4	0.0148	-0.0007	0.0321
	cohort2-cohort3	**0.0193**	**0.0038**	**0.0446**
	cohort2-cohort4	0.0065	-0.0131	0.0221
	cohort3-cohort4	**0.0124**	**0.001**	**0.0281**
Lammefjord	cohort1 - cohort2	**0.0826**	**0.0609**	**0.1242**
	cohort1- cohort3	0.0113	-0.0025	0.0349
	cohort2-cohort3	**0.0694**	**0.0229**	**0.1263**
Herslev	cohort1 - cohort2	0.0099	-0.0082	0.0426
	cohort1- cohort3	0.0063	-0.0012	0.0137
	cohort2-cohort3	**0.0138**	**0.0003**	**0.0261**

SUPPLEMENTARY TABLE S3 Genetic differentiation between females and males respectively within site estimated via G'_{ST} and the respective confidence intervals (CI). Values are significant when 0 is not included in the CI and are displayed in bold. No statistics can be calculated when less than two individuals are in one sample or when there are less than three samples per comparison. Hence, no results are available for Lynæs, for Lammefjord females and males in May and August, for Vellerup females in May and August and for Herslev males in August.

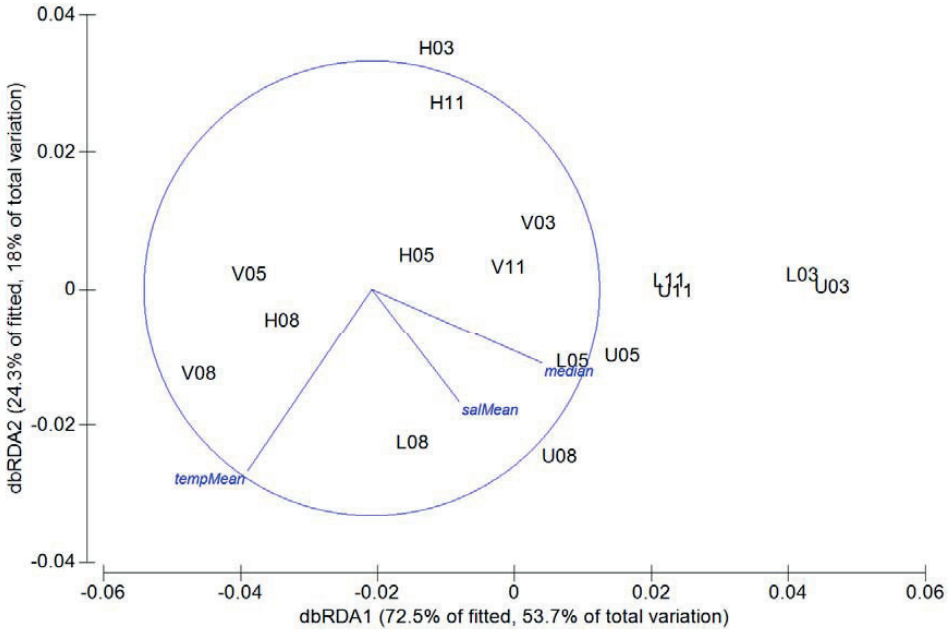
			G'_{ST}		
		comparison	actual	lower CI	upper CI
Lammefjord	Females	Mar - Oct	**0.213**	**0.155**	**0.299**
		Mar - Nov	**0.207**	**0.140**	**0.281**
		Mar - Feb	**0.123**	**0.042**	**0.230**
		Oct - Nov	-0.001	-0.112	0.144
		Oct - Feb	**0.114**	**0.059**	**0.181**
		Nov - Feb	0.002	-0.094	0.114
	Males	Mar - Oct	**0.298**	**0.198**	**0.508**
		Mar - Nov	**0.169**	**0.038**	**0.301**
		Mar - Feb	-0.027	-0.134	0.103
		Oct - Nov	-0.033	-0.132	0.143
		Oct - Feb	**0.180**	**0.042**	**0.403**
		Nov - Feb	0.051	-0.081	0.246
Vellerup	Females	Mar - Oct	**0.211**	**0.132**	**0.324**
		Mar - Nov	**0.115**	**0.053**	**0.169**
		Mar - Feb	-0.014	-0.082	0.067
		Oct - Nov	0.014	-0.060	0.094
		Oct - Feb	0.105	-0.035	0.300
		Nov - Feb	0.031	-0.058	0.174
	Males	Mar - May	**0.178**	**0.094**	**0.282**
		Mar - Aug	**0.146**	**0.113**	**0.202**
		Mar - Oct	**0.127**	**0.054**	**0.228**
		Mar - Nov	**0.068**	**0.034**	**0.105**
		Mar - Feb	**0.025**	**0.006**	**0.084**
		May - Aug	**0.517**	**0.411**	**0.598**
		May - Oct	**0.182**	**0.045**	**0.307**
		May - Nov	**0.273**	**0.126**	**0.412**
		May - Feb	**0.164**	**0.056**	**0.296**
		Aug - Oct	**0.186**	**0.066**	**0.346**
		Aug - Nov	-0.038	-0.120	0.089
		Aug - Feb	-0.005	-0.030	0.024
		Oct - Nov	0.106	-0.016	0.252
		Oct - Feb	0.060	-0.055	0.197
		Nov - Feb	-0.021	-0.069	0.035
Herslev		Mar - May	-0.007	-0.079	0.105

Females	Mar - Aug	0.044	-0.078	0.199
	Mar - Oct	0.018	-0.128	0.186
	Mar - Nov	0.005	-0.025	0.064
	Mar - Feb	0.011	-0.037	0.066
	May - Aug	-0.034	-0.160	0.121
	May - Oct	0.081	-0.070	0.332
	May - Nov	-0.019	-0.103	0.114
	May - Feb	-0.011	-0.056	0.102
	Aug - Oct	0.014	-0.173	0.219
	Aug - Nov	0.053	-0.068	0.204
	Aug - Feb	0.068	-0.048	0.212
	Oct - Nov	0.133	-0.020	0.361
	Oct - Feb	0.044	-0.046	0.167
	Nov - Feb	0.031	-0.015	0.105
Males	Mar - May	-0.012	-0.055	0.035
	Mar - Oct	-0.008	-0.064	0.123
	Mar - Nov	-0.020	-0.036	0.013
	Mar - Feb	-0.008	-0.018	0.010
	May - Oct	0.037	-0.051	0.174
	May - Nov	-0.024	-0.076	0.019
	May - Feb	-0.011	-0.098	0.071
	Oct - Nov	0.004	-0.066	0.137
	Oct - Feb	0.009	-0.057	0.084
	Nov - Feb	-0.004	-0.029	0.019

SUPPLEMENTARY TABLE S4 Genetic differentiation between females, males and all specimens within samples estimated via G'_{ST} and the respective confidence intervals (CI). Values are significant when positive and when 0 is not included in the CI and are displayed in bold. No statistics can be calculated when less than two individuals are in one sample or when there are less than three samples per comparison. Hence, no results are available for Lynæs except in October, for Lammefjord and Vellerup in May and August and for Herslev in August.

			G'_{ST}		
		comparison	actual	lower	upper CI
Lynæs	Oct	females - males	0.168	-0.017	0.319
		females - total	**0.100**	**0.010**	**0.245**
		males - total	**0.167**	**0.086**	**0.274**
Lammefjord	Mar	females - males	0.013	-0.020	0.045
		females - total	-0.016	-0.024	-0.007
		males - total	0.007	-0.034	0.069
	Oct	females - males	0.035	-0.028	0.137
		females - total	**0.050**	**0.012**	**0.113**
		males - total	**0.072**	**0.017**	**0.161**
	Nov	females - males	-0.005	-0.189	0.238

		females - total	**0.156**	**0.023**	**0.341**
		males - total	**0.061**	**0.011**	**0.115**
	Feb	females - males	0.088	-0.042	0.279
		females - total	0.085	-0.054	0.258
		males - total	-0.030	-0.098	0.096
Vellerup	Mar	females - males	-0.006	-0.040	0.026
		females - total	-0.018	-0.034	0.009
		males - total	-0.016	-0.038	0.016
	Oct	females - males	0.002	-0.101	0.166
		females - total	0.032	-0.027	0.100
		males - total	0.006	-0.067	0.101
	Nov	females - males	0.062	-0.018	0.174
		females - total	0.028	-0.006	0.124
		males - total	-0.004	-0.028	0.023
	Feb	females - males	-0.064	-0.141	0.101
		females - total	-0.019	-0.062	0.038
		males - total	-0.009	-0.039	0.055
Herslev	Mar	females - males	-0.016	-0.033	0.002
		females - total	-0.013	-0.026	0.001
		males - total	-0.019	-0.032	-0.009
	May	females - males	-0.055	-0.142	0.041
		females - total	-0.025	-0.088	0.026
		males - total	-0.022	-0.064	0.044
	Oct	females - males	-0.088	-0.195	0.052
		females - total	-0.053	-0.112	0.111
		males - total	-0.021	-0.072	0.051
	Nov	females - males	-0.019	-0.064	0.036
		females - total	0.003	-0.035	0.057
		males - total	-0.012	-0.030	0.012
	Feb	females - males	-0.019	-0.053	0.023
		females - total	-0.016	-0.055	0.109
		males - total	-0.008	-0.036	0.026



SUPPLEMENTARY FIGURE S1 Distance-based redundancy analysis (dbRDA): Ordination of the population genetic differences of *Pygospio elegans* between the four sites and time points(U, Lynæs; L, Lammefjord; V, Vellerup; H, Herslev; 3, March; 5, May; 8, August; 11, November) fitted to the significant predictor environmental parameters temperature, median grain size, and salinity. The parameters explain 74 % of the total variation in the population genetic structure, with 72 % explained by the first two axes as shown. Overlaid vectors indicate the loadings (importance) of the predictor parameters temperature, median grain size, and salinity on the two axes.

III

SEASONAL VARIATION IN DIVERSITY OF MARINE BENTHIC INVERTEBRATES LEADS TO A POSITIVE SPECIES-GENETIC DIVERSITY CORRELATION

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SEASONAL VARIATION IN DIVERSITY OF MARINE BENTHIC INVERTEBRATES LEADS TO A POSITIVE SPECIES-GENETIC DIVERSITY CORRELATION

K.E. Knott[1], A. Thonig[1,2], S. Heiskanen[1], B. Winding Hansen[2], G.T. Banta[2]

[1]Department of Biological and Environmental Science, University of Jyvaskyla, FI-40014 University of Jyvaskyla, Finland
[2]Department of Science and Environment, Roskilde University, DK-4000 Roskilde, Denmark

ABSTRACT

Species-genetic diversity correlations (SGDCs) are useful indicators of processes that simultaneously affect diversity at multiple biological levels. We combine spatial and temporal sampling of four study sites in the Danish Isefjord-Roskilde Fjord Estuary at four time points over one year to investigate the effect of seasonal variation on SGDCs. Species diversity was estimated as species richness from samples comprising 20,752 individuals representing 51 benthic invertebrate taxa. Genetic diversity was estimated for a single focal taxon, the polychaete *Pygospio elegans,* as mean allelic richness at seven microsatellite loci. Combining all samples, a significant positive correlation between species richness and allelic richness was found. Median sediment grain size and mean temperature had significant effects on species richness, whereas only mean temperature had a significant effect on allelic richness of *P. elegans*. Our results show that both the benthic community as a whole and populations of *P. elegans* respond similarly to seasonal environmental variation at the study sites. The results suggest that seasonal timing of reproduction and dispersal in this temperate marine habitat might have a greater influence on diversity than spatially varying environmental variables and highlight the benefits of also investigating temporal SGDCs. Because of seasonal changes in diversity, it is important that samples are compared on the same time scale when investigating SGDCs.

INTRODUCTION

Diversity can be measured within individuals, populations, and/or communities, but relationships between diversity at these different levels are unclear. Ecological and evolutionary processes can have similar effects on species diversity within communities and on genetic diversity within species, and thus, positive correlations between the different levels of biodiversity can occur (see Vellend 2003, Vellend *et al.* 2014). These "species-genetic diversity correlations" (SGDCs) can be useful indicators of the processes that simultaneously affect diversity at multiple biological levels. Moreover, identification of SGDCs is useful in an applied context if they allow the inference of one level of diversity based on that of another (Kahilainen *et al.* 2014). However, the sign of SGDCs can be difficult to both predict and interpret (Laroche *et al.* 2015). Positive relationships are expected when variation in diversity is mediated by factors affecting population sizes acting in the same way on individual species and on the entire community, for example via available habitat, environmental conditions or dispersal routes. Such factors are described as "site factors," and how they affect SGDCs can vary depending on whether the focal species and communities under study are ecologically similar (Lamy *et al.* 2016). On the other hand, biological interactions can disrupt potential species-genetic diversity correlations, for example in cases of competition or facilitation between species. Such interactions, described as "community factors" (Lamy *et al.* 2016), could lead to either positive or negative SGDCs. Several reviews of empirical studies (Kahilainen *et al.* 2014, Vellend *et al.* 2014, Whitlock 2014) have now emphasized that an expectation for positive SGDCs in most cases might be premature. Nevertheless, investigation of the factors explaining positive (or negative) SGDCs is fruitful for understanding the ecology of the focal species and community in question.

Most studies on the relationships between species diversity and genetic diversity have focused on terrestrial systems, and SGDCs in marine environments have received less attention (Messmer *et al.* 2012, Josefson & Göke 2013, Selkoe *et al.* 2016). Since SGDCs in *a* diversity (diversity at the local scale in a particular population or community) are expected to be more frequently positive in island-like systems due to clear limitations of area on population size (Vellend *et al.* 2014), positive SGDCs might be less likely in marine environments, where limits of area on population size can be hard to define. This is because oceans are environments of high connectivity, and both environmental conditions and behavioural characteristics of benthic marine organisms can increase their dispersal capabilities (Cowen & Sponaugle 2009), thereby counter-acting possible restrictions on population size defined by area. However, restrictions to dispersal in the marine environment are not always obvious, and there are many examples of species with limited actualized dispersal despite their potential for wider dispersal (Hellberg 2009, Weersing & Toonen 2009). Therefore, the connectivity and diversity of marine communities

might be affected more by environmental conditions than by area *per se*. For example, abiotic variables, such as water salinity (Bekkevold *et al.* 2005) or temperature (Banks *et al.* 2007), as well as different biotic factors (Cole 2010, de Juan & Hewitt 2011) are known to impact marine communities, particularly benthic macrofauna. Moreover, in some habitats, such as estuaries, fluctuations in abiotic conditions can be extreme, and dynamics in environmental conditions could also strongly influence diversity (Robinson *et al.* 2010, de Juan & Hewitt 2014).

At large spatial scales variation in species diversity is often accompanied by turnover in species composition (Vellend 2005), which is more appropriately described as β diversity (diversity between different populations or communities). SGDCs in β diversity are less commonly explored, but, like SGDCs of *a* diversity, these also vary in strength and sign (Kahilainen *et al.* 2014). SGDCs in β diversity might be particularly useful as indicators of dispersal or barriers to recruitment that organisms might face in new habitats or for species that show isolation by distance. For example, when examining several focal species, seascape genetic studies have indicated characteristics of the community, specifically biological interactions and the role of coral cover in Hawaiian coral reefs that promote high diversity and connectivity (Selkoe *et al.* 2016). At smaller spatial scales, when connectivity between populations is expected to homogenize populations and communities, SGDCs in β diversity are not expected (see Kahilainen *et al.* 2014).

Turnover in species composition can occur also within a population or community as a result of immigration of ephemeral species and succession over time (e.g. see Bracken & Williams 2017), but temporal variation in diversity is not typically explored through SGDCs. This could be for several reasons. Firstly, if limited resources restrict the scale of the study ,emphasis might be placed on spatial sampling rather than temporal sampling. Secondly, the site factors or community factors expected to affect diversity and drive SGDCs might not show temporal variation. Thirdly, researchers might simply assume that diversity (either species diversity or genetic diversity, or both) is not temporally variable. Nevertheless, temporal variation in species or genetic diversity can occur, particularly in seasonally dynamic environments (e.g. Lamy *et al.* 2013, de Juan & Hewitt 2014, Hewitt *et al.* 2016). Long-term environmental fluctuations (such as El Niño events and increasing global climate change) are also known to create temporal variation in species diversity (Cleary *et al.* 2006, Pauls *et al.* 2013). Therefore, studying temporal SGDCs might reveal concordant or conflicting responses to environmental variation in the focal communities. When SGDCs among temporal samples are analysed, the same methods used for analysing SGDCs among spatial samples typically are adopted (e.g. Cleary *et al.* 2006).

We expect that a combination of spatial and temporal sampling when investigating SGDCs has the potential to help clarify the most important site and community factors affecting diversity. In the present study, we examine the correlation between species diversity of benthic macrofauna at four sites in the Danish Isefjord-Roskilde Fjord estuary and genetic diversity of the polychaete

worm *Pygospio elegans* living at these sites. We include temporal sampling aiming to capture seasonal variation and evaluate whether different environmental variables can be related to the diversity patterns. *P. elegans* not only has broad environmental tolerances, capable of living at salinities ranging from 6-35 (Anger 1984, Thonig *et al.* 2016), but also shows variation in an important life history characteristic, the larval developmental mode, which is expected to impact its dispersal potential and population connectivity (Rasmussen 1973, Morgan *et al.* 1999). We have previously studied the population genetic structure of *P. elegans* at different spatial scales and found that populations in the Isefjord-Roskilde Fjord estuary show temporal genetic structure and chaotic genetic patchiness (Kesäniemi *et al.* 2012a, 2012b, 2014a, 2014b) as well as seasonal changes in genetic composition (Thonig *et al.* in review). A correlation between genetic diversity in *P. elegans* and overall species diversity of the benthic macrofauna community could indicate whether diversity at organismal (genetic) and community levels responds to common environmental variables or other common factors.

MATERIALS AND METHODS

Data collection

We assessed seasonal variation in species diversity of benthic macrofauna at four time points (March, May, August, and November, 2014) at four study sites (Lynæs, Lammefjord, Vellerup, and Herslev) in the Danish Isefjord-Roskilde-Fjord estuary. At each sampling, three replicate sediment cores were collected using a hand-held corer (15 cm diameter, 30 cm length). Samples were sieved using a 1 mm mesh and remaining material was fixed in 5 % buffered formaldehyde on site. In the lab, formaldehyde was removed in several washing steps using deionized water, and the samples were stained overnight with a 2 % Rose Bengal solution to better visualize the macrofauna. After removing the Rose Bengal solution, specimens were sorted and identified to the lowest reliable taxonomic level according to Barnes (1994) and Hayward & Ryland (1995), and we confirmed currently valid taxonomy using WoRMS (WoRMS Editorial Board 2017). Sorted specimens were stored in 95 % Ethanol.

The samples of benthic macrofauna were collected concomitantly with a field survey performed monthly in 2014/2015, during which environmental parameters were monitored, and population dynamics of the polychaete *Pygospio elegans* were followed at the four sites (Thonig *et al.* 2016). The environmental variables included sediment characteristics (median grain size, sorting, porosity, water content, carbon content and C/N ratio), water temperature and salinity. Sediment characteristics were determined from a mix of the top one cm of three replicate sediment cores per site and time. Temperature and salinity were logged every ten minutes during the whole period with data loggers and the mean and standard deviation was calculated

per month (Thonig *et al.* 2016). Also during the field survey, samples of *P. elegans* were collected each month and genotyped using seven microsatellite loci (Supplement 1). Population genetic structure of *P. elegans* using the monthly samples is described in Thonig *et al.* (in review). Genetic data collected at the four time points chosen for surveying the benthic community (March, May, August, November) were used in analysis of SGDC, described here.

Species diversity, genetic diversity, and SGDC

Abundance of each identified taxon was input into the software PRIMER-E v.6.1.16 (Clarke & Warwick 2001) for each core separately (3 replicate samples per location and sampling date). Counts were transformed using the 4th root to account for the high abundance of a single abundant taxon (i.e. *Hydrobia* spp.) and averaged over replicate sampling cores. Bray Curtis similarity was used when constructing a resemblance matrix, and temporal and spatial differences in species abundance were visualized in a non-metric multi-dimensional scaling (NMDS) plot with the default number of restarts (1000) using PRIMER-E. Species diversity was measured as species richness: the number of species present in each core was counted, and then averaged over replicate cores for each location and sampling date.

The allele frequencies of *Pygospio elegans* at each microsatellite locus and sampling date were calculated using Fstat v. 2.9.3.2 (Goudet 1995). These were input to PRIMER-E and a resemblance matrix was made using Euclidian distance. The spatial and temporal differences in allele frequencies were visualized in a NMDS plot in PRIMER-E. Genetic diversity was represented by allelic richness, calculated for each locus based on a sample size of 26 individuals using HP-Rare v1.1 (Kalinowski 2005) and then averaged over all loci.

A correlation between species diversity and genetic diversity (a SGDC) was calculated across all sites and time points using Spearman´s rank correlation coefficient using R v. 3.4.0 (R Core Team, 2017). Although our temporal samples represent repeated measures at the same site, and are possibly not independent, we included all time points in a single correlation analysis. Our previous analyses indicated significant genetic variation occurs among samples both spatially and temporally (Thonig *et al.* in review), although differentiation among all samples was not always statistically significant. Furthermore, preliminary analyses of the differences in species abundance and composition among samples indicated both spatial and temporal differentiation (data not shown). Therefore, we feel that the samples are sufficiently independent to be combined in a single correlation analysis. Variation in β diversity was not analysed, given the small overall spatial scale and our previous observations of chaotic genetic patchiness among *P. elegans* populations in the Isefjord-Roskilde Fjord estuary (Kesäniemi *et al.* 2014a, Thonig *et al.* in review).

Environmental impact on diversity

Generalized linear mixed models (GLMM) allow for the analysis of response variables that have different distributions than the normal distribution. These models can also account for dependence between samples by incorporating random effects in addition to fixed effects. In this study we used GLMM to investigate the effect of environmental parameters on both diversity measures, i.e., species diversity and genetic diversity, while accounting for repeated measures at the four sampling sites. Count data, such as species richness, are assumed to follow a Poisson or negative binomial distribution rather than a normal distribution. The negative binomial distribution is preferred in cases when over dispersion occurs, i.e. when the variance is larger than the mean, for example due to patchiness of species distributions, and is indicated by a small over dispersion parameter theta. We compared a log-linear model with a Poisson error term and a log-linear model with an error term following a negative binomial distribution for our response variable species richness. Since the latter resulted in a large estimate of theta (the over dispersion coefficient) we chose the Poisson distribution to model the error term. We checked for collinearity of our environmental variables using scatter plots and Pearson correlation coefficient, to reduce the number of explanatory variables. Accordingly, we removed porosity, sorting, and water content from the data set as they correlated strongly with median particle size ($r = 0.725$, -0.818, 0.775,respectively; p-values< 0.01). Additionally, the standard deviation of temperature was removed as it was closely correlated with mean temperature ($r = 0.905$; p-value < 0.001). Hence, the fixed effects of our explanatory variables were median particle size, organic content, C/N, mean temperature, mean salinity and standard deviation of salinity. We measured only one set of environmental variables per sampling; thus, the same environmental data was used for the three replicate measurements of species richness per sampling. As random effect we included sample, which represents the combination of time point and site, to account for the effect of season on the one hand and the repeated measure design of our study on the other hand. The GLMM was performed with glmmPQL in the R package MASS (R Core Team, 2017) according to the following equation:

$$\text{Log(SpeciesRichness)} = a + \beta_1 * \text{median particle size} + \beta_2 * \text{organic content} + \beta_3 * \text{C/N} + \beta_4 * \text{mean temperature} + \beta_5 * \text{mean salinity} + \beta_6 * \text{salinity SD} + \text{Poisson}(\lambda_{Sample}) + \text{Poisson}(\lambda_{Residual}).$$

Since our response variable allelic richness neither represents count data nor is normally distributed, we inspected it visually with a quantile comparison plot (qqp function in the R package car), which showed that it fit best to a log-normal distribution. For that reason, we used a log-linear model with a normally distributed error term. The explanatory variables were composed of the same fixed factors as for species richness, but included only site as a random

factor due to lack of replication within sample. The GLMM was performed with glmmPQL in the R package MASS (R Core Team, 2017) according to the following equation:

Log(AllelicRichness) = a + β_1*median particle size + β_2*organic content + β_3*C/N + β_4*mean temperature + β_5*mean salinity + β_6*salinity SD + Normal(0,σ^2_{Site}) + Normal(0,$\sigma^2_{Residual}$).

RESULTS

Species diversity, genetic diversity, and SGDC

We collected in total 20,752 individuals representing 51 benthic invertebrate taxa from samples taken from four locations in the Danish Isefjord-Roskilde Fjord estuary at four times of the year (See Figure 1; Supplementary Figure S1). The most abundant taxon at all sites was the gastropod *Hydrobia* spp. The focal species of our study, *Pygospio elegans*, was found at all sites, in 38 out of the 48 samples, and was the fourth most frequently found species. However, the presence of *P. elegans* was patchy, and it was not sampled in any of the replicate cores from Lynæs or Lammefjord in November, even though additional sampling at these sites in November yielded sufficient samples of *P. elegans* to use in the genetic analysis. Density of *P. elegans* was highest in May and lowest in November (see Thonig *et al.* 2016).

We visualized the spatial and temporal variation in species abundance of the benthic macrofauna using a NMDS plot (Figure 2A). The moderate stress value (0.15) indicates that the NMDS plot does a sufficient, but not perfect job in representing the relations between samples based on species abundance. The plot indicates good spatial differentiation (i.e., separate groupings) between all sites; Vellerup and Herslev were clearly distinct and not overlapping with other sites. Lynæs and Lammefjord were more similar to each other with some overlap, but differed from the other sites. Polychaetes were most abundant in Vellerup, while gastropods were most abundant in Lynæs and Lammefjord (Supplement Figure S1). Crustaceans and bivalves had relatively low abundances at all sites. No large temporal shifts in species abundance were observed, with the exception of November, which differed from the other times at all sites.

Species richness varied from a low of five species observed in March at Lynæs to a high of 29 species observed in August at Vellerup (Figure 1, Figure 3A). Higher species richness was generally observed at Vellerup, whereas the other sites had similar, lower levels of diversity. Temporal patterns at each site showed lowest richness in March, which then increased during the year. In Lammefjord and Vellerup, diversity reached a peak in August and then decreased in November. In contrast, diversity peaked in Lynæs in May and in November in Herslev.

Seasonal genetic variation in *Pygospio elegans* is described in Thonig *et al.* (in review). Allele frequencies of seven microsatellite loci from genotyped *P. elegans* were visualized in a NMDS plot (Figure 2B). Allele frequencies were similar in Lynæs and Lammefjord at all collection times excluding August at Lammefjord. Furthermore, allele frequencies in August differed markedly from those of samples taken at other times except for Lynæs. Temporal variation in allelic frequencies was greatest in Vellerup (Figure 2B). Allelic richness averaged over all loci ranged from 2.5 in March at Vellerup to 5.7 in August at Lammefjord (Supplementary Table S2). A seasonal pattern was observed in allelic richness, particularly for Lammefjord and Vellerup, and in general, highest values were observed at all sites in August (Figure3B).

There was a significant positive correlation between species richness and allelic richness (rho = 0.697, p-value = 0.003) (see Fig. 4).

Factors explaining the pattern

Environmental variables measured for each site and sampling time are reported in detail in Thonig *et al.* (2016). In general water temperature showed similar patterns at all sites, with highest temperatures in July and lowest temperatures in February. Salinity, in contrast differed between sites, being around 19-20 PSU at Lynæs, Lammefjord and Vellerup, and around 14 PSU at Herslev. Likewise, sediment characteristics differed between sites but did not show any or consistent seasonal patterns. Sediment was fine grained at Lynæs and Lammefjord, medium at Herslev and coarse at Vellerup. Water content and porosity was highest in fine sediment. Sorting of sediment was moderately well in Lynæs, only moderately in Lammefjord and Herslev and poorly at Vellerup. Organic content was highest at Lammefjord, followed by Lynæs, Vellerup and Herslev. At Vellerup we found the highest C/N, i.e. most refractory material, while more labile organic matter was present at Lammefjord, Herslev, and most at Lynæs (Thonig *et al.* 2016).

For species and allelic richness, the variation explained by the random factors sample and site is very low, indicating that most of the difference between sites and times that can be predicted is already captured with the fixed effects. Median sediment grain size and mean temperature had significant effects on species richness (Table 1). Considering that we used a log-linear model, an effect size of -0.5 of median grain size implies that per unit increase in grain size the species richness decreases 0.607 (= $e^{-0.5}$) fold. Since median grain size is determined as phi, i.e. $-\log_2$ of grain size in mm, sediment gets finer with increasing phi. Hence, higher species richness was found in coarse and - considering the correlation with sediment sorting - poorly sorted sediments. Furthermore, species richness increases 1.034 (= $e^{0.034}$) fold per degree Celsius. Allelic richness of *P. elegans* was also affected significantly by temperature, i.e., it increased 1.044 fold per degree (Table 1). Allelic richness was not significantly related to any of the other environmental variables investigated.

DISCUSSION

We found a positive correlation between species richness of the benthic macrofauna community in the Danish Isefjord-Roskilde Fjord estuary and genetic diversity of a focal species, the polychaete *Pygospio elegans*. Our study was conducted over a small spatial scale (maximum distance between sites ~30 km) and emphasized temporal sampling in addition to spatial sampling. This positive species-genetic diversity correlation (SGDC) was based on combining the data from all sites and collection times. Our results suggest that both the benthic community as a whole and populations of *P. elegans* are affected similarly by seasonal variation at the study sites. Seasonal changes in diversity of marine fauna are common, particularly at latitudes where temperature and other abiotic factors vary predictably (Valiela 2015). Moreover, because these temporal changes are associated with variation in food supply (e.g. vertical transport of matter originating from phytoplankton blooms, Cloern & Jassby 2010), many marine organisms have adapted to life in seasonal environments and time their reproductive events to follow seasonal variation (Coma *et al.* 2000, Smart *et al.* 2012). Similar life histories of *P. elegans* and the taxa comprising the benthic community likely lead to the observed relationship between species and genetic diversity.

When examining the role of abiotic environmental factors in explaining the patterns of diversity, we found that mean temperature and median sediment grain size helped explain the patterns of species richness. Species richness was higher at warmer temperatures and in coarser sediments (with greater porosity and water content and poorer sorting). Temperature is a good predictor of seasonal change, and seasonal variation in species richness has been documented for other benthic communities similar to what we observed here, e.g. in the Baltic Sea (Blomquist & Bonsdorff 1986, Bonsdorff & Blomquist 1989) and in the North Sea (Reiss & Kröncke 2004). Sediment factors, on the other hand, are not expected to vary seasonally, but represent habitat preferences of the benthic taxa that can also affect diversity. However, an indirect relationship between sediment factors and seasonal variation might exist, for example in the biotic environment (microbial or algal population dynamics) that was not measured during our study. Although salinity typically has a large role in explaining patters of species composition in the Baltic Sea on a large spatial scale (Zettler *et al.* 2014), salinity mean and standard deviation did not explain patterns of species richness in the present study. This could indicate that the differences in salinity among the four studied sites and the sampled seasons are not fluctuating at a level that alters this estuarine community (which is made up of euryhaline species generally tolerant to salinity fluctuations). Also, there might have been insufficient power for finding an effect of salinity due to the small number of studied sites. Robinson *et al.* (2010) also found little support for a role of salinity in driving SGDCs in estuaries in the southeastern United States. But, when comparing regions along

the North Sea-Baltic Sea transition, where salinity differences are more extreme and long-lasting, salinity significantly explained diversity patterns (Josefson & Göke 2013).

When analyzing genetic diversity of *P. elegans*, we found that, out of the environmental variables studied, only mean temperature had a significant effect explaining variation in allelic richness. Allelic richness increased in August when temperatures were warmer. Seasonal genetic variation in marine invertebrates is poorly studied, but has been observed in some lineages of the cryptic nematode *Pellioditis marina* as a result of (meta)population turnover (Derycke *et al.* 2006) and in the ascidian *Styela plicata* in North America resulting from seasonal patterns of recruitment (Pineda *et al.* 2016). Similarly, the variation in allelic richness of *P. elegans* is also likely explained by seasonal reproduction and recruitment of new, genetically differentiated cohorts that co-exist with older cohorts at the sites in August (see Thonig *et al.* 2016, Thonig *et al.* in review) and suggests that dispersal is the driving force behind the seasonal pattern. *Pygospio elegans* shows variation in larval developmental mode, producing planktonic, benthic, and intermediate larvae that differ in their capability for dispersal (Rasmussen 1973, Morgan *et al.* 1999, Thonig *et al.* 2016). In these study sites all types of larvae have been observed, except in Herslev, where only benthic and intermediate larvae were noted (Thonig *et al.* 2016). Most of the taxa sampled in the benthic community show life history strategies incorporating planktonic larvae and seasonal population dynamics, with an increased number of larvae present in summer (June, July and August) and reductions in population size in winter (Thorson 1946, Rasmussen 1973). Therefore, seasonal life histories and dispersal are the factors likely driving the observed SGDC.

Inter-annual temporal variation in SGDCs has been described for butterflies in rainforest and freshwater snails in a pond network (Cleary *et al.* 2006, Lamy *et al.* 2013), but seasonal variation in SGDCs has not been a focus in previous studies. Our finding of a significant SGDC with a combination of spatial and temporal sampling suggests that seasonal environmental change and associated life histories are relevant for understanding diversity patterns in temperate marine benthic communities. Considering the small geographic distances between our study sites and the negligible differences in temperature among sites (Thonig *et al.* 2016), temporal sampling was needed to reveal the effects of temperature (seasonality) on species and genetic diversity. Previously, Kesäniemi and colleagues (2014a) could not relate genetic diversity of *P. elegans* (local F_{ST}) to any environmental variables in a study in which *P. elegans* was collected from a large number of sites in the Isefjord-Roskilde Fjord estuary at a single time point (April). Due to limited resources, we could only sample four study sites at four different times, which prohibits us from investigating site-level diversity and SGDCs at each time point separately. A broader (spatial) study might indicate other environmental variables, both abiotic and biotic, that could affect diversity relationships. Nevertheless, our results indeed highlight an important temporal effect and help inform a relevant sampling scale for future larger scale studies. Namely, when investigating patterns of diversity, it

is important that samples are compared on the same time scale. Timing of sampling can have a significant effect on results of SGDCs and should be clearly reported, particularly for meta-analyses and when the samples used for calculating species richness and allelic richness are not collected concomitantly. Because of seasonal changes in diversity, Reiss and Kröncke (2005) have also cautioned against comparing diversity indices of different data sets collected in different seasons. In our study we saw clear evidence of a positive SGDC related to seasonal factors that affect diversity, most likely through seasonal reproduction and dispersal, and highlight the importance of life history strategies on broader ecological patterns that could be relevant also at other timescales.

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TABLES

TABLE 1 Environmental factors explaining Species Richness and Allelic Richness in the Isefjord-Roskilde Fjord estuary according to GLMM (see Materials and Methods for details)

SPECIES RICHNESS

Random effects (Poisson)					
	SD	Variance %			
sample	0.119	0.025			
Residual	0.746	0.975			
Fixed effects					
Groups	Value	SE	DF	t-value	p-value
(Intercept)	2.193	0.700	32	3.134	0.004
median grain size	-0.500	0.091	9	-5.515	**0.0004**
organic content	0.106	0.327	9	0.324	0.753
C/N	-0.011	0.055	9	-0.201	0.845
temperature mean	0.034	0.010	9	3.293	**0.009**
salinity mean	0.035	0.022	9	1.550	0.156
salinity SD	0.064	0.048	9	1.334	0.215

ALLELIC RICHNESS

Random effects (Normal)					
	SD	Variance %			
site	1.5E-06	0.000			
Residual	0.554	1.000			
Fixed effects					
Groups	Value	SE	DF	t-value	p-value
(Intercept)	-0.475	0.842	6	-0.564	0.593
median grain size	-0.101	0.096	6	-1.053	0.333
organic content	0.327	0.307	6	1.066	0.327
C/N	0.038	0.062	6	0.612	0.563
temperature mean	0.044	0.012	6	3.607	**0.011**
salinity mean	0.037	0.029	6	1.252	0.257
salinity SD	0.023	0.055	6	0.416	0.692

FIGURES

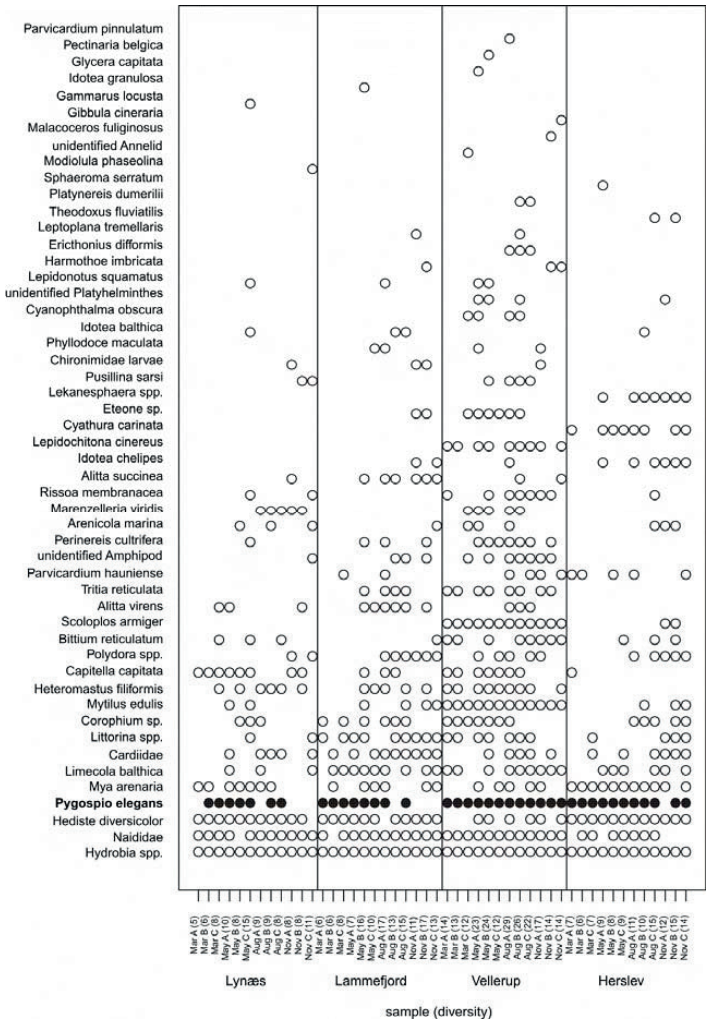

FIGURE 1 Benthic macrofauna present from four sites in the Danish Isefjord-Roskilde Fjord estuary at four time points during the year (a circle indicates that a taxon was present in a particular sample). The y axis lists the taxa observed ranked from least to most common (top to bottom) among the samples. The focal taxon, *Pygospio elegans*, is highlighted in bold. Samples are arranged on the x axis according to site, time point and replicate sample (A, B, C). The number of taxa observed in each replicate is shown in parentheses, e.g. Lynæs Mar A (5) means replicate A collected at Lynæs in March contained 5 taxa.

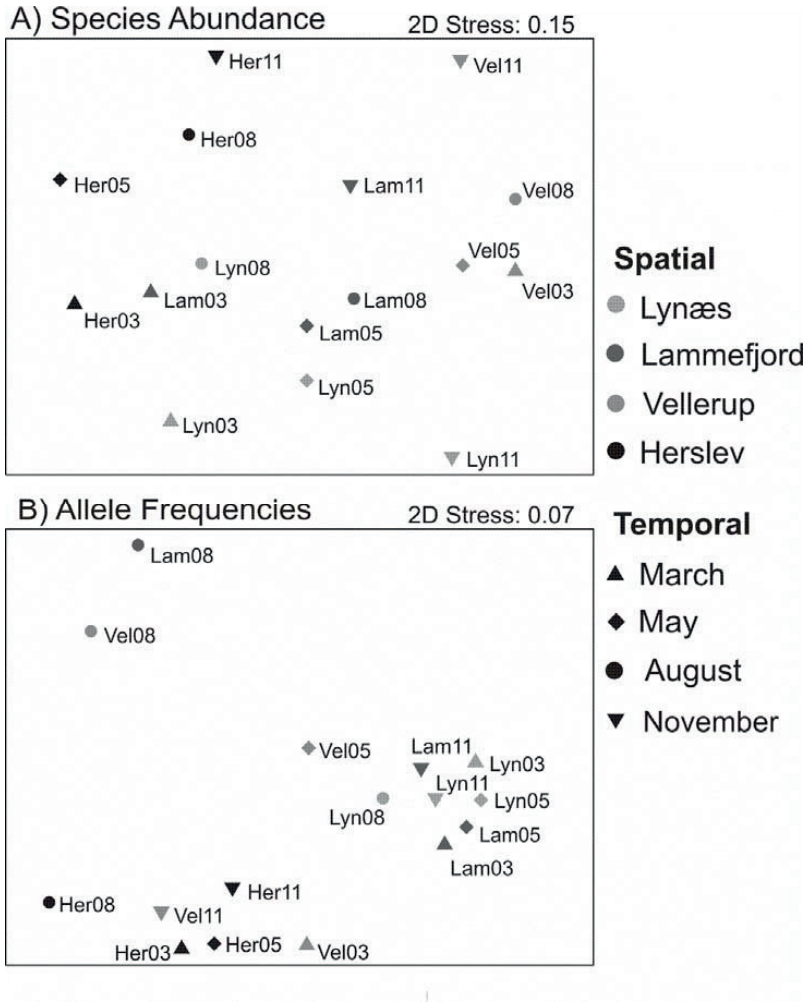

FIGURE 2 Non-metric multi-dimensional scaling (NMDS) plots of A) Species abundances of benthic macrofauna and B) Allele frequencies of *Pygospio elegans* sampled at four sites in the Danish Isefjord-Roskilde Fjord estuary at four times. Samples are coded with an abbreviated site name (Lynæs = Lyn, Lammefjord = Lam, Vellerup = Vel, and Herslev = Her) and number representing sampling time (March = 03, May = 05, August = 08, and November = 11) and with a symbol, with grey shading to indicate spatial sampling and different symbol shapes to indicate temporal sampling.

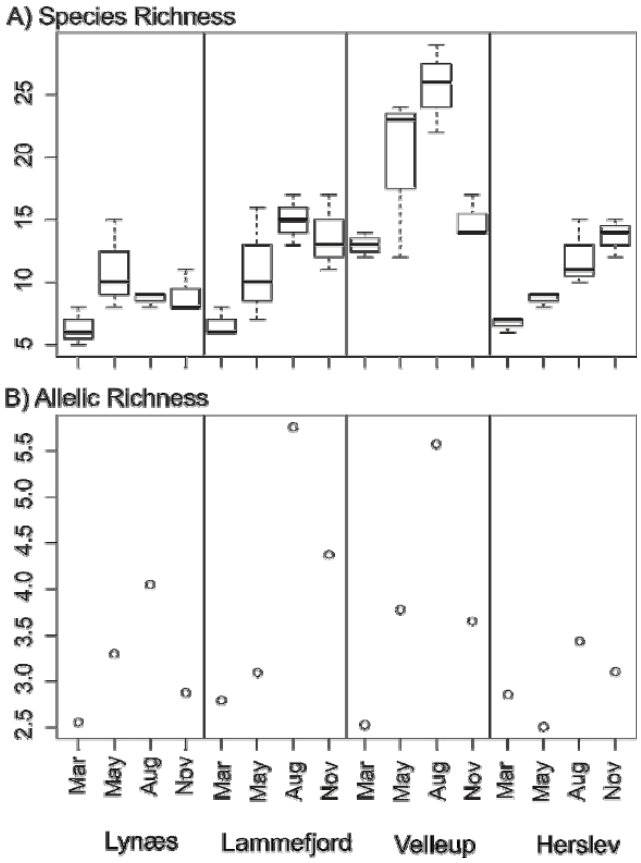

FIGURE 3 A boxplot of species richness (A) estimated for the benthic invertebrate communities and average allelic richness (B) of *Pygospio elegans* sampled from each sampling site and sampling time in the Isefjord-Roskilde Fjord estuary.

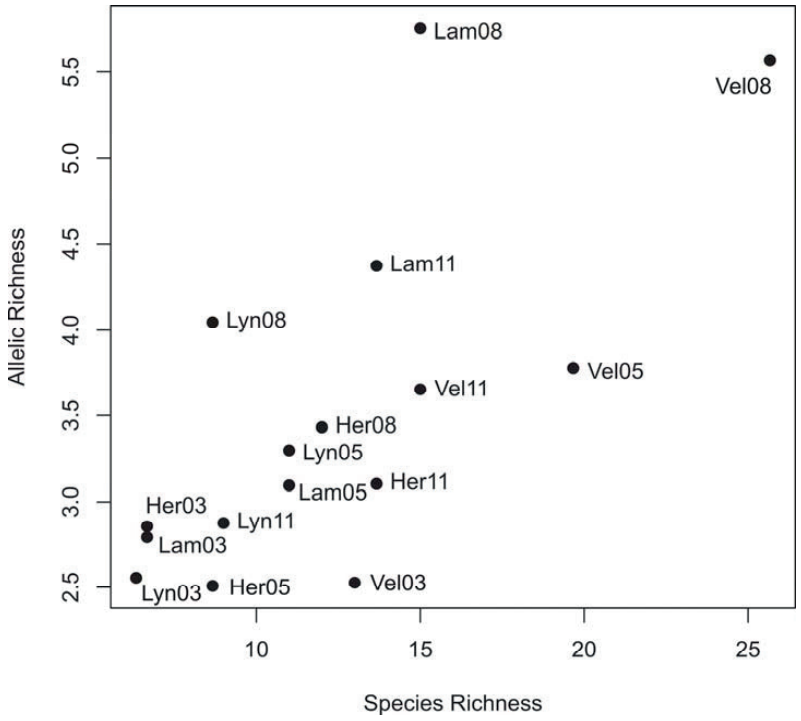

FIGURE 4 Scatter plot of average Species Richness at four sites in the Danish Isefjord-Roskilde Fjord estuary at four time points and average allelic richness of populations of *Pygospio elegans* from those sites showing a positive SGDC (Spearman rank: rho = 0.697, p-value = 0.003). Samples are coded with an abbreviated site name (Lynæs = Lyn, Lammefjord = Lam, Vellerup = Vel, and Herslev = Her) and number representing sampling time (March = 03, May = 05, August = 08, and November = 11).

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLE S1 Microsatellite loci, repeat found in reference sequence, the primers used for amplification, and GenBank Accession number. Loci marked with an asterisk were discarded from the study because they showed a high estimated null allele frequency (see Thonig *et al*. in review). The number of alleles and size-range observed in this study are shown. Loci Pe6, Pe7 and Pe19 were described in Kesäniemi *et al.* 2012. The loci were grouped into two multiplex panels: Multiplex 1 contained Loci Pe307, Pe309, Pe385, Pe6 and Pe7; Multiplex 2 contained Loci Pe19, Pe159, Pe234, Pe294 and Pe369.

Locus Name	Repeat sequence	Primer sequences	Gen Bank	No. of Alleles	Size Range (bp)
Pe307	(TG)6	F: AGCTAAATCTTGACACTGGCCT R: GAAGTCAGCCATCTTGGATTCT	xxx	12	181-202
Pe309*	(ATG)8	F: CCAGAGGAAATGATGTAGGCTC R: ATTCACACTTGACCATGACCAC	xxx	11	377-402
Pe385	(GGT)8	F: TCAATAGGAGAAGCACAACGAA R: CGCTGGTTATTTTAGGGATGAG	xxx	13	392-430
Pe6	(CA)28	F: ACTACGGAAACTGCCTGCAC R: ATATGGCCACCGAAACCTCT	GU321899	6	265-287
Pe7*	(CATA)13	F: CTCACCCTTTACACCCAAGG R: AGCGTCTGTTATGGGGTACAG	GU321900	38	124-255
Pe19	(GA)23	F: TATCCAACGCACACCTACCA R: TTGAGTGATGGTGCGAGGTA	GU321906	13	214-285
Pe159*	(GT)10	F: TTGGTTTGAGCAATGTGGAA R: GCCCTTTGCACTCATTGTTT	xxx	35	184-255
Pe234	(AG)6AA (AG)4	F: AGCAGTAAAAGCGGATCACAAC R: TGTCTCTGGCGTAATTTTCTCA	xxx	5	374-384
Pe294	(AG)5	F: AGTGGGTGTGTGAGAAGAGC R: AGTTGAGCCGTGATACAAAATC	xxx	5	231-239
Pe369	(GT)8	F: CTTTCTTCCCCAAGGCTTCT R: TTTCTCACCCTCCTGACCTG	xxx	17	190-227

SUPPLEMENTARY TABLE S2 Genetic diversity for each sample. Expected and observed heterozygosity (H_e and H_o), gene diversity, and inbreeding coefficient (F_{IS}) were calculated using Arlequin v.3.5.2 (Excoffier & Lischer 2010). F_{IS} values with a p-value smaller than 0.05 are indicated with *. Allelic richness and number of private alleles were determined with HP-Rare v1.1 (Kalinowski 2005). Relatedness was calculated using Coancestry v.1 (Wang 2011). N – number of individuals per sample.

Sample	N	He	Ho	Gene diversity	FIS	Allelic richness (N=26)	Private alleles (N=26)	Relatedness
Lynæs								
Mar	35	0.313	0.281	0.222	0.099	2.54	0	0.393
May	36	0.322	0.268	0.263	0.143*	3.29	0.1	0.226
Aug	29	0.315	0.283	0.239	0.077	4.02	0.01	0.246
Nov	38	0.337	0.320	0.289	0.051	2.86	0.1	0.311
Lammefjord								
Mar	31	0.330	0.347	0.276	-0.068	2.78	0	0.287
May	44	0.319	0.320	0.264	-0.022	3.07	0	0.344
Aug	41	0.416	0.348	0.416	0.165*	5.71	0.21	0.131
Nov	40	0.335	0.291	0.321	0.112*	4.33	0	0.184
Vellerup								
Mar	32	0.355	0.372	0.300	-0.056	2.52		0.297
May	37	0.330	0.251	0.299	0.226*	3.75	0.33	0.231
Aug	27	0.400	0.349	0.388	0.115*	5.51	0.37	0.147
Nov	33	0.333	0.262	0.276	0.201*	3.64	0	0.295
Herslev								
Mar	41	0.318	0.280	0.314	0.112*	2.85	0.04	0.287
May	37	0.369	0.330	0.192	0.085	2.5	0.07	0.320
Aug	34	0.429	0.421	0.358	0.003	3.41	0.03	0.216
Nov	43	0.336	0.309	0.283	0.072	3.09	0.3	0.272

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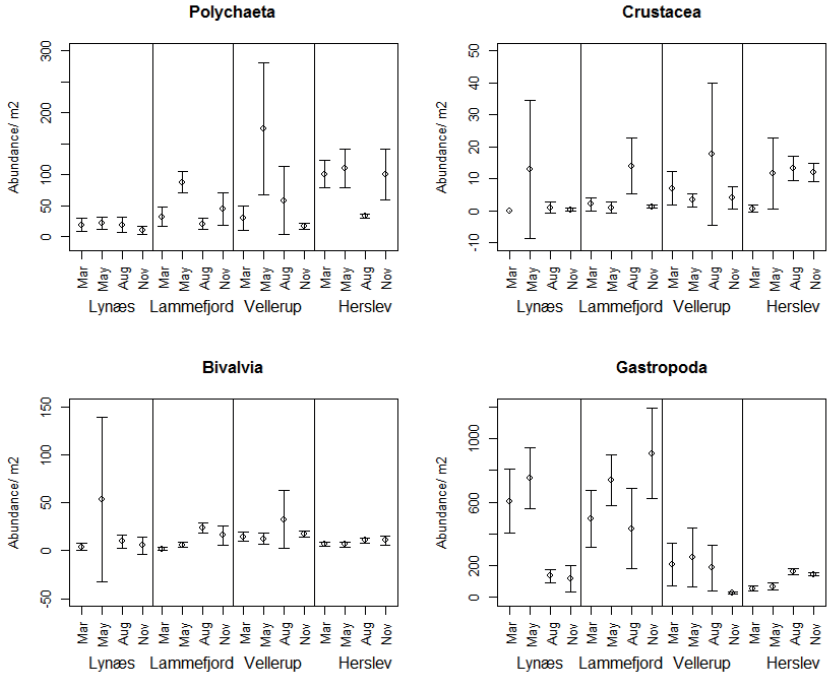

SUPPLEMENTARY FIGURE S1 Abundance / m² of different taxonomic groups sampled at each site and sampling time point: Polychaeta, Crustacea, Bivalvia and Gastropoda.

IV

ACUTE AND CHRONIC RESPONSE TO CHANGES IN SALINITY OF THE EURYHALINE POLYCHAETE *PYGOSPIO ELEGANS*

Thonig, A., Banta, G.T., Winding Hansen, B. & Knott, K.E. 2017.

Manuscript

ACUTE AND CHRONIC RESPONSE TO CHANGES IN SALINITY OF THE EURYHALINE POLYCHAETE *PYGOSPIO ELEGANS*

Thonig, A.[1,2], Banta, G. T.[2],Winding Hansen, B.[2], Knott, K. E.[1]

[1]Department of Biological and Environmental Science, University of Jyvaskyla, FI-40014 University of Jyvaskyla, Finland
[2]Department of Science and Environment, Roskilde University, DK-4000 Roskilde, Denmark

ABSTRACT

Estuaries are very harsh environments due to their fluctuations in salinity and other physico-chemical parameters such as temperature or oxygen. The distribution of species in an estuary is thus determined by the ability to cope with these abiotic conditions and their fluctuations as well as biological interactions, e.g. competition. The spionid polychaete *Pygospio elegans* is common in fully marine and brackish environments with salinities as low as 5. Moreover, it is commonly found in estuarine habitats that show strong salinity fluctuations. In this study we investigated the capacities of *P. elegans* to cope with changing salinities on an acute as well as a long-term time scale. The specimens investigated originated from a population experiencing salinities of on average 13.6 ± 2.1 and were thus exposed to salinity 15 as control and 5 and 30 as low and high salinity treatments, respectively. In the acute response we measured body volume, tissue water content as well as gene expression of seven genes of interest within 4 hours of exposure. As the long-term response we monitored growth, survival and reproduction within 6 weeks after gradual salinity change. Increase of body volume that could not be fully restored, increased mortality, and no clear change in expression levels in response to hyposmotic medium indicate that *P. elegans* might be a weak cell-volume regulator and cannot cope well with sudden drops in salinity. Gradual changes in salinity, in contrast, seemed to be less stressful. Although slightly increased mortality and reduced or delayed maturity were observed at salinity 5, the tested salinities seemed to be within the tolerance range for specimens from this population.

INTRODUCTION

Estuaries are some of the most challenging habitats to organisms due to their salinity fluctuations. Estuarine organisms are exposed to daily and seasonal changes in salinity due to tides, river runoff and evaporation. Additionally, rain storms or other weather related instances can change salinities suddenly and unpredictably in coastal areas. Also daily and seasonal fluctuations in other physiologically important factors such as temperature and dissolved oxygen are common in estuaries (Oglesby 1981, Richmond and Wooding 1996, Henry 2012). Due to these unpredictable physico-chemical conditions, the number of species able to inhabit estuaries is lower than that in stable marine or freshwater habitats, yet the abundance of a given species can be high (Remane 1934, Barnes 1989). Although mean salinity is often used to define species distribution limits, Whitfield (2012) proposed that species distributions might be more influenced by salinity fluctuations given that changes in salinity are considered a greater physiological challenge than a given mean salinity level, *per se*.

Organisms use different strategies to cope with salinity fluctuations including escaping and avoiding unfavourable salinities, e.g. by closing shells or burrows, as well as osmotic and ionic regulation and cell-volume regulation (Kinne 1966, Pechenik *et al.* 2000). Osmoregulators maintain a stable osmotic concentration of extracellular body fluids despite changing external ion concentration, which can be achieved by mechanisms such as low ion and water permeability, production of hyposmotic urine or isosmotic urine with different ionic composition, and active ion transport via e.g. $Na^+K^+Cl^-$ cotransporter, Na^+/H^+ exchanger, and Cl^-/bicarbonate exchangers as well as Cl^- and K^+ channels and Na^+K^+-ATPase (Smith 1970, Henry 2012, Hauton 2016). In contrast, in osmoconformers the extracellular body fluid is isosmotic to the ambient medium. Cell-volume regulation is essential to ensure enzyme function for both osmoconformers and –regulators, but particularly for organisms experiencing a variety of salinities. Decreasing ambient salinity results in water inflow and swelling of cells; these will subsequently decrease the amount of osmolytes to adjust osmolality. Likewise, in response to increasing ambient salinities cells will increase the amount of osmolytes to counteract shrinkage. Osmolytes that can be actively regulated include inorganic ions as well as organic molecules, such as free amino acids and can differ between species and even tissues (Deaton and Pierce 1994, Henry 2012, Hauton 2016). Most marine invertebrates are osmoconformers, however a variety of crustaceans osmoregulate below salinities of 26 (Henry 2012), as do some polychaetes below salinities of 14-18 (Fritzsche 1995). Osmoregulation at low salinities is especially common among invertebrates living in brackish or coastal environments. For example, the polychaetes *Hediste limnicola*, *H. diversivolor*, *Allita succinea* and *Marenzelleria viridis* osmoregulate at low salinities, while *Nereis vexillosa* and Arenicolidae are osmoconformers even at low salinities (Oglesy 1965, Smith 1970, Fritzsche 1995).

Coping with low or changing salinities is energy demanding, and hence, as a direct energetic trade-off due to increased regulatory activities (cell-volume, ionic, osmotic), can reduce the amount of energy available for growth, development and reproduction (Kinne 1966, Haunton 2016). The polychaete *H. limnicola,* for example, can survive in freshwater but cannot reproduce (Oglesby 1965). However, reduced growth rates or fecundity could also be due to indirect effects of coping with changing salinity such as decreased rate of energy intake, increased activity levels, decreased efficiency of digestion or assimilation or altered concentrations of growth and reproductive hormones that can occur in response to salinity fluctuation (Kinne 1966, Pechenik *et al.* 2000). The salinity tolerance range of an organism, i.e. where survival, growth and reproduction occurs, can be either narrow (stenohaline) or broad (euryhaline). This range is not only determined by the salinity gradient and exposure time, but also the genetic background, physiological condition, stage of the life cycle, size as well as previous salinity history and other environmental parameters, such as temperature (Kinne 1966, Costa 1980). The tolerance range of an organism in turn affects the dynamics of the population and distribution of the species, since reduced growth rates can also delay maturity, ageing and reproductive potential (Kinne 1966, Smyth and Elliot 2016).

Pygospio elegans is a euryhaline spionid polychaete, with a distribution ranging from full marine habitats to brackish habitats with salinities as low as 5. Anger (1984) found that reproduction and survival of *P. elegans* was highest in brackish water, for populations originating from both brackish and marine habitats. Moreover, *P. elegans* is also found in estuaries where in addition to low salinities also salinity fluctuations are common (Rasmussen 1973, Gudmundsson 1985, Morgan *et al.* 1999, Bolam 2004, Kesäniemi *et al.* 2012, Thonig *et al.* 2016). Nevertheless, abrupt drops in salinity might be a challenge for *P. elegans* and diminish local populations. The temporal and spatial variation in genetic structure of *P. elegans* populations in the Danish Isefjord-Roskilde Fjord estuary complex could be the result of salinity fluctuations that lead to the extinction of local populations (Kesäniemi *et al.* 2014a, Thonig *et al.* in review). Moreover, such heterogeneous unpredictable environments might favour different types of larvae at different occasions, since a seasonal switch in developmental mode was described in two estuarine habitats (Rasmussen 1973, Gudmundsson 1985, Thonig *et al.* 2016), while in habitats with constant salinities *P. elegans* produced either benthic or pelagic larvae. Similarly, Anger (1984) could not detect an influence of constant changes in temperature and salinity on the mode of development. This suggests that salinity changes are important for this organism´s fitness. In the present study we investigated the response of *P. elegans* to acute and long-term salinity changes to elucidate whether large and at times unpredictable salinity changes in its estuarine environment can be related to its observed genetic patchiness and developmental polymorphism.

MATERIALS AND METHODS

We performed both acute and long-term exposure experiments to investigate the temporal aspect of the physiological response of the polychaete *Pygospio elegans* to changing salinities. The acute experiments simulated sudden changes in salinity and here we recorded changes in body volume, water content and expression of selected genes after exposure to a change in salinity for a maximum of 4 hours. The long-term experiment lasted six weeks and investigated the effects of salinity changes on survival, growth and reproduction. Seawater of different salinities was produced by mixing natural seawater from a general tank at Roskilde University (salinity 34) with tap water. Specimens of *Pygospio elegans* were collected during summer in 2015 and 2016 at Herslev in the Roskilde Fjord in Denmark (N 55°40´ 41.29´´, E 11° 59´ 13.07´´) (see Supplementary Table S1 for setup and exact sampling dates). Roskilde Fjord is one part of the Isefjord-Roskilde Fjord estuary complex and represents a typical estuary that exhibits a decreasing salinity gradient from the entrance to the interior, as well as highest salinities in summer. Salinity is mainly influenced by freshwater runoff from land and by wind driven water movements, while tides in this microtidal estuary are of no importance (Rasmussen 1973). Salinity and temperature were monitored at this site previously, from March 2014 until February 2015 using data loggers (Fig. 1) (for details see Thonig *et al.* 2016). Salinity during this period was on average 13.6 ± 2.1. However, on several occasions salinity reached values beyond this range, and at extreme events dropped even below a salinity of 5. Decreases in salinity could last from several hours up to a couple of weeks and reductions by about 11 salinity units could be reached within 1-6 hours.

Body volume

Specimens were collected on the 11th of July and 1st of August 2015 at 22˚ C and salinity 15 (measured with a hand-held refractometer on site) and kept at 12˚ C and salinity 15 in the laboratory until needed. Experiments were performed in two blocks with newly collected individuals to ensure that the individuals were in good physiological condition. In each block five individuals were transferred from salinity 15 directly to 5, 30 and 15 (as a control). The experiment was performed at room temperature and the order of the salinity exposures was random. One individual at a time was placed in a 1.6 ml well of 11 mm diameter containing seawater of the respective salinity under a dissecting microscope (Nikon, RAMCON A/S Birkerød, DK). A cover slide was applied to avoid evaporation and a dark chamber surrounded the well to avoid light disturbance of the animal. A time lapse video was recorded, taking one picture per second for 30 seconds immediately and then after 5, 10, 20, 30, 45, 60, 90, 120, 150, 180, 210, 240 minutes of exposure. Several pictures per time point were taken to account for potential volume differences caused by movement of the individual. Additionally, pictures were taken before exposure as well as once

per day for one week after exposure. Only pictures in which worms were still and seemed relaxed were used. A camera mounted on the dissecting microscope and the software NIS BR v. 4.2 (Nikon, RAMCON A/S Birkerød, DK) were used to measure the length (L) and the width (W) of the 5th setiger for each picture automatically, at each time point for every individual. In cases when specimens curled up, manual measurement was needed and hence only 5 pictures for each time point were measured. We assumed an approximate cylinder shape of the worms and calculated the volume as $V = L * (W/2)^2 * \pi$ per picture and the average volume per time point per individual.

We used a linear mixed model to predict body volume with treatment as categorical fixed factor and individual ID as random factor to account for the repeated measures design and different initial size of the individuals. For that purpose we used the function lmer in the R package lme4 (R Core Team 2017). We conducted one set of planned contrasts so that each time point at salinity 5 and 30 were compared to the respective time point at salinity 15 to investigate the effect of salinity compared to the control treatment over time. Furthermore, we performed a second and third set of planned contrasts to compare each time point to the initial as well as the previous time point within each salinity treatment. P-values were derived from the Student t-distribution. The assumption of variance homogeneity between treatments for linear models was confirmed by Levene´s test and the residual plot. The QQ-plot of the residuals indicated that the error is not normally distributed and log-transformation could not improve this behavior. However, since size data usually fits a normal distribution best, we pursued a linear model design with untransformed response variable.

Tissue water content

Pygospio elegans were collected at Herslev on the 1st of August 2015 and on the 5th and 6th of September 2016 at about 22° C and salinity 14. The worms´ water content was determined in three replicates of a group of 28 to 32 individuals after five different treatments. Individuals were (1) taken from original salinity of 14 or exposed to (2) salinity 5 for 45 min, (3) salinity 5 for 4 hours, (4) salinity 30 for 45 min and (5) salinity 30 for 4 hours at 12° C in a 6 well plate. Wet weight was measured in mg to the third digit after removing surplus water with filter paper and dry weight was determined after two hours at 60° C and subsequent 30 min in a desiccator. The tissue water content was calculated as the percentage of weight loss per wet weight.

We used the function lm in R to fit a linear model for the response variables, % weight loss as well as dry weight, with treatment as categorical fixed factor (R Core Team 2017). Planned contrasts were defined to compare the effect of changing salinity to control salinity 15 and whether the effect differs between time points. Visual inspection of the QQ-plot of the residuals and the residual plot did not show major deviation from the assumed normal distribution of the residuals and variance homogeneity.

RNA expression

Individuals were collected on the 11th of July and 1st of August 2015 at 22° C and salinity 15, together with specimens used in the body volume experiment. In the laboratory, 10 individuals were separately transferred from the original salinity of 15 to each treatment in 24 well plates and kept at room temperature. The treatments included: (1) salinity 5 for 45 min, (2) salinity 5 for 4 hours, (3) salinity 30 for 45 min, (4) salinity 30 for 4 hours as well as (5) salinity 15 for 45 min and (6) salinity 15 for 240 min as controls. After exposure, individuals were preserved in RNAlater™ (Qiagen) and stored at -80° C until further processing. RNA was extracted in March/April 2017 from whole individuals using the Ambion RNaqueous Micro kit according to the manual. Samples were disrupted with a pestle after placed in the lysis solution and elution was performed in two steps with 10 µl elution buffer. DNA was removed using DNase I and samples were stored at -80° C. RNA concentration was measured with the Qubit RNA Assay Kit and Fluorometer 2.0 (Thermo Fisher Scientific). We performed cDNA synthesis using iScript cDNA synthesis Kit (BioRad) standardizing the RNA input to 50 ng. Primers for our targets of interest were designed based on the transcriptome data of *P. elegans* using Primer 3 (Heikkinen *et al.* 2017, GenBank: GFPL00000000.1, Table 1). We chose to measure the expression of genes involved in different aspects of the physiological response to salinity changes, including ion transport, amino acid and aerobic metabolism, cell signalling and formation of the cytoskeleton. The PCR product of each primer pair was verified using Sanger sequencing and blastx at NCBI.

Gene expression was measured using the QX200TM digital droplet PCR system from Bio-Rad. For that purpose a PCR reaction of 22 µl was prepared, containing 1x QX200TM ddPCRTM EvaGreen Supermix, 100 nM of forward and reverse primers and 2 µl of 1/10 diluted cDNA. The reaction mix was transferred into DG8TM Cartridges along with 70 µl QX200TM Droplet Generator Oil for EvaGreen and an emulsion of droplets each with a volume of about 1 nl was produced using the QX200TM Droplet Generator. Afterwards, droplets were carefully transferred into a 96 well plate which was then sealed using a PX1 PCR Plate Sealer (Bio-Rad). A C1000 Touch thermocycler (Bio-Rad) was used with the following program set with a ramp rate of 2° C/sec: initially 5 min at 95° C, followed by 40 cycles of 95° C for 30 sec, 60° C for 1 min and 72° C for 45 sec, finally 4° C for 5 min and 90° C for 5 min. Subsequently, the ddPCR plate was transferred to the QX200™ Droplet Reader that detects the fluorescence amplitude for each droplet. The R script ddpcRquant was used to correct the baseline of fluorescence intensities between samples and to determine the threshold between negative and positive droplets using extreme value theory on the negative controls that contained no cDNA (Trypsteen *et al.* 2015, R Core Team 2017). Subsequently Poisson statistics were used to calculate the absolute concentration of template in the initial sample from the ratio of positive and negative droplets. We used samples only when droplet numbers were larger than 6,000. On average samples contained 13,000 droplets. One

negative control per 7 samples was included on the plates. Due to the high reproducibility and repeatability of the ddPCR system (Huggett *et al.* 2013) no analytical replicates were included. The absolute expression data was not normalized to a reference gene since we controlled input RNA concentration in cDNA preparation and we assume that the baseline correction also normalizes sample to sample variation in the amount of input material. The genes IGF and carbonic anhydrase exhibited two data clouds with different fluorescence amplitude in the ddPCR analysis, indicating isoforms or different splice variants. We selected only the cloud with higher fluorescence for our analyses.

We fitted linear models for the expression of each gene of interest using treatment as a categorical fixed factor. We performed planned contrasts to investigate the difference 1) between salinity 5 and 30 to 15 at the respective time point and 2) between the time points 45 and 240 min after exposure at the respective salinities. The assumption of variance homogeneity was met for every gene according to Levene´s test and inspection of the residual plots. Normal distribution of the residuals was confirmed via QQ-plots of the residuals. Additionally, we performed a two-way PERMANOVA in PRIMER-E v.6 (Clarke and Gorley 2006) to analyse the effect of salinity and time on all genes of interest at the same time. Since the expression of the different genes was not on comparable scales, we normalized the data per gene by subtracting the mean and dividing by the standard deviation of the gene for each sample per gene (Clarke and Gorley 2006). Afterwards, a resemblance matrix based on Euclidian distance was created and used for the basis of the PERMANOVA analysis.

Long-term experiment

Worms were collected on the 8th of June 2015 at 19.5° C and salinity 15. We used six replicate beakers each with 30 randomly chosen individuals per salinity treatment: 5, 15 (control) and 30. Beakers had a diameter of 7.5 cm resulting in a density of 6790 individuals per m^2, which represented a density that at that time of the year was observed at the sampled site in the field (Thonig *et al.* 2016). Beakers were filled with 1.5 cm sediment from the field, which was sieved with a 1mm mesh, then frozen and rinsed with seawater of the respective salinity after thawing. Natural seawater of the respective salinity was added to the beaker up to 4 cm above the sediment. Water was gently replaced by freshly diluted seawater once a week, disturbing the sediment and specimens as little as possible. The worms were kept at 12˚ C, which represent the temperatures experienced in April/May and September/October when reproduction takes place (Thonig *et al.* 2016). According to Anger *et al.* (1986) *P. elegans* prefer static culturing conditions, hence no oxygen bubbling was applied, but a lid was placed on the beakers to avoid evaporation. The worms were fed three times a week with 100 µl of a 1:3 dilution of Invertfood (SeAquariums) that was added to the water and allowed to settle on the sediment surface. Worms were gradually acclimated to their treatment salinities by exposing them to salinity 10, 15 and 22 respectively for three days before adjusting the salinity to the final

value and starting the experiment. After 3 weeks and after 6 weeks three beakers of each treatment were sampled destructively. For that purpose the sediment was sieved with a 0.5 mm mesh and sand tubes of *P. elegans* as well as worms were removed. Afterwards, sediment was additionally checked using a dissecting microscope. We documented the number of survivors, reproductive individuals, asexually reproducing individuals, length from head until beginning of gills of each individual and the presence of larvae. Additionally, the product of number of survivors and mean size was calculated as a proxy for total worm biomass.

We fitted generalized linear models to the different response variables with planned contrasts between treatments: 1) salinity 5 and 30 compared to 15 at the respective time points and 2) between the time points 3 weeks and 6 weeks exposure at the respective salinities. Survivors, mean length and biomass fulfilled the assumptions of variance homogeneity and normal distribution for a linear regression. Number of asexually and sexually reproducing individuals were treated as count data so that we fitted a Poisson and a negative binomial regression. According to the Akaike information criterion (AIC) as well as a small over-dispersion parameter, theta, a negative binomial regression was the best choice for these response variables. The presence and absence of larvae represents binomial data, hence a logistic regression was used. The functions lm, glm.nb and glm in R were used to fit the models (R Core Team 2017). Similar to the RNA expression study, we performed a 2-way PERMANOVA in PRIMER-E v.6 to investigate the effect of salinity and exposure time on the overall performance, including all response variables measured (Clarke and Gorley 2006). For that purpose, we normalized the data and created a resemblance matrix based on Euclidian distance.

RESULTS

Body volume and tissue water content

Body volume in response to acute salinity change is very dependent on the individual, probably including size and condition of the individual, since 71 % of the variation could be explained by individual (Table 2 and Fig. 2). Significant volume differences due to acute salinity change could only be observed when exposed to salinity 5, but not to 30. Already five minutes after exposure to salinity 5 the volume of individuals was 1.7 fold larger than that of individuals at control salinity 15. This increase in volume reached its maximum 45 min to 3 hours after exposure, being about 2.3 fold larger, and dropped back to 1.6 fold increase after one day (1444 min) of exposure. Comparing volume during the treatment to the measurements made before exposure revealed that the volume at salinity 5 differed significantly from the initial measurement at all time points, peaking at 30-120 minutes after exposure with a 2.7 fold increase over initial size, and remained about 1.7 fold larger than the initial

measurement even after 7 days. Note that at the control salinity 15 the body volumes measured 1, 2, 3, 4, and 6 days after exposure were also (marginally) significantly higher than that measured initially. This increase might be the reason why we did not detect a significant difference between salinity 5 and 15 at these later time points anymore even though the volume at salinity 5 still differs from the initial size. There were, however, no significant changes in volume for worms at salinity 15 during the first 4 hours, in contrast to the largest changes seen for the worms at salinity 5. Comparing every time point to the previous one, revealed a significant increase from initial measurement to one minute after exposure and a significant decrease from day 1 to day 2 after exposure at salinity 5. There were no statistically significant changes in body volume at salinity 30. Five specimens died within one week at salinity 5: one after 4 days, one after 5 days and three after 6 days. One individual also died at salinity 30 after 5 days.

The tissue water content (measured as % weight loss) changed significantly when individuals were exposed to both salinity 5 or 30, but in opposite ways (Table 3 and Fig. 3). We observed an increase of 9.5 % when worms were transferred to salinity 5 and a decrease of 11.5 % when they were transferred to salinity 30. The effect weakened slightly over time but was significantly different from salinity 15 at 45 min and 240 min after exposure for both groups. At salinity 5 the water content decreased by 1.3 % from 45 min to 240 min after exposure and at salinity 30 the water content increased by 2.5 % from 45 min to 240 after exposure. We used the same model with dry weight as response variable to investigate whether size of the individuals differed between treatments. The dry weight of samples at salinity 5 was marginally smaller and the dry weight of samples at salinity 30 was significantly larger than the samples at salinity 15 processed the previous year (Table 3 and Fig. 3).

RNA expression

Gene expression of seven genes of interest is illustrated in Fig. 4. We observed high variation within treatments resulting in significant differential expression in only four comparisons in three genes (Table 4a). The expression of bicarbonate exchanger showed a significantly lower expression after 240 min at salinity 30 than after 240 min at salinity 15 (by -812.1 copies μl^{-1}). Although we did not expect any changes within the control treatment salinity 15 over time since no change in salinity occurred, the expression of bicarbonate exchanger also showed a significantly higher expression after 240 min compared to 45 min at salinity 15 (by 816.2 copies μl^{-1}). Expression of IGF was significantly lower after 45 min at salinity 30 than after 45 min at salinity 15 (by -632.6 copies μl^{-1}). Na^+K^+-ATPase was expressed in significantly lower amounts after 240 min at salinity 5 than after 240 min at salinity 15 (by -2746.3 copies μl^{-1}). Taking all genes together, we did not detect any overall effect on gene expression due to salinity (p-value 0.102), temperature (p-value 0.142) or an interaction between these two factors (p-value 0.634) using PERMANOVA (see Table 4b).

Long-term experiment

The performance of *P. elegans* after long-term changes of salinity can be found in Fig. 5 and Table 5a and 5b. The number of individuals at salinity 15 and 30 increased from the initial number of 30 specimens after 3 weeks and further after 6 weeks, while at salinity 5 the number of individuals decreased significantly between 3 weeks and 6 weeks of the experiment. Interestingly, the mean size of individuals at salinity 5 increased, but size remained constant at salinity 30 and even decreased at salinity 15 between 3 weeks and 6 weeks. Hence, an increase in biomass, i.e. survivors * mean length, from 3 to 6 weeks was only observed at salinity 30, while it remained constant at salinity 15 and decreased at 5. One source of the increase in number but decrease or stagnation in size, as seen at salinities 15 and 30, could be asexual reproduction. Individuals performing asexual reproduction were more frequent at salinities 15 and 30, especially at salinity 15 after 6 weeks. Likewise, the number of sexually reproducing individuals was higher at salinities 15 and 30 compared to 5, with a slight increase from 3 to 6 weeks. This is also evident in the presence of larvae, which were found only at salinities 15 and at 30 after 6 weeks. Even though these trends are visible, only a few differences were statistically significant. Moreover, we could only detect a marginally significant effect of salinity (p-value 0.094), while no effect of time point (p-value 0.872) or the interaction between salinity and time point (p-value 0.772) could be observed via PERMANOVA considering all response variables at once. Pairwise comparisons of salinity levels revealed a significant difference between salinity 5 and 15 (p-value 0.04) as well as between 5 and 30 (p-value 0.012) (see Table 5b).

DISCUSSION

The euryhaline polychaete *Pygospio elegans* inhabits full marine and brackish habitats as well as estuaries that are exposed to salinity fluctuations due to tides, freshwater runoff and rain storms. In this study we analysed the capability of *P. elegans* to cope with the acute and chronic changes in salinity on different levels. The population we studied is exposed to salinities of about 13.6 ± 2.1 during the year, therefore, we chose salinity 5 as hyposmotic exposure and 30 as hyperosmotic exposure in both acute and long-term exposure experiments. Considering that other annelids are known to osmoregulate below salinities of about 14-18 (Fritzsche 1995), this population of *P. elegans* might already express adaptations to low salinities.

Acute response: volume regulation and compatible solutes

Volume regulation of cells is essential, especially for osmoconformers but also for osmoregulators, for maintaining cell membrane integrity and enzyme

function. *Pygospio elegans* increased in volume as well as water content in response to the hyposmotic environment but restoration of initial size was initiated after 150 min of exposure. However, the initial volume could not be restored within seven days, when a 1.7 fold increase in size was still apparent. Similarly, Costa *et al.* (1980) described a volume increase of 50-70 % in coelomocytes of the polychaete *Glycera dibranchiata* after hyposmotic exposure within 2-10 minutes depending on the temperature. After 20-120 min the volume was restored only partly to about 40-60 % increase of the initial size. The osmoregulator *Alitta succinea* increased in wet weight for 0.75 to 2 hours after hyposmotic exposure, but reached an acclimation state after 22-26 hours with a weight only 10 % higher than the initial one (Dykens and Mangum 1984). *Nereis vexillosa*, an osmoconforming nereid, on the other hand was described as swollen, turgid and immobile after exposure to hyposmotic medium for seven days, which indicated a failure of volume regulation (Oglesby 1965). Since half of the individuals of *P. elegans* exposed to salinity 5 died within one week and the initial volume could only be restored partly within this time, *P. elegans* seems to be a weak volume regulator and is negatively impacted by abrupt drops in salinity.

The response of *P. elegans* to a hyperosmotic environment showed differences in the body volume and tissue water content experiments. No change in volume was observed at salinity 30, which is supported by Oglesby (1981) who proposed that inflow of water after hyposmotic exposure is usually greater than water loss after hyperosmotic exposure. Yet, a drop in tissue water content after exposure to salinity 30 was evident, and this change in water content was larger than after the transfer to salinity 5. Likewise, Fletcher (1974a) observed an increase in wet weight in hyposmotic medium and a decrease in wet weight in hyperosmotic medium in *Hediste diversicolor* and the weight change was slightly more pronounced in the hyperosmotic exposure. The acute exposure to a hyperosmotic medium of salinity 30 seems less stressful than the acute exposure to a hyposmotic medium of salinity 5 for our population of *P. elegans* adapted to salinity 15. The fact that individuals differ in their response to salinity changes becomes obvious in the volume experiment as individual explains 71 % of the variation. Although marginal difference in dry weight between the treatments and the control could be detected in the tissue water content experiment, we assume that different initial worm sizes did not cause the different results of the tissue water content and body volume experiment related to salinity exposure. Sample dry weights in both treatments were larger than in the control at salinity 15 to a similar extent, indicating that the treatments should be alike regarding possible biases introduced by size variation. The deviating results between body volume and tissue water content experiment may demonstrate that individuals respond in different ways to salinity changes, which is not only explained by size but also other factors, such as condition or previous experiences (Kinne 1966).

Volume regulation is achieved by changing the concentration of osmolytes, such as ions and free amino acids, in the cells. The concentration of free amino acids can be altered via amino acid transport or metabolism (Dykens

and Mangum 1984). Among other amino acids, concentrations of alanine, proline, taurine and glycine changed in polychaetes in response to salinity changes, although the free amino acid composition can differ between species and tissues (Costa 1980, Hoeger and Abe 2003, Blank 2004). The expression of alanine aminotransferase, which catalyses the synthesis of L-alanine from glutamate and pyruvate (Blank *et al.*2004), indeed decreased in *P. elegans* after 45 min in salinity 5 and increased after 45 min in salinity 30, indicating an adjustment of amino acid concentration in order to regulate the volume. However, these differences were not significant and reversed after 240 min of exposure. A decreased expression of enzymes involved in amino acid metabolism or amino acid transport was described in molluscs and crustaceans in hyposmotic medium after exposures ranging from 4 hours up to 10 days (Lockwood and Somero 2011, Zhao *et al.* 2012, Lv *et al.* 2013) as well as an increased expression in hyperosmotic medium (Li *et al.* 2014). However, Hu *et al.* (2015) described an increase in expression of an amino acid transporter in the shrimp *Litopenaeus vannamei* 24 hours after transfer to hyposmotic medium. Consequently, alterations in gene expression in response to salinity change likely varies among species. Furthermore, cell swelling after transfer to hyposmotic medium is expected to increase the expression of the proteins actin and tubulin, components of the cytoskeleton (Deaton and Pierce 1994, Lang 2007). Zhao *et al.* (2012) described such an increase in expression of actin and tubulin in the Pacific oyster in hyposmotic medium. Nevertheless, *P. elegans* did not show any significant change in tubulin expression. Thus, also the lack of a clear molecular response in alanine amino transferase and tubulin to salinity changes supports a weak cell-volume regulation in *P. elegans*.

Acute response: ionic and osmotic regulation

Osmoregulating organisms maintain the osmolality of their body fluid in hyposmotic medium via decreased permeability, hyposmotic urine and active ion transport (Smith 1970, Henry 2012). The activity of ion transport pathways during osmoregulation have been investigated in detail for crustaceans (Henry 2012). Accordingly, active uptake of NaCl takes place via cation/proton (Na^+/H^+) and anion/bicarbonate (Cl^-/HCO_3) exchangers in apical cell membranes. These transporters are coupled with the cytoplasmic carbonic anhydrase, which catalyses the production of the substrates for both transporter, H^+ and HCO_3^-, from water and CO_2. Additionally, $Na^+/K^+/2Cl^-$ cotransporter in combination with K^+ and Cl^- channels are also involved in NaCl uptake. Respectively, Lv *et al.* (2013), Towle *et al.* (2011) and Havird *et al.* (2013) described increased expression of carbonic anhydrase under hyposmotic conditions as well as its decreased expression under hyperosmotic conditions. In contrast, there were no clear changes in expression of Na^+/H^+ exchanger and anion/HCO_3 transporter in the shore crab *Carcinus maenas* within 24 hours of hyposmotic exposure (Towle *et al.* 2011). *Pygospio elegans* showed an opposite trend in its expression of carbonic anhydrase, with an increase in expression under hyperosmotic conditions and a decrease when under hyposmotic

conditions. The expression of bicarbonate exchanger showed a significant decrease after 240 min in salinity 30 compared to 15. This might be a consequence of the unexpected increase of expression of bicarbonate exchanger in the control salinity 15 over time.

In both routes of NaCl uptake, via the Na^+/H^+ and Cl^-/HCO_3 exchanger or the $Na^+/K^+/2Cl^-$ cotransporter, Na^+K^+-ATPase is needed in the basolateral membrane to transfer ions into the extracellular fluid and to maintain the electrochemical gradient within the cell (Henry 2012). Accordingly, the expression of Na^+K^+-ATPase was described to increase under hyposmotic (Lovett *et al.* 2006, Towle *et al.* 2011, Havird *et al.* 2013, Hu *et al.* 2015) as well as hyperosmotic conditions (Havird *et al.* 2013). However, we detected a significant decrease in expression of Na^+K^+-ATPase in *P. elegans* 240 min after transfer to salinity 5 compared to 15, which is in agreement with the results of Zhao *et al.* (2006), Lockwood and Somero (2011) and Lv *et al.* (2013).

A change in salinity is expected to invoke a cellular osmoregulatory response, which is initiated via cell signalling pathways and is energy demanding. Accordingly, an increased expression of signalling proteins, such as IGF and EIF (Zhao *et al.* 2012, Li *et al.* 2014) as well as enzymes involved in the electron transport chain or citrate cycle, such as ATP-Synthase, cytochrome C oxidase and malate dehydrogenase, are expected in response to changing salinities (Lockwood and Somero 2011, Towle *et al.* 2011). Surprisingly, *P. elegans* showed no differential expression in ATP-Synthase in response to altered salinities, while the expression of IGF decreased 45 min after transfer to salinity 30 compared to 15.

There is no clear, sustained pattern apparent in the gene expression of *P. elegans* in response to salinity changes. One reason could be a too short exposure time of maximum 4 hours, particularly as two out of three of the observed significant differences occurred only 4 hours after exposure, at the end of our experiment. Similar studies were able to detect changes in expression over time periods ranging from 2 hours up to 10 days after exposure. Additionally, immediate responses to salinity changes can also be regulated on translation or protein level. So, longer exposure time might have been needed for observing a clearer gene expression response. Nevertheless, Havird *et al.* (2013) found in their meta-analysis that a response of Na^+K^+-ATPase and carbonic anhydrase could be seen already 1 hour after exposure. Most of the previous studies on gene expression responses following salinity stress have been performed with crustaceans and a few molluscs, however. Even though those studies included both osmoconformers and osmoregulators it is not clear whether similar results should be expected for polychaetes, considering that even within crustaceans there are inconsistent gene expression responses. Additional variation can also be introduced via inconsistencies during RNA extraction and cDNA synthesis (Huggett *et al.* 2013). However, replication of these procedures on individual basis was not possible due to the small size of our specimens.

The expression study does not give any clear indication that *P. elegans* osmoregulates at low or high salinities. However, to clarify whether *P. elegans*

acts as an osmoconformer or osmoregulator, we would also need measurements of the osmolality of the body fluid in response to changing ambient salinities. Among polychaetes both osmoconformers such as Arenicolidae, *Alitta virens* and *Nereis vexillosa*as well as osmoregulators at low salinities such as *Marenzelleria viridis*, *Hediste diversicolor*, H. *limnicola* and *Alitta succinea* can be found (Smith 1955, Hohendorf 1962, Oglesby 1965, Dykens and Mangum 1984, Fritzsche 1995, Bastrop *et al.* 1997).

The distribution of a species in the estuary is determined by its ability to cope with low salinities as well as the competition with other species (Oglesby 1965). Hereby, the lack of mechanisms to regulate the osmotic concentration of extracellular fluid does not prevent organisms from occurring in estuaries, since osmoregulators such as *Carcinus maenas* and *H. diversicolor* as well as osmoconformers such as the bivalves *Mytilus edulis* and *Crassostrea gigas* are commonly found (Henry 2012, Fritzsche 1995). Although the latter two species represent strong volume regulators, in contrast to *P. elegans*, high summer mortality due to sudden rain falls have been described for these economically important species (Zhao *et al.* 2012). During our field survey 2014/2015 in the Isefjord-Roskilde fjord estuary complex many dead specimens were found on three occasions (Thonig *et al.* 2016). One of these, namely at Lammefjord in January, dead individuals were found directly after a low salinity event (from salinity 22 down to 2). Yet, at Lynæs in July no drop in salinity was detected before sampling and unfortunately no salinity data are available for Lammefjord in July. The high mortality we observed at these occasions could be due to senescence or salinity induced stress (Thonig *et al.* 2016). If *P. elegans* does not exhibit any behaviour to avoid exposure to acute changes in salinity such as digging deeper into the sediment or closing their tubes these events might thus diminish local populations.

Chronic exposure: tolerance range and impact on population dynamics

We analysed ecological consequences of chronic exposure of *P. elegans*, originating from a salinity of about 14, to different salinities over a period of six weeks. Survival and mean body length were similar after six weeks at salinity 15 and 30, hence also their product - our proxy for biomass - did not differ. There were no negative population-level effects of a long-term increase in salinity on this population of *P. elegans*. Number of individuals showing signs of asexual reproduction and number of reproductively mature individuals, however, were higher in salinity 15. Egg strings were found in two beakers after six weeks at salinity 30 while only in one beaker egg strings were found at salinity 15. Our results are supported by Anger (1984) who observed highest reproduction of *P. elegans* in brackish water for populations originating from both brackish and marine habitats. She detected a similar response for survival, being highest in brackish water compared to full strength seawater regardless of the original salinity. Likewise, *Capitella* sp. I showed higher fecundity at intermediate salinities (Pechenik *et al.* 2000).

In contrast, survival, biomass, sexual maturity and asexual reproduction were lower in salinity 5 compared to 15 and 30. Although these responses did not differ significantly when taken separately, PERMANOVA indicated a significant difference in the overall response between salinity 5 and 15 as well as 5 and 30. According to Anger (1984), salinity 10 is well within the tolerance range of *P. elegans* originating from salinities 10-32, but experiments exposing *P. elegans* to lower salinities have not been performed previously. Reduced fecundity is expected to be a response to altered salinity, with sexual reproduction being suppressed more so than asexual reproduction (Kinne 1966). Moreover, decreased growth and development in juveniles and longer time to produce broods are expected to be sub-lethal effects of altered salinity for different polychaetes (Kinne 1966, Qui and Qian 1997, Pechenik *et al.* 2000). Before we started the long-term experiment we characterized a subsample of 57 individuals, which had a mean length of 1588 µm, and included only 2 reproductively mature females. Compared to this initial sample, specimens exposed to salinity 5 did grow in the first three weeks and also in the following three weeks, although to a lesser degree. Furthermore, juveniles matured into ripe males and gravid females even though they did not produce egg strings during the experiment. Although the number of survivors was indeed lower in salinity 5 than in 15 and 30, a minimum of 27 (of the original 30) were still present after six weeks. Thus, salinity 5 might not represent an optimal salinity for *P. elegans*, but it is still within the tolerance range based on our results. Indeed, populations of *P. elegans* are present in salinities as low as 5-8, e.g. at Hanko and Fårö, northern Baltic Sea (Kesäniemi *et al.* 2012, 2014b). To see whether the population we tested could persist at a salinity of 5, even longer exposure including reproductive success and a turnover of generations would be necessary. Moreover, to investigate whether populations that are able to persist at salinities of 5 show adaptations to these low salinities, their response to long-term exposure of salinity 15 and 30 needs to be investigated as a comparison. Such an experiment lasting over 14 months was performed by Anger (1984) for populations originating from salinities 10, 16 and 32, and indicated that no adaptations are present yet, since similar survival and reproduction rates were found. For the persistence of a population the tolerance ranges of all life stages are of importance and usually early life stages exhibit the narrowest tolerance ranges (Kinne 1966, Fritzsche 1995, Qui and Qian 1997, Pechenik *et al.* 2000). For example, Smith (1964) demonstrated that larvae of *H. diversicolor* show different salinity tolerances in seawater of salinity 20 and 5. In this respect the viviparous lifestyle of *H. limnicola* might serve as an adaptation since juveniles are released at a stage with full osmoregulatory capacity (Oglesby 1965), while egg capsules seem to slow the rate of salinity changes when changes cannot be avoided (Richmond and Woodin 1996). Whether or not the larvae of *P. elegans* have different salinity tolerance ranges is unknown. However, only brooded larvae (in egg capsules) have been observed in the population studied here (Thonig *et al.* 2016) and other populations found at lower salinities (e.g. Kesäniemi *et al.* 2014a, b). Thus, we could hypothesize that

protecting larvae in egg capsules until a later developmental stage might serve as an adaptation against low salinities and salinity fluctuations.

In summary, we can see from our long-term experiment that the population of *P. elegans* we studied can survive and probably be sustained at both extreme salinities (5, 30) tested if transferred gradually, although with a somewhat lowered fitness at salinity 5. Abrupt changes in salinity that can occur in estuaries or shores, with low water levels and sudden rain storms seem to be more detrimental, and such episodes might lead to the extinction of local populations. These metapopulation dynamics, with extinction and re-colonization of subpopulations might be the reason for the observed chaotic genetic patchiness and developmental polymorphism in the Isefjord-Roskilde-Fjord estuary complex (Kesaniemi *et al.* 2014a, Thonig *et al.* 2016).

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TABLES

TABLE 1 Genes of interest for the analysis of RNA expression after acute salinity changes: target name, assumed function, primer sequences and reference contigs to *P. elegans* transcriptome (Heikkinen *et al.* 2017).

Target of interest	Function	Primers	Transcript
1) Alanine Aminotransferase	Amino acid metabolism	F: CAGCCCATCACCTTCCTCAG R: ACCGATACTGCCTCCTTTGC	TR58395|c1_g5_i1
2) ATP Synthase	Aerobic metabolism	F: AGCGTATCACCACCACCAAG R: ACCCAGCTCAGCAATACCAC	TR94952|c0_g2_i1
3) Bicarbonate Exchanger	Ion transport	F: TGCGGACGAGTCACTGAAAA R: ACGCAGGCAAAGTACAGGAA	TR53126|c2_g1_i2
4) Carbonic Anhydrase	Ion transport (indirect)	F: ATGGCCCTAACACATGGAGC R: GTTTATCAGCTTCCCGGCCT	TR56167|c0_g1_i2
5) Insulin-like growth factor (IGF)	Cell signaling	F: GACGACACCAACCCATCTGT R: GCACTTTCATGGCTGTCCTC	TR63322|c1_g1_i5
6) Na^+K^+-ATPase	Ion transport	F: CCTCGAACAAACATGGCGTC R: GCTCGTCCATCTCCATCTCC	TR66219|c3_g1_i2, TR66219|c3_g1_i1
7) α-Tubulin	Cytoskeleton	F: CATCCTGACGACACACACCA R: TCAGATCCACATTGAGGGCG	TR41952|c0_g1_i2

TABLE 2 Linear mixed model with response variable body volume and explanatory variable treatment as categorical fixed effect and individual as random effect. Three different planned contrasts were conducted: 1) comparing volume at every time point to the initial measurement of the respective salinity, 2) comparing volume at every time point to the previous time point of the respective salinity and 3) Comparing salinity 5 and 30 to salinity 15 at the respective time point

Random effects:			
Groups	Variance	SD	% variation
ID (Intercept)	1.55E+16	124328899	0.7171
Residual	6.10E+15	78093457	0.2829

Fixed effects:	Within salinity, time point to initial measurement			
	Estimate Δ	Std. Error	t-value	p-value
15-0 (Intercept)	207117401	46428723	4.461	***0.000***
treatment15-1	31556117	34924456	0.904	0.366
treatment15-5	-3155206	34924456	-0.09	0.928
treatment15-10	-3639240	35933690	-0.101	0.920

treatment15-20	2515979	35943725	0.07	0.944
treatment15-30	17781573	34924456	0.509	0.611
treatment15-45	2344953	35939828	0.065	0.948
treatment15-60	16924624	34924456	0.485	0.628
treatment15-90	9415090	37152663	0.253	0.800
treatment15-120	5188128	35943725	0.144	0.886
treatment15-150	16383185	35933667	0.456	0.649
treatment15-180	-8543498	37157981	-0.23	0.818
treatment15-210	21486677	35933667	0.598	0.550
treatment15-240	17657084	37162540	0.475	0.635
treatment15-1440	65231592	38675272	1.687	*0.092*
treatment15-2880	68526370	38675899	1.772	*0.077*
treatment15-4320	85805020	37163155	2.309	***0.021***
treatment15-5760	73109011	37165688	1.967	***0.050***
treatment15-7200	34883786	35943725	0.971	0.332
treatment15-8640	66142285	35943725	1.84	*0.066*
treatment15-10080	49594408	35933690	1.38	0.168
treatment30-0	-14685057	65660130	-0.224	0.823
treatment30-1	25642908	35944518	0.713	0.476
treatment30-5	-28677940	34924456	-0.821	0.412
treatment30-10	-9000518	37177974	-0.242	0.809
treatment30-20	12514207	34924456	0.358	0.721
treatment30-30	10096463	34924456	0.289	0.773
treatment30-45	-27135805	37171515	-0.73	0.466
treatment30-60	-5138695	37169944	-0.138	0.890
treatment30-90	-6466178	38705046	-0.167	0.867
treatment30-120	-14134325	38689091	-0.365	0.715
treatment30-150	10158359	37170499	0.273	0.785
treatment30-180	3511374	35944281	0.098	0.922
treatment30-210	-5363908	35937071	-0.149	0.882
treatment30-240	-7153027	40623190	-0.176	0.860
treatment30-1440	5108600	35944518	0.142	0.887
treatment30-2880	-8046342	37169864	-0.216	0.829
treatment30-4320	3514748	38695682	0.091	0.928
treatment30-5760	30128220	37177960	0.81	0.418
treatment30-7200	34351243	38705046	0.888	0.375
treatment30-8640	-7404863	37178960	-0.199	0.842
treatment30-10080	15114746	37170815	0.407	0.684
treatment5-0	-28697401	66209880	-0.433	0.665
treatment5-1	149465344	35947366	4.158	***0.000***
treatment5-5	169889419	36953645	4.597	***0.000***
treatment5-10	229266047	36953645	6.204	***0.000***
treatment5-20	262138247	35947366	7.292	***0.000***

treatment5-30	305358756	36947197	8.265	***0.000***
treatment5-45	298875015	36947197	8.089	***0.000***
treatment5-60	318458499	35947366	8.859	***0.000***
treatment5-90	318010370	35947366	8.847	***0.000***
treatment5-120	311586587	35947366	8.668	***0.000***
treatment5-150	291746268	35947366	8.116	***0.000***
treatment5-180	295367722	35947366	8.217	***0.000***
treatment5-210	278722120	35947366	7.754	***0.000***
treatment5-240	279644514	36952624	7.568	***0.000***
treatment5-1440	259714636	36948922	7.029	***0.000***
treatment5-2880	162522739	38019718	4.275	***0.000***
treatment5-4320	162124851	39500051	4.104	***0.000***
treatment5-5760	141768965	43918382	3.228	***0.001***
treatment5-7200	152721935	41413190	3.688	***0.000***
treatment5-8640	133596016	41406009	3.226	***0.001***
treatment5-10080	126848107	43920013	2.888	***0.004***

Fixed effects:	Within salinity, time point to previous time point			
	Estimate Δ	Std. Error	t-value	p-value
15-0 (Intercept)	207117401	46428723	4.461	***0.000***
treatment15-1	31556117	34924456	0.904	0.366
treatment15-5	-34711324	34924456	-0.994	0.321
treatment15-10	-484034	35933690	-0.013	0.990
treatment15-20	6155219	36939368	0.167	0.867
treatment15-30	15265593	35943725	0.425	0.671
treatment15-45	-15436619	35939828	-0.43	0.667
treatment15-60	14579671	35939828	0.406	0.685
treatment15-90	-7509534	37152663	-0.202	0.840
treatment15-120	-4226962	38141769	-0.111	0.912
treatment15-150	11195056	36939374	0.303	0.762
treatment15-180	-24926683	38132933	-0.654	0.513
treatment15-210	30030176	38132933	0.788	0.431
treatment15-240	-3829593	38015484	-0.101	0.920
treatment15-1440	47574508	40808195	1.166	0.244
treatment15-2880	3294778	42096589	0.078	0.938
treatment15-4320	17278650	40487579	0.427	0.670
treatment15-5760	-12696009	39206891	-0.324	0.746
treatment15-7200	-38225225	38007903	-1.006	0.315
treatment15-8640	31258499	36813609	0.849	0.396
treatment15-10080	-16547877	36939368	-0.448	0.654
treatment30-0	-14685057	65660130	-0.224	0.823
treatment30-1	25642908	35944518	0.713	0.476
treatment30-5	-54320848	35944518	-1.511	0.131
treatment30-10	19677422	37177974	0.529	0.597
treatment30-20	21514725	37177974	0.579	0.563
treatment30-30	-2417744	34924456	-0.069	0.945
treatment30-45	-37232268	37171515	-1.002	0.317
treatment30-60	21997110	39213466	0.561	0.575

treatment30-90	-1327482	40675326	-0.033	0.974
treatment30-120	-7668148	42121625	-0.182	0.856
treatment30-150	24292685	40676243	0.597	0.551
treatment30-180	-6646985	38012162	-0.175	0.861
treatment30-210	-8875282	36942851	-0.24	0.810
treatment30-240	-1789119	41398548	-0.043	0.966
treatment30-1440	12261627	41577531	0.295	0.768
treatment30-2880	-13154942	38008806	-0.346	0.729
treatment30-4320	11561091	40505992	0.285	0.776
treatment30-5760	26613472	40677849	0.654	0.513
treatment30-7200	4223023	40506410	0.104	0.917
treatment30-8640	-41756106	40505918	-1.031	0.303
treatment30-10080	22519609	39201004	0.574	0.566
treatment5-0	-28697401	66209880	-0.433	0.665
treatment5-1	149465344	35947366	4.158	***0.000***
treatment5-5	20424076	35943358	0.568	0.570
treatment5-10	59376628	36813609	1.613	0.107
treatment5-20	32872200	35943358	0.915	0.361
treatment5-30	43220509	35936925	1.203	0.230
treatment5-45	-6483741	36813609	-0.176	0.860
treatment5-60	19583485	35936925	0.545	0.586
treatment5-90	-448129	34924456	-0.013	0.990
treatment5-120	-6423783	34924456	-0.184	0.854
treatment5-150	-19840319	34924456	-0.568	0.570
treatment5-180	3621454	34924456	0.104	0.917
treatment5-210	-16645601	34924456	-0.477	0.634
treatment5-240	922394	35943416	0.026	0.979
treatment5-1440	-19929878	36944920	-0.539	0.590
treatment5-2880	-97191896	38167857	-2.546	***0.011***
treatment5-4320	-397889	40487986	-0.01	0.992
treatment5-5760	-20355885	45961878	-0.443	0.658
treatment5-7200	10952969	47399431	0.231	0.817
treatment5-8640	-19125919	45324892	-0.422	0.673
treatment5-10080	-6747909	47425616	-0.142	0.887

Fixed effects:	Within time point, salinity 5/30 compared to salinity 15				Estimated total size
	Estimate Δ	Std. Error	t-value	p-value	
15-0 (Intercept)	207117401	46428723	4.461	***0.000***	207117401
treatment15-1	31556117	34924456	0.904	0.366	238673518
treatment15-5	-3155206	34924456	-0.09	0.928	203962195
treatment15-10	-3639240	35933690	-0.101	0.920	203478161
treatment15-20	2515979	35943725	0.07	0.944	209633380
treatment15-30	17781573	34924456	0.509	0.611	224898974
treatment15-45	2344953	35939828	0.065	0.948	209462354
treatment15-60	16924624	34924456	0.485	0.628	224042025
treatment15-90	9415090	37152663	0.253	0.800	216532491
treatment15-120	5188128	35943725	0.144	0.886	212305529
treatment15-150	16383185	35933667	0.456	0.649	223500586

treatment15-180	-8543498	37157981	-0.23	0.818	198573903
treatment15-210	21486677	35933667	0.598	0.550	228604078
treatment15-240	17657084	37162540	0.475	0.635	224774485
treatment15-1440	65231592	38675272	1.687	*0.092*	272348993
treatment15-2880	68526370	38675899	1.772	*0.077*	275643771
treatment15-4320	85805020	37163155	2.309	***0.021***	292922421
treatment15-5760	73109011	37165688	1.967	***0.050***	280226412
treatment15-7200	34883786	35943725	0.971	0.332	242001187
treatment15-8640	66142285	35943725	1.84	*0.066*	273259686
treatment15-10080	49594408	35933690	1.38	0.168	256711809
treatment30-0	-14685057	65660130	-0.224	0.823	192432344
treatment30-1	-20598267	66208333	-0.311	0.756	218075252
treatment30-5	-40207791	65660130	-0.612	0.541	163754404
treatment30-10	-20046335	67418463	-0.297	0.767	183431826
treatment30-20	-4686830	66207903	-0.071	0.943	204946551
treatment30-30	-22370166	65660130	-0.341	0.733	202528807
treatment30-45	-44165815	67418174	-0.655	0.513	165296539
treatment30-60	-36748376	66881536	-0.549	0.583	187293649
treatment30-90	-30566325	68921828	-0.443	0.658	185966166
treatment30-120	-34007511	68268693	-0.498	0.619	178298019
treatment30-150	-20909883	67414330	-0.31	0.757	202590703
treatment30-180	-2630185	67413087	-0.039	0.969	195943718
treatment30-210	-41535643	66742182	-0.622	0.534	187068436
treatment30-240	-39495168	70022249	-0.564	0.573	185279317
treatment30-1440	-74808050	68261281	-1.096	0.274	197540944
treatment30-2880	-91257770	68914740	-1.324	0.186	184386002
treatment30-4320	-96975329	68922227	-1.407	0.160	195947092
treatment30-5760	-57665848	68083085	-0.847	0.397	222560564
treatment30-7200	-15217600	68277737	-0.223	0.824	226783587
treatment30-8640	-88232205	67424356	-1.309	0.191	185027481
treatment30-10080	-49164719	67414516	-0.729	0.466	207547090
treatment5-0	-28697401	66209880	-0.433	0.665	178420000
treatment5-1	89211825	65660130	1.359	0.175	327885344
treatment5-5	144347224	66207704	2.18	***0.030***	348309419
treatment5-10	204207886	66745580	3.059	***0.002***	407686047
treatment5-20	230924867	66207903	3.488	***0.001***	440558247
treatment5-30	258879782	66204211	3.91	***0.000***	483778756
treatment5-45	267832660	66745421	4.013	***0.000***	477295015
treatment5-60	272836474	65660130	4.155	***0.000***	496878499
treatment5-90	279897879	66871933	4.186	***0.000***	496430370
treatment5-120	277701057	66207903	4.194	***0.000***	490006587
treatment5-150	246665682	66202443	3.726	***0.000***	470166268
treatment5-180	275213818	66874888	4.115	***0.000***	473787722
treatment5-210	228538041	66202443	3.452	***0.001***	457142120
treatment5-240	233290028	67415139	3.46	***0.001***	458064514

treatment5-1440	165785642	68258689	2.429	***0.016***	438134636
treatment5-2880	65298968	68920900	0.947	0.344	340942739
treatment5-4320	47622429	68921544	0.691	0.490	340544851
treatment5-5760	39962553	71545339	0.559	0.576	320188965
treatment5-7200	89140748	69394394	1.285	0.199	331141935
treatment5-8640	38756329	69389400	0.559	0.576	312016016
treatment5-10080	48556298	70912481	0.685	0.494	305268107

TABLE 3 Linear model with response variable water content dry weight and explanatory variable treatment as categorical fixed effect. We conducted planned contrasts to compare the effect of salinity 5 and 30 to salinity 15 and investigate whether the effect changes over time.

Weight loss [%]					
Residuals					
	Min	1Q	Median	3Q	Max
	-0.758	-0.41488	0.05885	0.29056	0.7048
Coefficients					
		Estimate	Std. Error	t-value	Pr(>|t|)
15-0 (Intercept)		78.788	0.307	256.296	***0.0000***
15 to 5		9.537	0.377	25.329	***0.0000***
5-45 to 5-240		1.274	0.435	2.931	***0.0150***
15 to 30		-11.532	0.377	-30.628	***0.0000***
30-45 to 30-240		-2.535	0.435	-5.830	***0.0002***

Dry weight [mg]					
Residuals					
	Min	1Q	Median	3Q	Max
	-0.6497	-0.351	-0.1593	0.465	0.8543
Coefficients					
		Estimate	Std. Error	t-value	Pr(>|t|)
15-0 (Intercept)		2.321	0.327	7.100	***0.000***
15 to 5		0.832	0.400	2.077	*0.065*
5-45 to 5-240		-0.815	0.462	-1.763	0.108
15 to 30		0.908	0.400	2.267	***0.047***
30-45 to 30-240		0.283	0.462	0.612	0.554

TABLE 4a Linear model fitting absolute gene expression of seven genes of interest to the categorical fixed factor treatment. Planned contrasts were performed (1) between 45 and 240 min exposure within each salinity and (2) between salinity 5/30 and salinity 15 at the respective time points.

Alanine Aminotransferase

Residuals:

Min	1Q	Median	3Q	Max
-181.99	-70.26	-13.12	54.97	291.42

Coefficients:

	Estimate	Std. Error	t-value	Pr(>|t|)
15-45 (Intercept)	2605.600	350.000	7.444	***0.000***
15-45 to 15-240	433.200	495.000	0.875	0.385
5-45 to 5-240	567.000	508.600	1.115	0.270
30-45 to 30-240	-639.600	495.000	-1.292	0.202
15-45 to 5-45	-268.100	495.000	-0.542	0.590
15-45 to 30-45	361.600	495.000	0.730	0.468
15-240 to 5-240	-134.300	508.600	-0.264	0.793
15-240 to 30-240	-711.200	495.000	-1.437	0.157

ATP-Synthase

Residuals:

Min	1Q	Median	3Q	Max
-1751.3	-669.9	-162.5	640	2378

Coefficients:

	Estimate	Std. Error	t-value	Pr(>|t|)
15-45 (Intercept)	23658.500	3180.400	7.439	***0.000***
15-45 to 15-240	203.500	4497.800	0.045	0.964
5-45 to 5-240	4776.300	4621.000	1.034	0.306
30-45 to 30-240	-2302.900	4497.800	-0.512	0.611
15-45 to 5-45	-7307.000	4497.800	-1.625	0.110
15-45 to 30-45	-478.500	4497.800	-0.106	0.916
15-240 to 5-240	-2734.300	4621.000	-0.592	0.557
15-240 to 30-240	-2984.900	4497.800	-0.664	0.510

Bicarbonate Exchanger

Residuals:

Min	1Q	Median	3Q	Max
-92.18	-30.8	-9.75	19.45	346.38

Coefficients:

	Estimate	Std. Error	t-value	Pr(>|t|)
15-45 (Intercept)	570.560	207.740	2.747	***0.008***
15-45 to 15-240	816.160	293.790	2.778	***0.008***
5-45 to 5-240	539.480	301.840	1.787	0.080
30-45 to 30-240	15.730	293.790	0.054	0.958
15-45 to 5-45	-138.970	293.790	-0.473	0.638
15-45 to 30-45	-11.660	293.790	-0.040	0.968
15-240 to 5-240	-415.650	301.840	-1.377	0.174
15-240 to 30-240	-812.090	293.790	-2.764	***0.008***

Carbonic Anhydrase

Residuals:

Min	1Q	Median	3Q	Max
-219.541	-42.234	-3.515	40.09	197.007

Coefficients:

	Estimate	Std. Error	t-value	Pr(>|t|)
15-45 (Intercept)	1929.600	223.950	8.616	***0.000***
15-45 to 15-240	32.720	316.720	0.103	0.918
5-45 to 5-240	363.550	325.390	1.117	0.269
30-45 to 30-240	296.880	316.720	0.937	0.353
15-45 to 5-45	-212.830	316.720	-0.672	0.505
15-45 to 30-45	198.010	316.720	0.625	0.535
15-240 to 5-240	118.000	325.390	0.363	0.718
15-240 to 30-240	462.170	316.720	1.459	0.150

IGF

Residuals:

Min	1Q	Median	3Q	Max
-91.07	-38.78	-10.85	13.8	244.72

Coefficients:

	Estimate	Std. Error	t-value	Pr(>|t|)
15-45 (Intercept)	1825.450	194.560	9.383	***0.000***
15-45 to 15-240	-260.390	275.150	-0.946	0.348
5-45 to 5-240	-192.200	290.000	-0.663	0.511
30-45 to 30-240	-178.900	275.100	-0.650	0.518
15-45 to 5-45	-114.250	282.690	-0.404	0.688
15-45 to 30-45	-632.570	275.150	-2.299	***0.026***
15-240 to 5-240	-46.050	282.690	-0.163	0.871
15-240 to 30-240	-551.110	275.150	-2.003	0.050

NaK-ATPase

Residuals:

Min	1Q	Median	3Q	Max
-490.22	-127.59	-13.75	119.18	488.14

Coefficients:

	Estimate	Std. Error	t-value	Pr(>|t|)
15-45 (Intercept)	7060.600	757.300	9.323	***0.000***
15-45 to 15-240	1477.200	1071.000	1.379	0.174
5-45 to 5-240	-988.700	1100.300	-0.899	0.373
30-45 to 30-240	355.900	1071.000	0.332	0.741
15-45 to 5-45	-280.500	1071.000	-0.262	0.794
15-45 to 30-45	-345.800	1071.000	-0.323	0.748
15-240 to 5-240	-2746.300	1100.300	-2.496	***0.016***
15-240 to 30-240	-1467.200	1071.000	-1.370	0.177

Tubulin					
Residuals:					
	Min	1Q	Median	3Q	Max
	-109.558	-41.11	-9.352	33.462	160.235
Coefficients:					
		Estimate	Std. Error	t-value	Pr(>|t|)
15-45 (Intercept)		1583.150	190.050	8.330	***0.000***
15-45 to 15-240		-205.120	268.770	-0.763	0.449
5-45 to 5-240		-92.820	292.030	-0.318	0.752
30-45 to 30-240		-145.220	268.770	-0.540	0.591
15-45 to 5-45		-147.480	276.130	-0.534	0.596
15-45 to 30-45		-146.590	268.770	-0.545	0.588
15-240 to 5-240		-35.180	285.070	-0.123	0.902
15-240 to 30-240		-86.690	268.770	-0.323	0.748

TABLE 4b Effect of salinity, time and their interaction on the expression of all our genes of interest in total according to PERMANOVA.

Source	df	Sums of Squares	Mean Square	Pseudo-F	p-value (permutations)	Unique permutations
Salinity	2	21.946	10.973	1.6052	0.102	999
Time	1	10.476	10.476	1.5325	0.142	999
Time x Salinity	2	10.827	5.4134	0.79191	0.634	994
Residuals	51	348.63	6.8359			
Total	56	392				

TABLE 5a Different models fitting parameters of the long-term experiment to the categorical fixed factor treatment. Planned contrasts were performed (1) between 45 and 240 min exposure within each salinity and (2) between salinity 5/30 and salinity 15 at the respective time points.

LINEAR REGRESSION

Survivors				
Residuals:				
	Min	1Q	Median	3Q	Max
	-6	-1.5	1.333	2.25	4
Coefficients:				
	Estimate	Std. Error	t-value	Pr(>|t|)
15-3 (Intercept)	32	2.113	15.147	***3.48E-09***
15-3 to 15-6	4.667	2.988	1.562	0.1443
5-3 to 5-6	-2.667	2.988	-0.893	0.39
30-3 to 30-6	2	2.988	0.669	0.516
15-3 to 5-3	-1.333	2.988	-0.446	0.6633
15-3 to 30-3	1.333	2.988	0.446	0.6633
15-6 to 5-6	-8.667	2.988	-2.901	***0.0133***
15-6 to 30-6	-1.333	2.988	-0.446	0.6633

LINEAR REGRESSION

Mean Length [µm³]				
Residuals:				
	Min	1Q	Median	3Q	Max
	-229.46	-84.65	-27.54	80.24	311.84
Coefficients:				
	Estimate	Std. Error	t-value	Pr(>|t|)
15-3 (Intercept)	1981.760	93.930	21.098	***0.000***
15-3 to 15-6	-212.630	132.840	-1.601	0.135
5-3 to 5-6	46.836	132.837	0.353	0.731
30-3 to 30-6	5.097	132.837	0.038	0.970
15-3 to 5-3	-133.000	132.840	-1.001	0.336
15-3 to 30-3	-193.950	132.840	-1.460	0.170
15-6 to 5-6	126.460	132.840	0.952	0.360
15-6 to 30-6	23.780	132.840	0.179	0.861

LINEAR REGRESSION

Biomass (Survivors*Mean Length) [µm³]					
Residuals:					
	Min	1Q	Median	3Q	Max
	-12366.6	-1178.2	544.9	2754	9832.4
Coefficients:					
		Estimate	Std. Error	t-value	Pr(>|t|)
15-3 (Intercept)		63493.900	4034.900	15.736	***0.000***
15-3 to 15-6		491.000	5706.300	0.086	0.933
5-3 to 5-6		-3776.000	5706.000	-0.662	0.521
30-3 to 30-6		3294.000	5706.000	0.577	0.574
15-3 to 5-3		-6633.400	5706.300	-1.162	0.268
15-3 to 30-3		-3777.000	5706.300	-0.662	0.521
15-6 to 5-6		-10900.300	5706.300	-1.910	0.080
15-6 to 30-6		-973.900	5706.300	-0.171	0.867

LOGISTIC REGRESSION

Larvae					
Deviance Residuals:					
	Min	1Q	Median	3Q	Max
	-1.4823	-0.67541	-0.00008	-0.00008	1.4823
Coefficients:					
		Estimate	Std. Error	t-value	Pr(>|t|)
15-3 (Intercept)		-0.693	1.225	-0.566	0.571
15-3 to 15-6		0.000	1.732	0.000	1.000
5-3 to 5-6		0.000	8781.000	0.000	1.000
30-3 to 30-6		20.260	6209.000	0.003	0.997
15-3 to 5-3		-18.870	6209.000	-0.003	0.998
15-3 to 30-3		-18.870	6209.000	-0.003	0.998
15-6 to 5-6		-18.870	6209.000	-0.003	0.998
15-6 to 30-6		1.386	1.732	0.800	0.423

NEGATIVE BINOMIAL REGRESSSION Theta: 2.35

Asexual Reproduction

Deviance Residuals:

Min	1Q	Median	3Q	Max
-1.88832	-0.84446	-0.07126	0.40386	1.36745

Coefficients:

	Estimate	Std. Error	t-value	Pr(>|t|)
15-3 (Intercept)	0.981	0.516	1.899	0.058
15-3 to 15-6	1.322	0.665	1.989	***0.047***
5-3 to 5-6	-18.204	5442.460	-0.003	0.997
30-3 to 30-6	-0.182	0.637	-0.286	0.775
15-3 to 5-3	-2.079	1.187	-1.752	0.080
15-3 to 30-3	0.811	0.681	1.191	0.234
15-6 to 5-6	-21.605	5442.460	-0.004	0.997
15-6 to 30-6	-0.693	0.619	-1.120	0.263

NEGATIVE BINOMIAL REGRESSSION Theta: 3.07

Sexual Reproduction

Deviance Residuals:

Min	1Q	Median	3Q	Max
-2.1246	-1.3149	-0.1705	0.7215	1.3188

Coefficients:

	Estimate	Std. Error	t-value	Pr(>|t|)
15-3 (Intercept)	1.204	0.457	2.636	***0.008***
15-3 to 15-6	0.182	0.633	0.288	0.773
5-3 to 5-6	0.288	0.895	0.322	0.748
30-3 to 30-6	0.251	0.686	0.366	0.714
15-3 to 5-3	-1.204	0.807	-1.493	0.135
15-3 to 30-3	-0.357	0.678	-0.526	0.599
15-6 to 5-6	-1.099	0.742	-1.481	0.139
15-6 to 30-6	-0.288	0.642	-0.448	0.654

TABLE 5b Effect of long-term salinity change, time and their interaction on the overall performance of *P. elegans* (survival, growth, reproduction) according to PERMANOVA.

Main effects

Source	df	Sums of Squares	Mean Square	Pseudo-F	p-value (permutations)	Unique permutations
Salinity	2	34.295	17.147	1.7368	*0.094*	998
Time	1	4.0589	4.0589	0.4111	0.872	999
Time x Salinity	2	13.169	6.5845	0.66691	0.772	999
Residuals	12	118.48	9.8731			
Total	17	170				

Pairwise comparisons within main effect salinity

Groups	t	p-value (permutations)	Unique permutations
5 to 15	1.6007	***0.04***	987
5 to 30	1.7511	***0.012***	985
15 to 30	0.5682	***0.923***	983

FIGURES

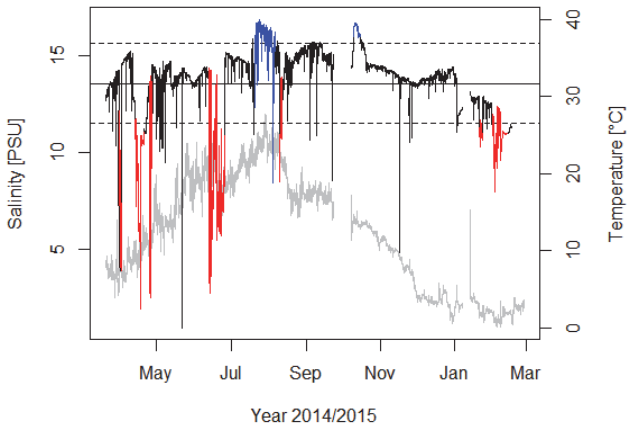

FIGURE 1 Salinity (black) and temperature (grey) profile at Herslev from March 2014 until February 2015. The solid line is the mean salinity during this period while the dotted line indicates ± 1 SD. Extreme events with reduced salinity are highlighted in red, with elevated salinity in blue.

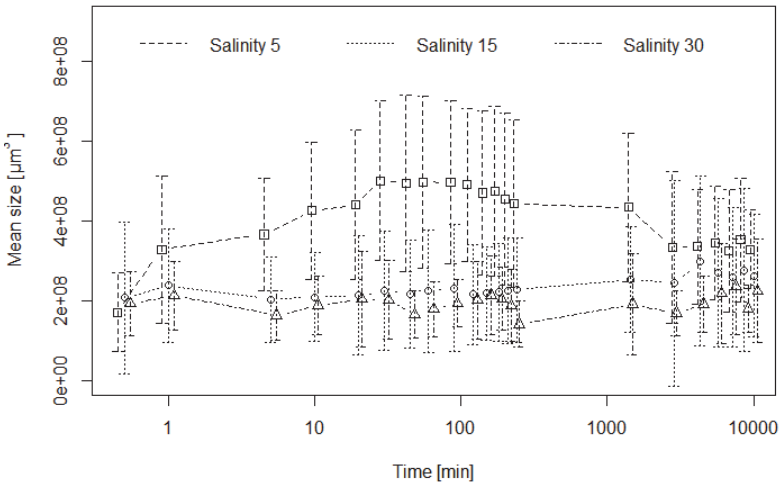

FIGURE 2 Body volume in mean and standard deviation after exposure to salinity 5, 15 and 30 over a period of one week. At salinity 5 the volume at each time point is significantly larger than at the initial measurement, while at salinity 15 only the volume after 3 days is significantly larger than at the initial measurement. The volume at salinity 5 differs from the one at salinity 15 from 5 min until 1 day after exposure.

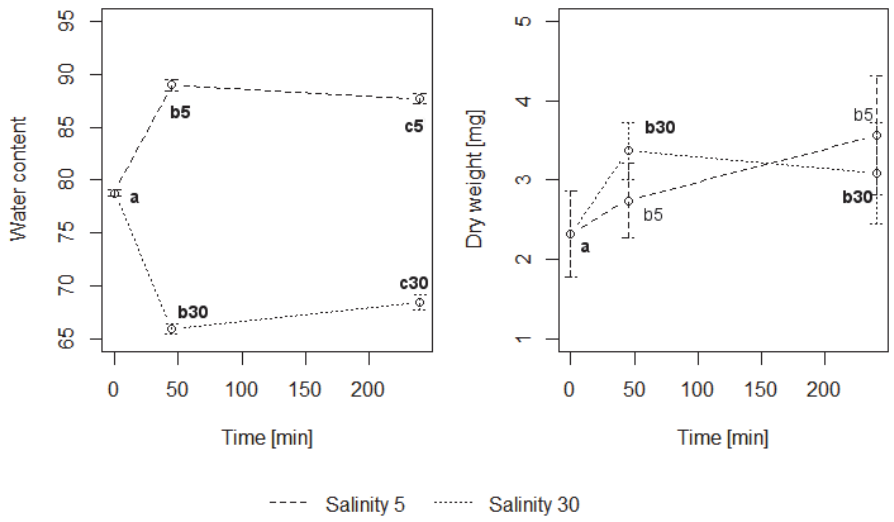

FIGURE 3 Mean and standard deviation of tissue water content (% weight loss per wet weight) and dry weight for the different salinity treatments at two time points. Bold fonts indicate significant differences and normal fonts indicate marginally significant differences from salinity 15 at time point zero and to the previous time point at the respective salinity.

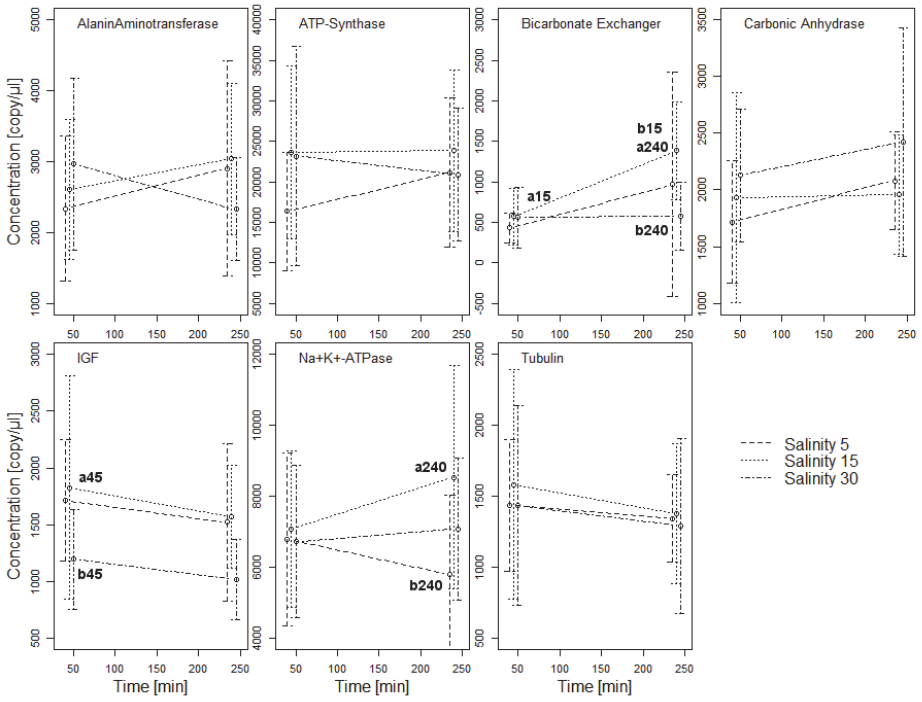

FIGURE 4 Mean and standard deviation of the absolute gene expression (copies/µl) of 7 genes of interest in response to changing salinities at two different time points (45 min, 240 min). Significant differences are indicated with letters and a number, which indicates whether the differences occurred between time points within salinity (45, 240) or between salinities within time point (5, 15, 30).

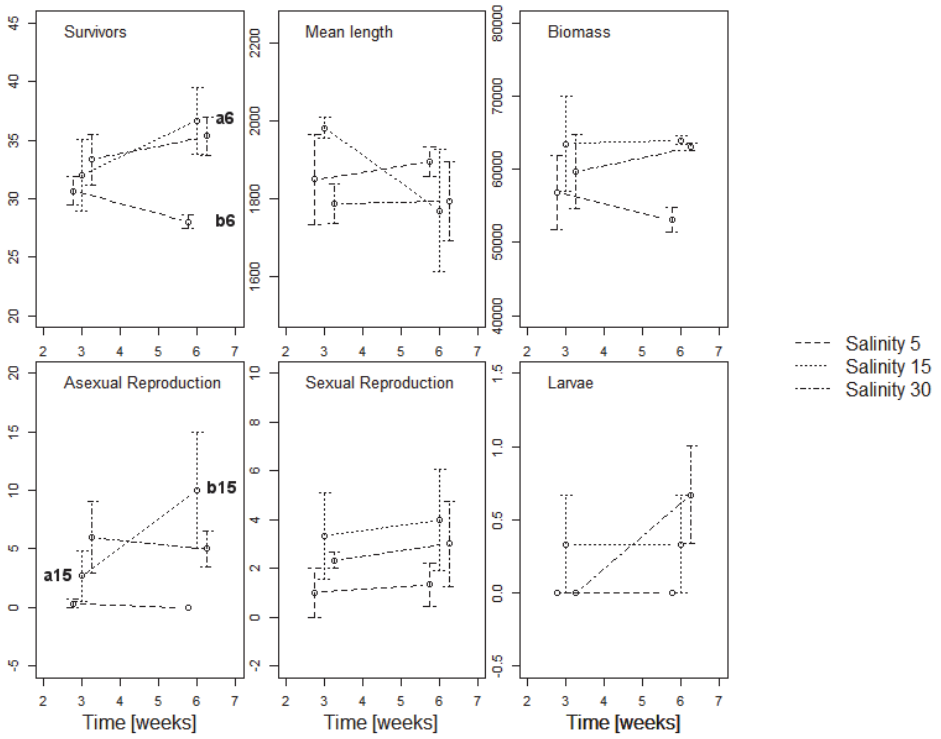

FIGURE 5 Mean and standard deviation of different ecological responses to long-term changes in salinity on *P. elegans* at two different time points, 3 weeks and 6 weeks. Significant differences are indicated with letters and a number, which indicates whether the differences occurred between time points within salinity (45, 240) or between salinities within time point (5, 15, 30).

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLE S1 Number of individuals per treatment and sampling date for each experiment.

Body volume

		Sampling date	
		11.07.15	01.08.15
Salinity	5	5 Ind.	5 Ind.
	15	5 Ind.	5 Ind.
	30	5 Ind.	5 Ind.

Tissue water content

		Time point			Sampling date
		0 min	45 min	240 min	
Salinity	5	-	3 x 30 Ind.	3 x 30 Ind.	05./06.07.16
	15	3 x 30 Ind.	-	-	01.08.15
	30	-	3 x 30 Ind.	3 x 30 Ind.	05./06.07.16

RNA Expression

		Time point		Sampling date
		45 min	240 min	
Salinity	5	3 + 7 Ind.	3 + 7 Ind.	11.07. + 01.08.15
	15	3 + 7 Ind.	3 + 7 Ind.	11.07. + 01.08.15
	30	3 + 7 Ind.	3 + 7 Ind.	11.07. + 01.08.15

Long-term experiment

		Time point			Sampling date
		Initially	3 weeks	6 weeks	
Salinity	5	-	3 x 30 Ind.	3 x 30 Ind.	08.06.15
	15	1 x 50 Ind.	3 x 30 Ind.	3 x 30 Ind.	08.06.15
	30	-	3 x 30 Ind.	3 x 30 Ind.	08.06.15