

# Amphipods in estuaries: the sibling species low salinity switch hypothesis

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## Abstract

A novel low salinity switch hypothesis is proposed to account for the speciation of an obligate estuarine (oligohaline) amphipod, *Orchestia aestuarensis*, from a closely-related one, *Orchestia mediterranea*, found in both estuarine and marine conditions (euryhaline). The underlying genetic mechanisms could involve:

1. A dimorphic allele, or linked set of alleles, carried by the euryhaline amphipod which controls the ability to breed in low salinity conditions in estuaries and which is selected for in these conditions, producing the oligohaline amphipod.
2. A genetically-assimilated gene or genes, controlling the ability to breed in low salinity conditions in estuaries, which is/are “switched on” by low salinity conditions.
3. Allopatric speciation from a euryhaline to an oligohaline amphipod species where low salinity conditions is the selective switch.

It is possible that other estuarine, sibling, amphipod pairs have evolved by salinity switching. In the North Atlantic coastal region, this could include: *Gammarus tigrinus*/*G. daiberi* and *G. salinus*/*G. zaddachi* (Amphipoda, Gammaridae).

## Key Words

Low salinity switch hypothesis, sibling species amphipods, evolution, estuaries, *O. mediterranea*, *O. aestuarensis*

## Introduction

The Amphipoda are one of 16 orders of the Crustacea (Horton et al., 2016). Over nine thousand (9,980) species of amphipods have been described to 2016 (Arfianti et al. 2018) occurring in a wide range of habitats: marine, estuarine, freshwater, terrestrial and in special association with other animals, such as parasites.

The Amphipoda contain species which extend from the marine environment into estuaries and freshwater. In colonising estuaries, the immigrant species demonstrate varying degrees of physiological acclimatisation or adaptation to the reduced salinities, which is the defining feature of estuaries.

We review the literature pertinent to sibling species pairs in estuaries and propose the low salinity switch hy-

pothesis to explain their evolutionary origin. Our review focuses on the euryhaline talitrid: *Orchestia mediterranea* A. Costa, 1853 (Crustacea, Amphipoda, Talitridae) and its oligohaline sibling, *Orchestia aestuarensis*, Wildish, 1987. A description of the kinds of estuaries that amphipods might encounter as they colonise them is given. Study methods are reviewed which can examine the inference that the oligohaline amphipod evolved by salinity switching from its euryhaline sibling.

## Types of Estuaries

An estuary is that part of the hydrological system where freshwater and marine waters mix. Classification of estu-

aries is based either on how they geologically form or how and where, the waters mix within them (Odum 1971).

The classification based on how and where fresh and saltwater mix within the estuary is of most use in this study. Odum (1971) recognised salt wedge, partially mixed and well mixed estuaries. Specialised salinity conditions were present in fjords, consisting of a deep basin of very saline, sometimes stagnant seawater. Hypersaline estuaries in the tropics are formed when evaporation exceeds the freshwater inflow. There have been attempts to classify well mixed estuaries by the salinity conditions within the estuary, coupled with the benthic fauna found throughout its length. Thus, the Venice System (Den Hartog 1960; 1963a) is based on salinity in relation to benthic macro-invertebrate distribution. The Venice System is applicable to coastal plain, well-mixed (lowland) estuaries of the North Atlantic coast of the Old World. The biologically-based (fish and macro-invertebrates) salinity zones, derived by Bulger et al. (1993), apply to partially mixed estuaries of the North Atlantic coast in the New World. Due to the varied physical and hydrological conditions in estuaries, a universal classification, based on salinity distribution, applicable to the varied geological types of estuaries, is unlikely to be achieved.

In this study, we have relied on direct measurement of chlorinity or salinity in the estuary, accounting for tidal effects to correlate with amphipod distribution. Up until the 1980s, salinity was measured by titrating the halide content with silver nitrate, yielding a "chlorinity" as parts per thousand (ppt), which, if multiplied by 1.80655, gives salinity as ppt. Assuming that typical, undiluted seawater has a salinity of 35g/kg or 35 ppt, the earlier chlorinity measurements can be converted to a percentage of 35 ppt salinity seawater and this is the most useful way of expressing it if salinity tolerance experiments are contemplated. From the 1980s onwards, salinity measurements were increasingly made by electrical conductivity methods and the results expressed as practical salinity units (PSU). Such measurements may be converted from the relationship: 35 PSU = 100% seawater.

## Low salinity switch hypothesis

The hypothesis, proposed here, is that low salinity acts as an environmental switch by "choosing" the new phenotype, which can live and reproduce at lower salinities, from amongst adjacent euryhaline, sibling populations which are adapted to live and reproduce in a higher range of salinities. Thus, the new phenotype is "induced" by the appropriate salinity conditions in a new estuary from a euryhaline, sibling population reaching the appropriate salinity conditions. Specific mechanisms which could control the appearance of an oligohaline from a euryhaline amphipod in low salinity locations in estuaries include:

1. By a polymorphic allele or linked set of alleles, carried at low frequency within a population of the

euryhaline amphipod which is selected for in low salinity conditions. The alleles control an ability to survive and breed in low salinity conditions. This is the combined switch mechanism of West-Eberhard (1989), with an allelic and low salinity switch.

2. After initial polyphenism by the ancestral euryhaline amphipod, the ability to breed at low salinities became fixed by genetic assimilation (Whitman and Agrawal 2000). The same gene or genes are then switched on by the appropriate low salinity conditions.
3. By regular allopatric or parapatric speciation mechanisms which occur within estuaries. This involves mutation and/or random genetic drift amongst the parental euryhaline amphipod population and selection in low salinity conditions of a more oligohaline amphipod in the appropriate salinity conditions. Kolding (1985) reports on two guilds of *Gammarus* species in the Baltic Sea which have evolved by allopatric speciation in this way over a period of ~ 4000 years.

The null hypothesis for each of the hypothetical mechanisms above would be that genetic change and natural selection were not involved in the phenotypic changes observed.

## Criteria for selection of the estuarine sibling salinity switch pair:

### *O. mediterranea*/*O. aestuarensis*

*Orchestia mediterranea* A. Costa, 1857 and *O. aestuarensis* Wildish, 1987 were selected, based on the following biological/geological criteria:

- both sibling species occur in the same estuary, but only one is found in the brackish part (oligohaline) and the other in a wider range of estuarine and fully marine habitats (euryhaline).
- based on morphological characters, it is difficult to distinguish the siblings taxonomically.
- in field conditions, the two siblings are reproductively isolated.
- each sibling tolerates a different range of salinities in estuaries.
- genetically, the siblings are closer to each other than to other congeneric species and they may be able to hybridise.
- because the oligohaline sibling may be of more recent origin, recent geological history may be of importance in determining which physical factors, such as salinity changes, were the ultimate cause of sibling speciation.

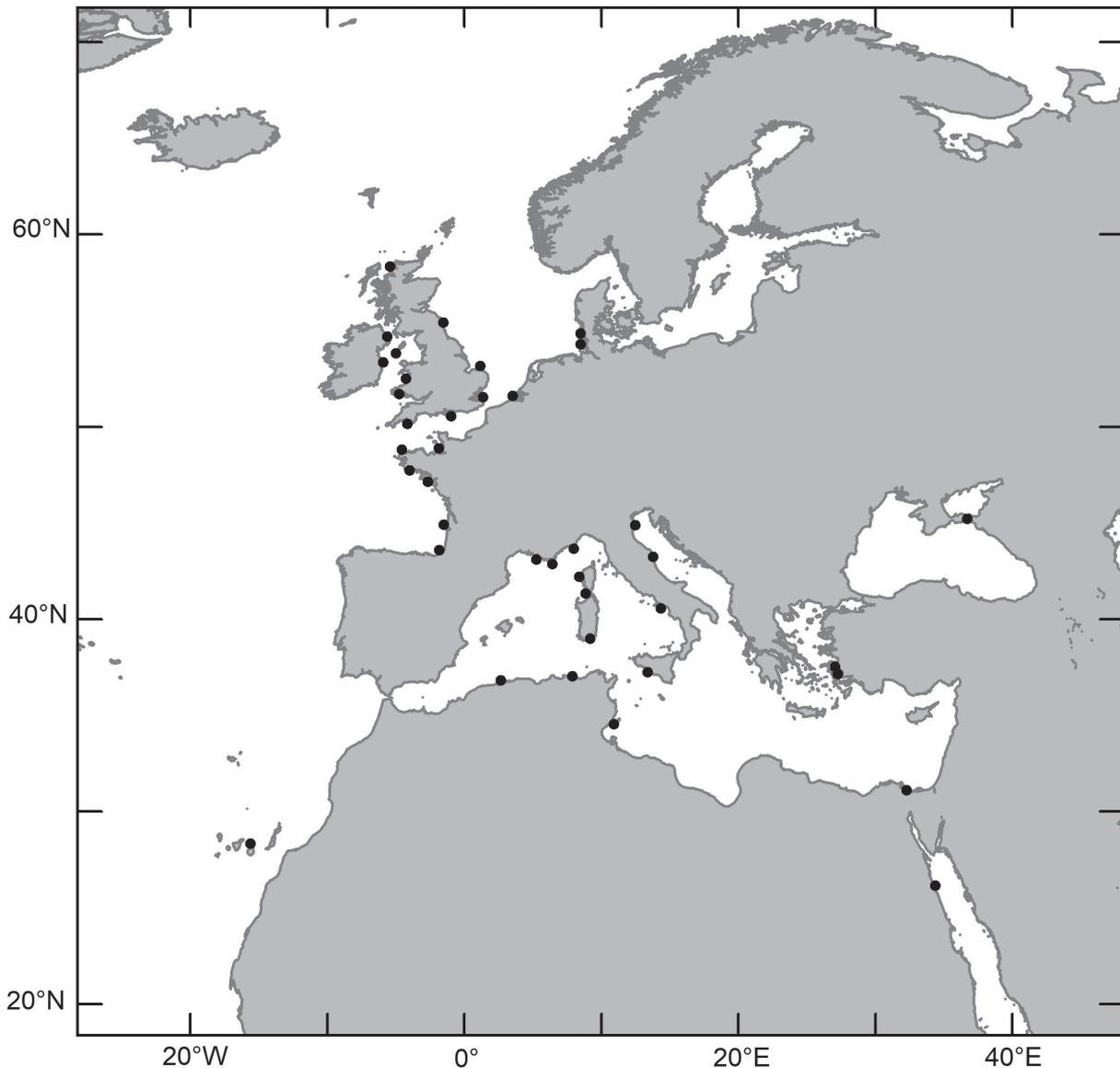
In what follows, we review, in the same order as listed above, the literature available for each of the six criteria as it pertains to the evolution of *O. aestuarensis* and *O. mediterranea* in estuaries.

## Geographical distribution

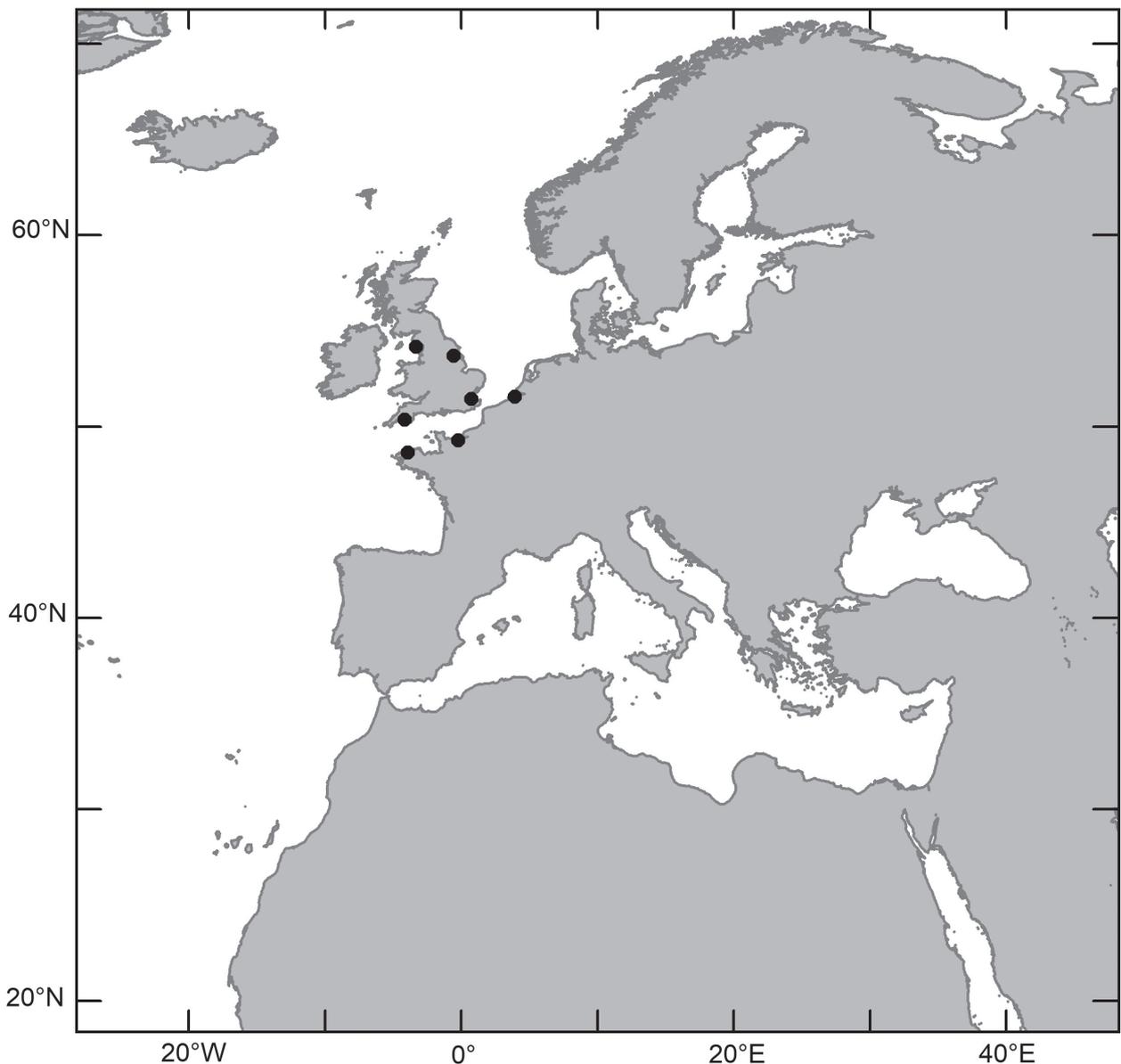
A synonymy list for *O. mediterranea* is given in Wildish (1969) from which the distribution data (Fig. 1) is taken. Summarising, it is a marine, eulittoral species found on north-eastern Atlantic coastal beaches (absent on north-western Atlantic beaches) with a northern limit on the German coast at Busum and Hallig Hooge (Schellenberg 1942). It is present on the British, Irish and continental European coasts and, thus, on suitable coastal beaches in the North Sea, Irish Sea, Bay of Biscay, Mediterranean and Black Seas. It is absent in the Baltic Sea. The most southerly records are from coastal Tunisia and Algeria in the Mediterranean Sea and from Kossier in the Red Sea (Ruffo 1938a, b). There are reports of *O. mediterranea* from the Canary and Azores archipelagoes (Dahl 1967). It is also present in estuaries and detailed distribution data within the Deltaic estuaries of

Holland are given by Den Hartog (1963b) and in the Medway Estuary, Britain, by Wildish (1970a).

*O. aestuarensis* was originally described as a morph of *O. mediterranea* and not given specific status until 1987 (Wildish 1987). To date, it has been identified in the following estuaries: Tamar, Medway (Wildish 1969), Duddon (Bradley 1975) and recently in the Humber Estuary (Bratton, personal communication) – all in Britain, Penzé Estuary, Brittany and Orne Estuary, Normandy on the Atlantic coast of France (Ginsburger-Vogel 1991) and four locations (Goeree, Philipsland, Mastgat, Schouwen) in the Deltaic area of the Netherlands (Stock 1995). The distribution (Fig. 2) likely represents an incomplete picture because of the difficulty in finding *O. aestuarensis* in the restricted area of occurrence in part of the mesohaline section of estuaries (see below). No records of occurrence have been found outside estuaries.



**Figure 1.** Geographical distribution of *O. mediterranea*, from Wildish (1969).



**Figure 2.** Geographical distribution of *O. aestuarensis* for sampling locations known to 2019.

To date, the British estuaries, Tamar, Medway and Duddon are the only ones where comprehensive estuarine distribution data has been collected. Results are summarised in Wildish (1987) and show that, in the Medway and Tamar, the most landward populations of *O. aestuarensis* have female-biased sex ratios and few intersexes, whereas in the most seaward population, intersexes are common. In the high eulittoral, both species occur in discrete colonies separated by natural barriers with respect to estuarine penetration, such as soft mud or quay walls, which are unsuitable substrates. In the Tamar Estuary the most seaward *O. aestuarensis* consisted of a mixed population with *O. mediterranea* and/or hybrids between the two. Mixed populations were not found in the Medway and Duddon estuaries. Similar estuarine penetration data were not collected for the French estuaries studied by Ginsburger-Vogel (1991), but in the one location sampled

in each of the Penzé and Orne estuaries, this author described mixed species populations.

Mixed species populations occur only at the interface between *O. aestuarensis* and *O. mediterranea* distribution in the Tamar Estuary. Landwards of this interface location, only *O. aestuarensis* occurs and seaward only *O. mediterranea*. This distribution is interpreted to be governed by a low salinity switch at a location in the estuary where both species can co-exist. In the Medway Estuary, *O. aestuarensis* is present from above the Medway bridge, up-estuary for ~2.5 km (approximately one half of the mesohalinity). Estuarine penetration terminates in the Medway where the highwater salinity is 31% of seawater (Fig. 3). *O. mediterranea* replaces *O. aestuarensis* below the Medway bridge where the salinity is 52% seawater and is present throughout the rest of the Estuary, into the Thames and North Sea/Strait of Dover, where suitable substrates and beaches are present.

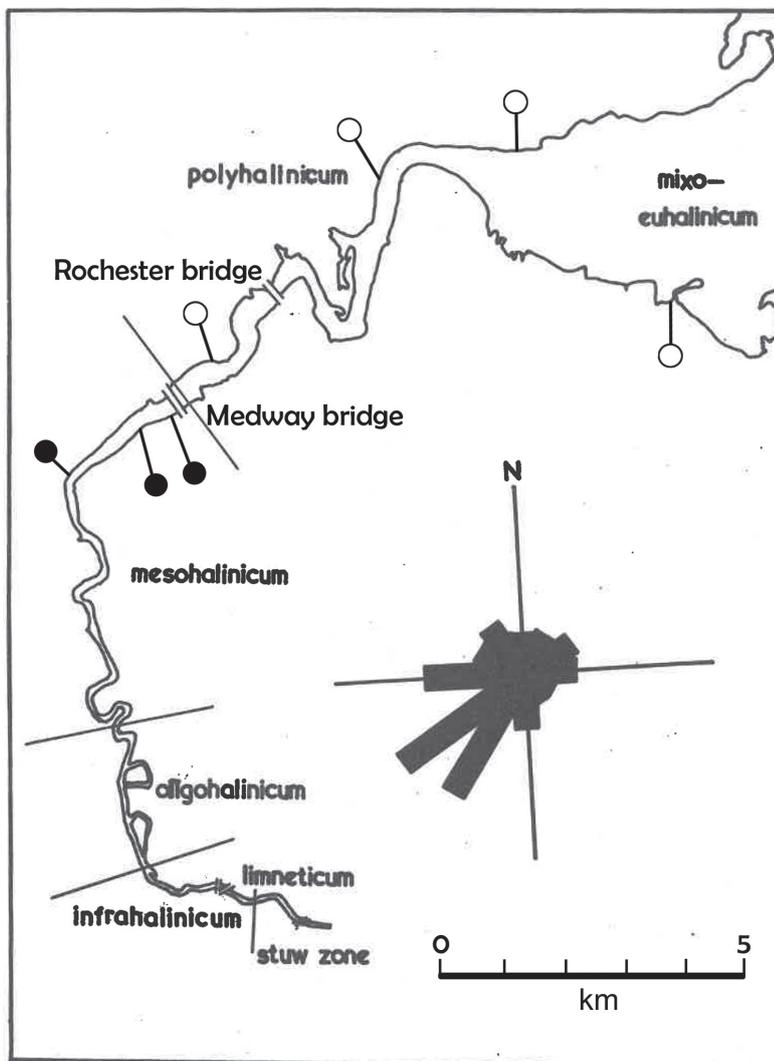


Figure 3. Distribution of *O. aestuarensis* (closed circles) and *O. mediterranea* (open circles) in the Medway Estuary, from Wildish (1969).

### Taxonomic similarity between *O. aestuarensis* and *O. mediterranea*

Morphologically, *O. aestuarensis* is close to *O. mediterranea*, although it can be distinguished by a number of minor taxonomic characters (Wildish 1987). Some examples include: male gnathopod 2 propodus palm with a proximal notch in *aestuarensis*, which is absent in *mediterranea*; male gnathopod 2 hinge tooth near the propodus articulation in *mediterranea* which is absent in *aestuarensis*; fewer third uropod peduncle spines and second antenna flagellum segments in *aestuarensis* (both are allometric characters). If a large population sample is available, *O. aestuarensis* can be recognised by the presence of a female-biased sex ratio and the occurrence of intersexes, versus a balanced sex ratio and few or no intersexes in *O. mediterranea*. Dorsal epidermal pigment patterns are similar in both species, except that *O. aestuarensis* has two elliptical holes in the solid mid-dorsal line which lack pigment in each body segment.

*O. mediterranea* lacks the pigment perforations in the mid-dorsal line. Pigment pattern differences are recognised in the field, but can be best seen with the aid of a binocular microscope after preservation in formalin-based solution.

### Reproductive isolation

Wildish (1970b) and Ginsburger-Vogel (1991) show that hybridisation between *O. aestuarensis* and *O. mediterranea* does occur in laboratory cultures. Whether hybridisation occurs in the wild at mixed-species locations in estuaries is uncertain and requires molecular genetic studies for confirmation. Both hybridisation experiments were conducted without considering the effect of salinity and temperature on phenotypic expression. In gammarids, salinity and temperature in conjunction with microsporidian endoparasites, are known to influence the occurrence of intersexes and sex ratio bias in the host population (Bulnheim 1978). The culture salinity was not stated during hy-

bridisation experiments (Wildish 1970b); they were conducted with undiluted locally available seawater of  $Cl = 16.2\text{‰}$  (84% of 35‰ salinity seawater) at 20–24 °C. The salinity and temperature during the hybridisation experiments conducted by Ginsburger-Vogel (1991) was also not stated, but it is assumed that relatively high salinity seawater was used. Due to (1) the uncertainty regarding the salinity of seawater employed during culture and (2) the unrealistically high salinities probably used, the hybridisation experiments should be repeated at appropriate salinity and temperature conditions for the estuary studied.

The hybridisation studies in the Medway Estuary suggest that reproductive isolation between the two species is incomplete. The source of the two populations used in the British hybridisation experiments were ~8 km apart along the estuarine gradient: the most landward colony sampled was in the mesohaliniacum and the most seaward in the polyhaliniacum (Wildish 1970b). A physiological method of reproductive isolation is unnecessary if mechanisms 1 or 2 (see section on low salinity switch hypothesis) were involved in the origin of *O. aestuarensis* in estuaries. This is because the low salinity switch operates to exclude hybridisation. If dispersed landwards, *O. mediterranea* females, at or downstream of the mixed interfacial location, would be unable to produce broods successfully because of the low salinity conditions (Wildish 1970b). Male *O. mediterranea* landward immigrants from the same downstream locations, which can survive and presumably breed in low salinities, were unable to produce a viable brood when mated with a female *O. aestuarensis* (Wildish 1970b). Regarding seaward dispersal of *O. aestuarensis*, the males when mated with *O. mediterranea* females, were able to produce broods. Presumably, this is a way of maintaining the rare allele or gene within the genotype of *O. mediterranea*.

## Physiological ecology

Species of *Orchestia* are sensitive to salinity conditions in estuaries and in lowland estuaries the high tide salinity may represent the salinity distribution limits (Table 1). Within the Medway Estuary, *O. gammarellus* is the least sensitive to low salinity and penetrates to the oligohaliniacum where the high-water salinity is ~ 5% of full-strength seawater. The sparse populations of *O. gammarellus* in the 5–10% seawater section of the estuary are likely

non-breeding immigrants dispersed from lower in the estuary (breeding absent in this part of the estuary, Morrith and Stevenson 1993).

Experiments with diluted seawater and *O. mediterranea* showed that lethality (lethal concentration at which 50% of those tested die, LC50) was dependent on the inter-moult stage, with a wide range of 48-hour LC50s from 2–15% of 35 ppt seawater (Wildish 1970a). The pre-moult stage was most susceptible to low salinity. These results suggest that direct lethality was not the primary cause of limited landward penetration in the estuary. Preliminary experiments to determine the effect of dilute seawater on fertility of *O. mediterranea* were conducted by Wildish (1970 a) with three seawater dilutions (14, 42 and 84% of 35 ppt seawater). Dilute salinity affected the inter-moult stage timing in breeding females, which increased the time between broods, as dilution increased. Both this and a loss of eggs and selective female mortality at low salinities reduced population fertility in low salinity conditions. These effects were present at both 14 and 42%, but not at 84% seawater (Wildish 1970a).

Great strides have been achieved in understanding the physiology of ionic and osmotic regulation in talitrids (Morrith and Spicer 1998). In vitro-cultured, early embryos of *O. gammarellus* were killed at salinities below 40% seawater, yet if eggs developed in the marsupium (= brood pouch) of a living female, normal broods were produced at salinities down to 10% seawater (Morrith and Stevenson 1993). The explanation for these apparently conflicting results was that female *O. gammarellus* were able to control the osmotic concentration of the marsupial fluid, thereby allowing egg development at lower salinities (Morrith and Spicer 1998). Similar experiments have not been conducted with *O. mediterranea* or *O. aestuarensis*, but it is proposed that marsupial fluid control was absent in the former species. This is consistent with the preliminary fertility experiments with *O. mediterranea* (Wildish 1970a) and that this species depends on frequent tidal wetting to maintain the marsupial fluid osmo-concentration. Further experiments with both species are clearly needed to clarify this. *O. aestuarensis*, which extends into the Medway Estuary at lower salinities than its sibling, is proposed to have developed a rudimentary form of marsupial fluid control that allows successful broods to be produced down to salinities of 31% seawater. *O. mediterranea* can only produce broods consistently in salinities > 52% seawater.

**Table 1.** Distribution limits at high water of *Orchestia* in two lowland estuaries: Deltaic Area, The Netherlands (Den Hartog 1963b) and Medway Estuary, UK (Wildish 1987). The chlorinity values (parts per thousand) used in both reports have been converted to salinity (parts per thousand) by multiplying by 1.80655.

Species	Deltaic Area		Medway estuary	
	Salinity (ppt)	Percent of full-strength seawater (S = 35ppt)	Salinity (ppt)	Percent of full-strength seawater (S = 35ppt)
<i>O. gammarellus</i>	3.25 > 29.81	9.3–100	1.81 > 29.81	5.2–100
<i>O. mediterranea</i>	9.03 > 29.81	25.8–100	18.07 > 29.81	51.6–100
<i>O. aestuarensis</i>	?	?	10.84–18.07	31–51.6

## Genetic distance between siblings

The Barcode of Life Data System (BOLD, [www.boldsystems.org](http://www.boldsystems.org), Ratnasingham and Herbert 2007) was searched for published and publicly available mitochondrial CO1 sequences of *Orchestia* in May 2020. The only oligohaline species, *O. aestuarensis* (N = 37) was compared with all the euryhaline *Orchestia* species. The closest species was *O. mediterranea* (N = 24), its sibling, with a mean CO1 genetic distance of 10.2%. The other mean interspecific genetic values were much higher: 16% with *O. montagui* Audouin, 1826 (N = 80), 19.8% with *O. gammarellus* (Pallas, 1766) (N = 244) and 21.0% with *O. stephensi* Cecchini, 1928 (N = 3)

## Geological background

The importance of the geological background in the study of *O. mediterranea* in estuaries of the coasts of the northeast Atlantic and Mediterranean Sea is that it provides a timescale for the evolution of its sibling species *O. aestuarensis*.

The Pleistocene history of the Thames and Medway estuaries in Britain are discussed by Bridgland and D'Olier (1995).

None of the glacials, including the last, the Devensian (115,000 to 11,700 years BP), reached the southern estuaries (e.g. Medway, Tamar) in Britain (Lee 2011), which suggests an estuarine fauna that is at least 400,000 years old. By contrast, the Duddon Estuary in the Lake District of north-western and the Humber Estuary in north-eastern Britain was ice-covered during the Devensian glacial (Lee 2011) and its fauna therefore extirpated, with a replacement age of < 10,000 years.

## Discussion

### *O. aestuarensis*/*O. mediterranea* sibling species pair in estuaries

All three of the proposed mechanisms of *in situ* evolution of *O. aestuarensis* from *O. mediterranea* in estuaries avoid the need for natural dispersal by the low salinity sibling from one estuary to another. Hypothetical mechanism 3 involves *de novo* evolution in each new estuary colonised by *O. mediterranea*. It seems to be less likely in view of the phenotypic similarities found in the few populations so far studied. The oligohaline *O. aestuarensis* in northern Britain would have diverged more recently (< 10,000 years) than southern species (> 400,000 years). Population divergence estimates, based on molecular genetic methods, such as the mitochondrial CO1 gene (Knowlton and Weight 1998), could indicate a temporal difference in the date of origin. Results expected would be for similar ages from different geographical samples, if mechanisms 1 and 2 were operating and variably divergent times for each estuary if mechanism 3 were op-

erating. For the latter, northern populations would have diverged more recently than southern ones.

For the ability to breed at low salinity (< 52‰ seawater), we propose that a dimorphic allele or linked set of alleles is selected for by low salinity conditions, a gene(s) is/are switched on by low salinity conditions or allopatric or parapatric speciation occurs in each new estuary, colonised by *O. mediterranea*. The minor morphological and dorsal pigment pattern differences with *O. mediterranea* in *O. aestuarensis* (Wildish 1987) are linked to genes or alleles governing the ability to breed at low salinities.

In a laboratory intrapopulation cross with males and females of *O. mediterranea* at Upnor Castle (polyhalinicum), Wildish (1970b) found that a male and female and 11 intersexes were produced with *aestuarensis* pigmentation patterns in the first generation, F<sub>1</sub> (N = 50). Ginsburger-Vogel (1991) also found a form of *O. mediterranea* in a Sijean salt marsh, on the French Mediterranean coast, which sometimes spontaneously produced the “*aestuarensis*” dorsal pigment pattern phenotype. Both observations suggest that *O. mediterranea* does carry alleles or genes characteristic of *O. aestuarensis*, supportive of either mechanism 1 or 2.

Histological observations and grafting experiments undertaken by Ginsburger-Vogel (1991) showed that intersexuality and female-biased sex ratios, as occur in wild populations of *O. aestuarensis*, are caused by the physiological activities of microsporidians within the host. We suggest that the relationship between the microsporidian(s) and its oligohaline amphipod host are symbiotic. This is because the microsporidian is provided with food and lodging by the host, but in return – perhaps by secreting appropriate hormone mimics – changes genetic males to females. Female biasing increases the fertility of a given population (Wildish 1971). The selective advantage here is not to the individual, but to the population. Such an adaptation may be of importance to small, precarious populations isolated in estuaries, like *O. aestuarensis*.

In an earlier paper (Wildish 1987), the genetic mechanism hypothesised to account for the evolution of *O. aestuarensis* included a superior ability to survive and breed in lower salinity conditions which was linked to the occurrence of intersexes/female bias. Ginsburger-Vogel (1991) showed that the occurrence of intersexes/female bias in *O. aestuarensis* is not linked to the ability to breed in low salinities, but independently results from co-evolution of an endosymbiotic protozoan and its host, *O. aestuarensis*. The enduring commonality between the two evolutionary mechanisms is that both are controlled by the same salinity switch. This leaves superior ability to breed and female biasing in low salinity conditions as two independent selective factors in the evolution of *O. aestuarensis*.

The genetic changes hypothesised to underlie low salinity switching in *Orchestia* occurred in the geological (recent) past, likely in different environments than those of today. It is therefore problematic to use direct experimentation to test the natural selection hypothesised to be involved. Instead, we have proposed inductive inference

**Table 2.** Putative sibling species pairs of Gammaridae associated with estuaries from the North Atlantic Ocean. Taxonomic names as in World Register of Marine Species (WoRMS), accessed April 2019, CO1 data from BOLD accessed May 2020, N = number of oligohaline/euryhaline individuals.

Estuary	Putative Sibling Species Pair		Reference	Mean % COI genetic distance	N
	Brackish / estuarine (oligohaline)	Marine / Estuarine (euryhaline)			
Canadian estuaries	<i>Gammarus tigrinus</i> Sexton, 1939	<i>Gammarus lawrencianus</i> Bousfield, 1956	Steele and Steele (1991)	24.86	172/70
North American estuaries	<i>Gammarus daiberi</i> Bousfield, 1969	<i>Gammarus tigrinus</i> Sexton, 1939	Bousfield (1973)	14.54	4/172
Deltaic Area, Holland	<i>Gammarus zaddachi</i> Sexton, 1912	<i>Gammarus salinus</i> Spooner, 1947	Den Hartog (1964)	14.62	60/82
Deltaic Area, Holland	<i>Gammarus salinus</i> Spooner, 1947	<i>Gammarus locusta</i> (Linnaeus, 1758)	Den Hartog (1964)	26.35	82/94
Deltaic Area, Holland	<i>Echinogammarus marinus</i> (Leach, 1815)	<i>Echinogammarus obtusatus</i> (Dahl, 1938)	Den Hartog (1964)	33.29	36/31

methods to do this. Strong evidence in support of low salinity switching will be provided if all the individual criteria, zoogeographic, physiological, ecological and genetic, reviewed above, support it.

### Amphipod sibling species pairs in estuaries: a possible general salinity switch hypothesis

Other species of amphipods are known to be sensitive to salinity conditions and some are confined to estuaries as sibling species (e.g. some gammarids) (Table 2). Amongst the Gammaridae of the North Atlantic region, we found five pairs of potential sibling species in estuaries and obtained their mitochondrial CO1 sequences from BOLD and compared interspecific genetic distances, as in *Orchestia*. Two species pairs, *G. zaddachi*/*G. salinus* and *G. daiberi*/*G. tigrinus*, were found to be genetically closer to each other than to other congeners (Table 2) and, thus, they are putative candidates for evolution by salinity switching. For two other species pairs of *Gammarus* and one pair of *Echinogammarus* (Table 2), there was no evidence that they were closer genetically than others within the same genus. Consequently, they are eliminated as candidates for evolution by salinity switching. We recognise that the genetic test we have used herein is not definitive on its own, because evolutionary pathways of species with reduced genetic distance between siblings could be directed by physical or biological factors other than salinity.

Genetic relatedness and ecological data (that the oligohaline amphipod is limited to low salinities in estuaries), together with other related biological data, are suggestive that low salinity conditions directed the evolution in these amphipods.

This study illustrates that limited ecological/genetic data are useful in identifying pairs of sibling species of estuarine amphipods which may have evolved by salinity switching. Using this method, three sibling species pairs were identified: *O. aestuarensis*/*O. mediterranea*, *G. zaddachi*/*G. salinus* and *G. daiberi*/*G. tigrinus*. Further studies, based on the six biological/geological criteria used herein to infer salinity switch evolution in *O. aestuarensis*/*O. mediterranea*, could

be applied to the two selected *Gammarus* sp. siblings. The location of a gene(s) for salinity switching would ultimately confirm the hypothesis.

We suspect that there will be other sibling species pairs of amphipods which have evolved in this way in other estuaries throughout the world and that our study is a step forward in shedding light on estuarine amphipod evolution.

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