DOTTORATO DI RICERCA IN ETOLOGIA, ECOLOGIA, ANTROPOLOGIA E BIOSISTEMATICA (XXVII CICLO)

Behavioural, ecological and physiological plasticity of East African fiddler crab (genus *Uca*): a paradigm of terrestriality
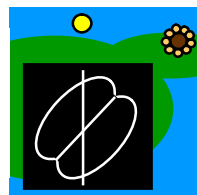
Tesi di

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CICLO XXVII

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Behavioural, ecological and physiological plasticity of East African fiddler crabs (genus *Uca*): a paradigm of terrestriality

Settore Scientifico Disciplinare BIO 05/BIO 07

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Anni 2011/2014

Un Granciu si picava
di educari li figghi
e l'insosizzunava
di massimi e cunsigghi,
'nsistennu: "V'àju dittu
di caminari drittu".
Chiddi, ch'intenti avìanu
l'occhi in iddu e li miri,
cumprendiri 'un putìanu
 "drittu" chi vulia diri;
sta idìa tra la sua cera
d'unni pigghiarla 'un c'era.
Iddu amminazza, sbruffa,
l'arriva a castïari,
ma sempri fici buffa:
mittennulu a guardari,
vidinu cosci e gammi
storti, mancini e strammi.
Alza l'ingegnu un pocu
lu chiù grannuzzu e dici:
"Papà, lu primu locu
si divi a cui ni fici:
vaìti avanti vui
ca poi vinemu nui".
 "'Nzolenti! Scostumati!"
grida lu patri, "oh bella!
A tantu vi assajati?
L'esempiu miu si appella?
Jeu pozzu fari e sfari,
 cuntu nun n'aju a dari;
si aviti chiù l'ardiri
birbi, di riplicari..."
Seguitau iddu a diri,
Seguitar' iddi a fari...:
 tortu lu patri, e torti
li figghi sinu a morti.

Giovanni Meli, 1814

Sommario

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Chapter 1 : Introduction

1.1 Fiddler crabs and Mangroves

Brachyuran crabs represent key models for investigating the process of terrestrialization (Bliss & Mantel, 1968). Paleontological, as well as biological evidence from extant forms, corroborates the hypothesis that they are at the dawn of land invasion, with the first fossil records of crab with a semiterrestrial life style going back no more than to the Cenozoic period (Greenaway, 1999; Naruse et al., 2003). The Ocypodidae family as well as Gecarcinidae and Sesarmidae families, has been considered as paradigmatic taxa for studying the evolutionary transition from water to land that they experienced, as they emerged from ancestor aquatic environments.

I focused my research on Fiddler Crabs (genus *Uca*), members of Ocypodidae family (Crane, 1976), the most recent marine animals to have invaded land (Rosenberg, 2001).

They spend the first part of their life as aquatic plankton and only settle in the intertidal zone after their last larval moult. Adults live in burrows on intertidal muddy and sandy substrata, within dense, mixed-age, mixed-sex and mixed-species assemblages (Crane, 1975). Each adult defends his or her own burrow and a small area around it. They are active on the surface during low tide, feeding on algae, bacteria and detritus in the topsoil (Crane, 1975). It is thought that fiddler crabs can live for up to seven years and adults of the largest species can reach a body size of about 5 cm (Crane 1975). The crabs grow by moulting which, under favourable conditions, they do about every eight weeks. Fiddler crabs are highly social animals with a rich behavioural repertoire. They communicate by visual (Zeil & Hofmann, 2001; Zeil & Hemmi, 2005) and vibratory signals; they have complex territorial interactions (Booksmythe et al., 2010) and flexible courtship and mating systems (deRivera & Vehrencamp, 2001). Some species carry individually distinct colour patterns (Detto, 2007) and some others even build mud or sand structures (Burford, 2001) as homing aids and to enhance or limit social interactions. As their common name suggests, one of the most obvious behaviours in a fiddler crab colony is claw waving (Pope, 2005): males wave their enlarged claw to attract females for mating and to repel intruders from their territory. The massive claw can weigh half a male's body weight and is also used as a weapon. Interestingly, handedness differs among species: in most species there are equal numbers of left- and right handed males, but in a few species virtually all the males are right-handed (Vernberg & Costlow, 1966). It is not yet clear what determines handedness nor what are its social consequences.

Fiddler crabs have two distinct mating strategies (deRivera & Vehrencamp, 2001; Ribeiro et al., 2005, 2010), with some species exhibiting

both forms. In one strategy, females leave their burrows and move through the assemblages visiting many males before choosing a mate. Males wave vigorously to attract these females to their burrow, where mating takes place underground
and where the female will incubate her eggs. In the other strategy, mating takes place at the entrance to the females' burrow, and it is the males that have to search for and locate the females.

During the evolution of fiddler crabs, intertidal environments as mangroves forests have played the role of ecological bridges able to connect the sea to the land (Little, 1990). Actually, Fiddler crabs constitute one of the most characteristic groups of animals associated with intertidal environment, in particular with mangrove forest in tropical and subtropical areas (Crane, 1975)

Mangrove forests cover a considerable part of the worlds' tropical and subtropical coasts. They are among the most productive ecosystems worldwide, providing substantial goods and services for people and the entire planet (Alongi, 2002).

A major unsolved enigma concerns how they maintain such enormous primary productivity in habitats exposed to extreme fluctuations in salinity and high hypoxia. Their high productivity is paradoxical because mangroves grow in low oxygen sediments, which should impair nutrient uptake (Duke et al., 2007; Cannicci et al., 2008; Kristensen, 2008). In particular, nutrient and water uptake by mangrove roots are strongly impaired by the scarcity of oxygen in the sediment. Soil hypoxia is caused by a combination of factors including periodic flooding, high oxygen demands by soil microorganisms and slow oxygen diffusion rates in the fine grain, highly impermeable sediment. During low tide burrowing by crabs enhances growth of the microphytobenthos (Kristensen & Alongi, 2006; Smith et al., 2009; Bartolini et al., 2011) and enhances sediment oxygenation (Tomilson, 1995; Donato et al., 2011; Andreetta et al., 2014). This is critical to the high productivity and carbon sink capacity of these forests (Lovelock et al., 2006; Hogarth, 2007).The diffusivity of oxygen is low in water, but production remains high during high tide when the sediments are flooded.

My thesis aims to clarify process of terrestrialization in Ocypodidae from several points of view: behavioural, ecological and physiological. Although Ocypodidae show terrestrial behaviour, they have been considered for long time as simple amphibious crabs because of their gill chambers were found barely vascularised and articulated (Farrelly & Greenway, 1993) Currently that definition is outdated by recent anatomical and physiological data. As a matter of fact, respirometric approaches (Fusi et al., 2014) revealed that *Uca urvillei* individuals have a good oxygen intake, linked with an high dissolved oxygen rate in the arterial haemolymph, in aerial medium. I, thus, investigated the terrestrialization in Ocypodidae, starting with the description

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and study of the peculiar behaviour, carried out by three species of fiddler crabs (*Uca urvillei, Uca chlolophthalmus* and *Uca annulipes*), related to their ability to seal their burrow as the tide rises(Chapter 2). I tried to understand if that could be considered a true adaptation to terrestrial life. I wondered if this behaviour could be a way to trap an oxygenated air reserve during the high tide and if there were differences among species and between sexes. The recorded data have been used also to determine if this behaviour build an air reservoir useful for the entire mangrove ecosystem, that is if that reservoir could guarantee an oxygenated soil to the mangrove root system (Chapter 3).

I investigated the bimodal breathing system of both sexes of two different species of fiddler crabs, *Uca urvillei* and *Uca annulipes,* from a physiological point of view (Chapter 4), that is recording the level of oxygen consumption in both aquatic and subaeral conditions. For both species I also conducted behavioural observations (Chapter 4), testing in laboratory their levels of disturbance during partial and forced submersion (restlessness, capsizing time, escape reaction) and their degree of preference for the subaeral condition with respect to aquatic condition during a simulated high tide phase.

Finally (Chapter 5), I investigated the spatial management of own territory in males of *Uca urvillei* species, observing in nature couples of neighbours residents to test the occurrence of the "dear enemy effect" (Fisher, 1954), that is the behavioural characteristic whereby residents are less aggressive towards neighboring territory owners than towards strangers (non neighbors). I tried to find correlation between defensive behavior of the territory and spatial and temporal patterns of activity.

1.2 Study species

I focused my theis on three species of fiddler crabs, both belonging to genus *Uca* of Ocypodidae family: *U. urvillei* (H. Milne-Edwards, 1852)*, U. cholophthalmus* (H. Milne-Edwards, 1837) and *U. annulipes* (H. Milne-Edwards, 1852).

The study species, as all fiddler crabs, are characterized by sexual dimorphism. In fact the males are usually larger than females and have an enlarged major claw, used during waving display to attract a female (Crane, 1975). The major claw is also used in aggressive interactions between males. Both species show digging behaviour to build a burrow in which the crabs can find a refuge during the high tide. Just before high tide comes, fiddler crabs occupy the burrow closing it with a "cap" made with mud or sand. In this way some air is trapped in the burrow also when the high tide comes (Chapter 3, present thesis).

From a physiological point of view, fiddler crabs are characterized by a bimodal

breathing system that allows to breath oxygen both atmospheric and solved in water (Fusi et al., 2014).

That is possible thank to a branchial chamber with highly convoluted vasculature, that works as a true lung (Chapter 3, present thesis).

U. urvillei (Fig.1) is the biggest among the study species (carapace width: 25.5-34mm males, 11.5-16.5 mm females) (Crane, 1976), and its colour is mainly blue/black with males having a white/orange major claw.

Figure 1 *Uca urvillei* specimen, male.

In the Indo-Pacific area, it inhabits the muddy substrate typical of the mangrove belts dominated by *Ryzophora* spp.; its geographical range is from Somalia to South Africa, Madagascar, the coast of Pakistan and western India (Vannini & Valmori, 1981). *U. cholophthalmus* (Fig.2) is characterized by green/black color with males having a red/orange major claw. The carapace length is 10.6.6-20 mm males, 9.6-18 mm females (Crane 1976).

Figure 2 *Uca cholophthalmus* specimen, male.

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The species inhabits muddy substrate and its geographical range is from Somalia to South Africa; Mauritius and Madagascar (Vannini & Valmori, 1981).

The last study species, *U. annulipes* (Fig.3), lives mainly on sandy substrate, that characterizes the landward belt of the mangrove forest, dominated by *Avicennia* spp.. Its carapace breadth is : 15-19 mm males, 11-15 mm females (Crane 1976) and the major claw of males is white.

Figure 3 *Uca annulipes* **specimen, male.**

Their geographical range is from Somalia to South Africa, Indonesia and Philippines (Vannini & Valmori, 1981).

1.3 Research period and study area

My research is the result of four fieldtrips conducted from 2012 to 2014. The sampling area was the mangrove forest in the Umngazana Estuary, South Africa (31°42' S, 29°25' E, Fig.4).

Umngazana River is situated in the Eastern Cape Province, South Africa, and it flows through 275 km^2 of catchment for 150 km before emerging into the Indian Ocean.

Figure 4 Map of South Africa showing the Umngazana Mangrove Forest

The estuary is approximately 5.3 km in length and is permanently open to the sea.

The Umngazana forest is the third largest mangrove area in the South Africa and covers approximately 118 ha (Sgwabe et al., 2004); it includes *Avicennia marina* and *Bruguiera gymnorrhiza,* but is dominated by *Rhizophora mucronata* trees.

Rainfall occurs throughout the year with more summer (November-January, 115.6 ± 3.4 mm) than winter rainfall (46.6 ± 3.1 mm) (Rajkaran & Adams, 2012). Annual minimum temperature ranges from 10.5 to 22.4 °C and maximum temperature between 18.7 and 28.2 °C.
Experiments were conducted at the Department of Zoology and Entomology, Rhodes University, Grahamstown, ZA.

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Chapter 2: Air storing behaviour in East African fiddler crabs: a comparative approach

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Abstract

Fiddler crabs (*Uca* spp.) are ubiquitous in mangrove forests. Vegetation, substratum, food, salinity, tidal exposure and presence of other species are considered as the environmental drivers that play a major role in the their distribution. At the same time, the mangrove ecosystem shows a high degree of interactions between macrofauna and plants.

We investigated the peculiar behaviour of air-storing in fiddler crabs, that are able to seal their burrows, trapping air bubbles during high tide. This behaviour is a key adaptation to a terrestrial life style. We recorded the volume of air trapped by fiddler crabs (*Uca urvillei, Uca chlolophthalmus* and *Uca annulipes*), focusing our work on differences among species and between sex. We also recorded the oxygen level saturation of air trapped by two species (*U. urvillei* and *U. annulipes)* inhabiting two different substrates. Results showed how fiddler crabs are able to trap high volumes of well oxygenated air in their burrows, during high tide. Differences among species and between sexes, are likely linked with differences in behavioural and biological traits.

2.1 Introduction

Mangroves forests are ecological bridges between the land and the sea, dominated by salt tolerant trees which evolved from rainforest trees over 50 million years ago (Duke, 1995; Ellison et al., 1999) and that are unique in their adaptation to the distinct environmental requirements of the intertidal habitat (Tomlison, 1986).

In this ecosystem, terrestrial plants have re-adapted to marine life, and marine invertebrates colonized intertidal and terrestrial habitats. They can be considered as evolutionary hotspots for the presence of many different niches

and the high daily and monthly variability in physical conditions (Cannicci et al., 2012).

These intertidal forests harvest a wide macro-faunal assemblage mainly consisting of marine taxa, which developed evolutionary trends from marine to semi-terrestrial and terrestrial life styles, such as snails, crabs and even fish (Cannicci et al., 2012).

In mangrove forests, crabs invaded a wide range of terrestrial and arboreal micro-habitats, playing a critical role in major mangrove ecosystem functions. In fact, all present-day brachyuran families with terrestrial and semi-terrestrial species, of non-freshwater origin, have mangrove representatives (Hartnoll, 1988; Schubart et al., 2006).

Fiddler crabs (*Uca* spp.) are ubiquitous in mangrove forests. Vegetation, substratum, food, salinity, tidal exposure and presence of other species are considered as the environmental components that play a major role in fiddler crabs distribution. At the same time, the mangrove ecosystem shows a high degree of interactions between macrofauna and plants (Aspey, 1978; Icely & Jones, 1978; Rabalais & Cameron, 1985; Ewa-Oboho, 1993; Thurman, 1998; Nobbs, 2003; Ribeiro et al., 2005; César et al., 2005, Bezerra et al., 2006). Organic matter, temperature, salinity, water content and granulometric composition of sediments are the substratum characteristics that deeply influence the fiddler crabs distribution in the mangrove forests (Frith & Brunenmeister, 1980; Ewa-Oboho, 1993; Reinsel & Rittschof, 1995; Mounton & Felder, 1996; Bezerra et al., 2006).

Fiddler crabs are specialized as detritivores, and different species of the genus *Uca* have morphological and behavioural adaptations for handling particles of a certain size of substratum and food (Crane, 1975). Facilitative interactions between crabs and low to middle marsh vegetation (primarily *Spartina alterniflora*) have been described by Bertness (1985) and Montague (1980; 1982). In the *Uca* feeding process, the ingested food is selected by the mouth parts. In this process, the crab takes a single grain of sand into the bucal cavity, where adhering organic matter is scoured off and the cleaned grain rejected (Miller, 1961). The mangrove ecosystem shows a high degree of coevolutive interactions between macrofauna and plants.

Plant production is affected by crab burrowing through moderating soil conditions by increasing soil aeration, by affecting the oxidation-reduction potential, and by accelerating the decomposition of plant debris (Montague, 1980 1982). Moreover, plants leaving in soft sediments facilitate crab burrowing by stabilizing the substratum, but in areas where the vegetation forms dense root mats, crab burrowing is effectively prevented (Bertness, 1985, Nomann & Pennings, 1998).

Focalizing our study on fiddler crabs, we wanted to investigate the role played by them in the aeration of the soil. We studied three congeneric species

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of *Uca* to record the quantity and the quality of the air trapped in the soil because of crabs burrowing activity. The study species were *U. urvillei* (H. Milne-Edwards, 1852)*, U. chlorophthalmus* (H. Milne-Edwards, 1837) and *U. annulipes* (H. Milne-Edwards, 1852) [see Chapter 1]and the data sampling was conducted from November 2012 to March 2013, during the spring tide periods. All samplings were conducted during afternoon high tide. The study area was the mangrove forest in the Umngazana Estuary, South Africa [see Chapter 1].

2.2 Materials and methods

2.4.1 Preliminary observations

We conducted preliminary observations during the low tide in the morning, to identify species and sex of burrow owners. We could thus mark the burrows by mean of long sticks, positioned close to their openings. In this way we were reasonably sure to assign the collected data to proper species and sex.

2.4.2 Measurement of burrow air volume

During the high tide, we positioned above the burrow cap an upset graded beaker, totally submerged and filled with water. We have been careful that any atmospheric air bubble was not trapped into the beaker. After that, we holed the burrow, trapping the burrow air into the beaker. Once all the air was trapped, we closed it by mean of a box full of water. So we were able to record how much air was contained in the beaker .We collected air samples from burrows of *U.urvillei* (32 males, 32 females), *U. chloropthalmus* (32 males, 32 females) and *U. annulipes* (32 males, 32 females).

2.4.3 Measurement of oxygen concentration in burrow-trapped air

We also analyzed the oxygen concentration in the burrows of *U. urvillei* (living on muddy substrate) and *U. annulipes* (living on sandy substrate). We collected air samples at the flloding tide (during the first hour of advance of the front of tide) and at the ebbing tide (during the first hour of retire of the front of tide), to observe how the oxygen concentration of the air trapped into the burrows varied during the submersion phase, using the same protocol used for the air volume. Once the air was in the submersed beaker, we transferred a small quantity of air from the beaker to a vial chamber (2 ml volume) by mean of a syringe. All the sampling protocol was conducted underwater and in absence of atmospheric air. Once trapped the air in the vial chambers, samples were rapidly transported in our field laboratory to record the oxygen concentration. Each vial chamber was equipped with an oxygen sensor (Sensor Type PSt3 PreSens, Regensburg, Germany), and connected to a single channel oxygen transmitter Fibox 3 (PreSens, Regensburg, Germany) through optical SMA fiber.

For each sample, the Fibox run for 60 seconds, recording the oxygen concentration every 5 seconds. After every recording, each vial chamber was filled with atmospheric air. These samples have been analyzed by mean of Fibox to obtain a reliable threshold value of oxygen concentration.

We collected 40 air samples from *U. urvillei* burrows (20 males, 20 females) at the beginning of high tide and 39 samples at the end of high tide (20 males, 19 females). Also from *U. annulipes* burrows, we collected 40 air samples (20 males, 20 females) at the beginning of high tide and 39 samples at the end of high tide (20 males, 19 females).

2.5 Data analysis

Our data were tested with Permutational Analysis of Variance (PERMANOVA), using PRIMER 6 & PERMANOVA + (Anderson et al. 2008); matrices from both data sets, air volume into the burrows and O_2 content in burrow air, were obtained with Euclidean Distances resemblance.

A one-way PERMANOVA was utilized to test differences between substrates (mud and sand) in the air volume contained into the burrows, with the only factor Substrate (two levels) orthogonal and fixed. For this analysis, we joined data of *U. chlorophthalmus* and *U. urvillei* individuals to obtain the "mud" data set, while *U. annulipes* represented the "sand" one; the two data sets were analysed with the multivariate Levene test (using the PERMDISP routine) to test homogeneity of the variance among samples; when variance within factors differed significantly, the data were transformed with square root or log (x+1) transformations.

A full factorial 2-way PERMANOVA was utilized to test differences in the air volume trapped into the burrows among species and between sexes, with both factors Species (three level) and Sex (two levels) fixed and orthogonal.

A full factorial 3-way PERMANOVA was utilized to test differences in O_2 concentration into the burrows at the beginning and at the end of high tide between species and sexes: all the three factors, Time (two levels: high tide beginning and end), Species (two level) and Sex (two levels) were fixed and orthogonal. All these analyses were conducted using 9999 permutations of the residuals under a reduced model (Anderson, 2001).

2.6 RESULTS

2.6.1 Air volume trapped into the burrows

All three focus species of fiddler crabs store about 70 ml of air in their burrows during the high tide (Fig.1). The burrows dug into the mud did not contain different air volumes with respect to the burrows dug into the sand (one-way PERMANOVA test, pseudo-$F_{1, 190}$= 0.01; P= 0.91).
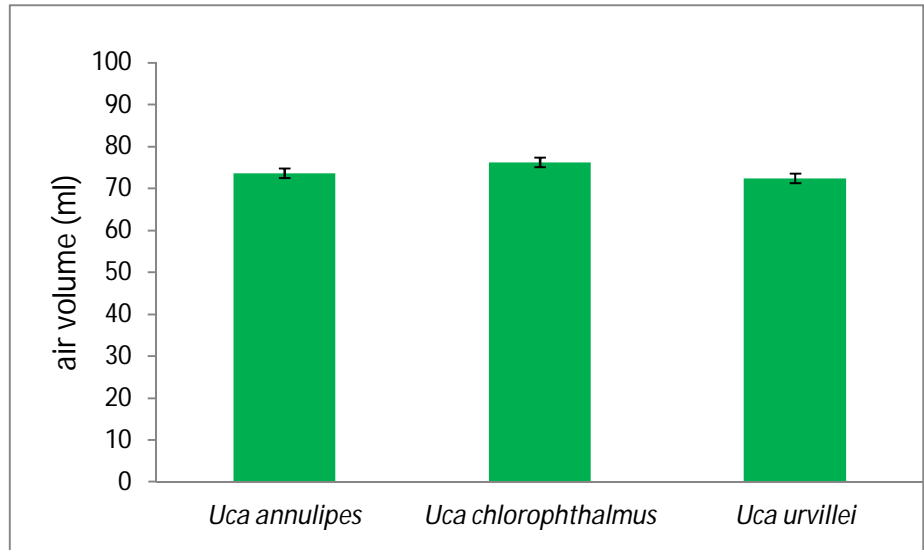
Figure 1 Mean volume (±SE) of air trapped in burrows by the three studied species of fiddler crabs

The full factorial 2-way PERMANOVA test showed no differences among species; it revealed instead significant differences for factor Sex and its interaction with the factor Species (Tab. 1).

Table 1 PERMANOVA test about air volume trapped by the three studied species of fiddler crabs. Factors abbreviation: Sp= Species, Se= Sex. Degree of freedom (df), mean square values (MS) and Pseudo-F values are reported. Probability level of Pseudo-F are indicated as **: p<0.01.

Source	df	MS	Pseudo-F	
Sp	2	243.44	0.17	
Se	1	26508	19.01	**
Sp × Se	2	14480	10.38	**
Res	186	1394.3		
Total	191			

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We conducted the Pair Wise test for the interactions between species and sex. Considering separately the three species, only *U. annulipes* showed differences between sexes: the males trapped a major air volume (around 100ml) with respect to the females (around 45 ml), (t=6.10 ; P=0.0001). There were no differences between sexes in *U. urvillei* (males around 70 ml; females around 74 ml) and in *U. chlorophthalmus* (males around 85 ml; females around 67 ml). The Pair Wise test individuated differences also among males: the *U. annulipes* males resulted to trap larger volumes of air than the *U. urvillei* males (Pair Wise test, t= 3.52; P= 0.0005). The opposite trend was shown for females: both *U. urvillei* and *U. chlorophthalmus* had more air in their burrows than the *U. annulipes* ones (respectively t= 3.41; P= 0.0022; and t= 2.49; P= 0.0145, Fig. 2).
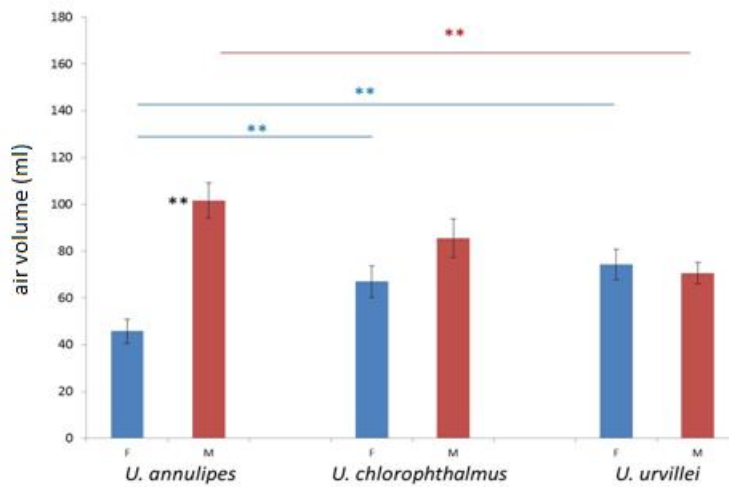
Figure 2. Mean volume (±SE) of air trapped in burrows by both sexes of three species of fiddler crabs, *U urvillei*, *U. chlorophthalmus* and *U. annulipes* (F = females, M = males) Probability level of Pseudo-F resulting from Pair Wise test are indicated as **: p<0.01.

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2.6.2 Burrow oxygen concentration

All the factors, Species, Sex and Time, resulted to have a significant relevance in determining the oxygen concentration (Tab. 2). Moreover, the PERMANOVA test showed significant differences for the interaction of the factors Species/Sex and Species/Time (Tab. 2). We conducted Pair Wise tests to individuate these differences. The oxygen concentration resulted to be higher in the burrows of *U. annulipes* males than both in the burrows of females of the same species (t= 5.66; P= 0.0001) and in the ones of males of *U. urvillei* (t= 4.56; P= 0.0001). We did not individuate significant differences between females of the two species, nor between sexes of *U. urvillei* (Fig. 3).

Table 2. PERMANOVA test results about oxygen concentration of air trapped by two species of fiddler crabs. Factors abbreviation: Sp= Species, Se= Sex, Ti= Time. Probability level of Pseudo-F are indicated as *: p<0.05 and as **: p<0.01.

Source	Df	MS	Pseudo-F	
Sp	1	2.91E-05	12.155	**
Se	1	6.79E-05	28.357	**
Ti	1	5.16E-05	21.571	**
sp × se	1	1.41E-05	5.8757	*
sp × ti	1	1.10E-05	4.5907	*
se × ti	1	8.81E-07	0.36813	
sp × se × ti	1	7.25E-07	0.30278	
Res	150	2.39E-06		
Total	157			
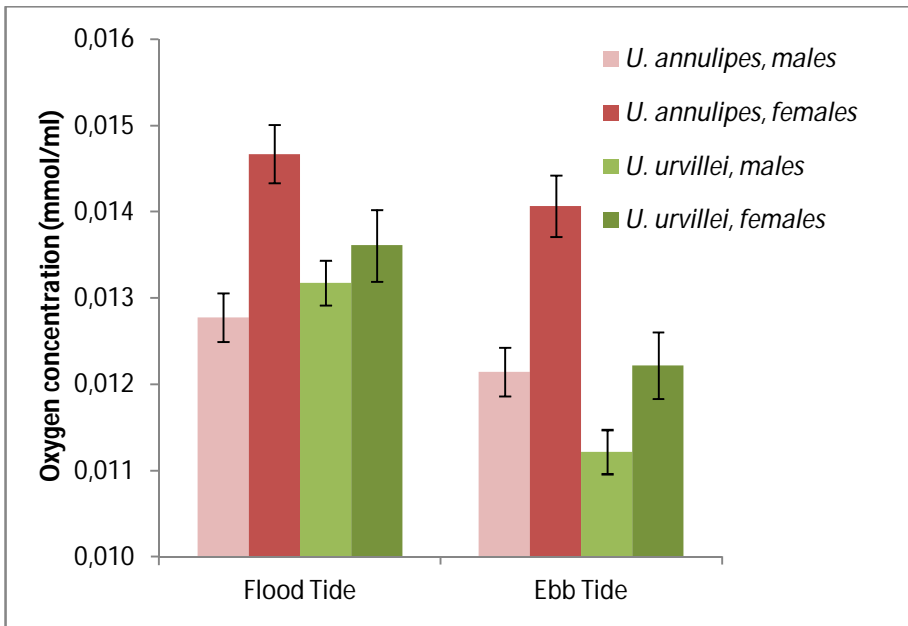
Figure 3. Oxygen concentration in both sexes of *U. urvillei* and *U. annulipes* burrows during flood and ebb tide phase

2.7 Discussion

Our results showed as the tree species of fiddler crabs investigated did not store different volumes of air in their burrows, independently on substrate (sand vs. mud) and species. All species in fact stored an average volume of about 70 ml of air during the high tide phase, confirming a strong relationship between fiddler crabs and sub-aerial environment as suggested by physiological (Fusi et al., 2014) and behavioural studies (Barnwell, 1966, 1968) .

Nevertheless our analysis revealed significant differences for factor Sex and its interaction with the factor Species.

Among the three focus species, only *U. annulipes* showed differences between sexes: the males trapped a volume almost double respect to the females. In *U. urvillei* and *U. chlorophthalmus*, males and females stored a comparable volume of air. Furthermore the air trapped by *U.annulipes* males was more than the air trapped by the other two species. An opposite trend was recorded among females of the three species. In fact, both *U. urvillei* and *U. chlorophthalmus* females showed a higher volume of trapped air with respect to *U. annulipes* females.

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In 2001, De Rivera and Vehrencamp presented a comparative study on mate searching in fiddler crabs. They found that female searching is associated with high burrow density, small body size, large substrate granulometry (i.e. sand), display complexity and short eyestalk. All these factors characterized the species *U. annulipes*. In detail, high density and large soil size lead females to mate and incubate in male's burrow. After oviposition, the male leaves the burrow which is then used by the mated female (Christy 1987; Christy & Salmon 1991). This frame could explain the difference between sexes recorded in *U. annulipes*. In fact, for hosting a female, a male should dig a greater burrow in order to trap a volume of air adequate for females with eggs and to provide a better thermal environment for incubation, as found also in U. beebei (Christy, 1987) and in *U. mjoebergi* (Reaney & Backwell, 2007). In *U. mjoebergi* (Reaney & Backwell, 2007), females chose larger males as sexual partner at the start of mating period. Since male size is positively correlated to burrow width, females select wide burrows and incubate at lower temperatures. This would slow down the developmental rates of larvae. In contrast, females that mate late in the mating period, select small males as mate. In *U. annulipes*, Backwell and Passmore (1996) found that, at the start of the mating period, females select the larger males in the population. Instead, towards the end of the sampling period, females are less selective. They also suggested that in *U. annulipes* females, mate choice is a two-stage process. First, females select males based on male size. They then decide whether or not to mate with a male based on burrow features. So burrow volume could represent one of features valued by females. The previous frame is not the case of *U. chlorophthalmus* and *U. urvillei*. In fact, bigger species with lower density and living on finer-grained soil as mud are characterized by male searching (Ribeiro et al., 2010). In this case, females do not mate and incubate inmale burrow and that could explain why there is no difference between sexes in trapped air volume of both *U. urvillei* and *U. chlorophthalmus.*

We also recorded as the air trapped in the burrows presents high level of oxygen concentration during the high tide phase. The mean level of oxygen saturation was in fact is over 80% (~ 0,015 mmol ml^{-1}) for both *U. urvillei* and *U. annulipes* at the beginning of the high tide. At ebbing tide, the oxygen concentration decreased at almost 70% for *U. urvillei* and at 79 % for *U. annulipes*.

The oxygen concentration in the males burrows of *U. annulipes* resulted to be higher than in the burrows of females of the same species. We did not individuate significant differences between females of the two species, nor between sexes in *U. urvillei*. Independently of species and sex, the volume of air trapped into the burrows represented a remarkable source of atmospheric oxygen for fiddler crabs, allowing the avoidance of hipoxic conditions during the high tide.

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Chapter 3: Oxygen Storage by Air-Breathing Crabs drives Mangrove Productivity

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Abstract

Mangrove forests perform critical ecological services for mankind, including buffering tsunamis. Their exceptionally high productivity is paradoxical because they grow in low oxygen sediments, which should limit nutrient uptake and production. During low tide, crab burrowing enhances sediment oxygenation, but production remains high when the sediments are flooded during tidal inundation. This is anomalous because oxygen diffusivity is low in water. Corrosion casting techniques and Micro Computed Tomography show that fiddler crabs (*Uca* spp.) have a well-developed lung and breathe air. During high tide, they trap air in their burrows at concentrations greater than in agricultural soil. This provides an enormous source of oxygen to mangrove roots, allowing their high productivity. Adaptations for air-breathing in fiddler crabs drastically affect the evolutionary and ecological success of mangroves.

Mangrove forests cover a considerable part of the worlds' tropical and subtropical coasts (Giri et al., 2011). They are among the most productive ecosystems worldwide (Alongi, 2002), providing substantial goods and services for people and the entire planet (Donato et al., 2011). A major unsolved enigma concerns how they maintain such enormous primary productivity in habitats exposed to extreme fluctuations in salinity and high hypoxia. In particular, nutrient and water uptake by mangrove roots are strongly impaired by the scarcity of oxygen in the sediment. Soil hypoxia is caused by a combination of factors including periodic flooding (Golley et al., 1962), high oxygen demands by soil microorganisms and slow oxygen diffusion rates in the fine grain, highly impermeable sediment (Bertics & Ziebis, 2009). Since only a steady provision of oxygen to the roots can sustain mangrove performance (Colmer & Voesenek, 2009), it is generally assumed that they rely on diffusion and internal storage of oxygen in the aerenchyma to satisfy their oxygen requirements (Duke et al., 2006). Diffusion alone cannot, however, fulfil the oxygen requirements of the submerged root system (Duke et al., 2007). Thus, pressurised venting (Evans et al., 2009) and aerial root photosynthesis (Kitaya et al., 2002) have been proposed as additional root oxygenation mechanisms, particularly at high tide, but this has never been demonstrated. Other oxygen sources do exist for mangrove roots, however. Mangrove forests are generally characterised by enormous numbers of crabs and their burrowing behaviour enhances oxygen penetration and diffusion in the soil through bioturbation (Tomlinson, 1995; Kristensen & Alongi, 2006; Smith et al., 2009; Bartolini et al., 2011). Since the dominant mangrove crab families Ocypodidae and Sesarmidae, are considered to be amphibious, their contribution to oxygen provision to the soil is thought to be restricted to low tide, when the upper parts of the burrows are filled with air, while at high tide the burrows are assumed to be flooded with water. Here we overthrow this perception, showing that the behaviour and respiratory physiology of these crabs lead to air-mediated oxygenation of the soil during

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both tidal phases, massively increasing oxygen availability to mangrove roots. We base this new understanding on (a) the respiratory apparatus of mangrove-dwelling fiddler crabs of the genus *Uca*, and (b) measurements of the volumes of oxygenated air trapped in *Uca* burrows during high tide along the tropical and sub-tropical east coast of Africa.

Casts of branchiostegal circulatory system were obtained by perfusion of polyurethane based casting resin into the heart or infrabranchial sinus of Kenyan specimens of *Uca vocans* [e.g. see supplementary materials]. Some casts were examined with a Philips XL20 SEM, while others were analysed through Micro Computed Tomography (MicroCT). The 3D reconstruction of the image stacks produced by MicroCT was created using Imaris 6.4. software (Bitplane, Switzerland).

Micro Computed Tomography (Micro CT) provided very fine resolution of the 3D morphology of the branchiostegal circulatory system in the East African mangrove crab *Uca vocans*. The branches of afferent sinuses interdigitate with those of the efferent sinuses (Fig. 1), forming a branchiostegal lung with highly convoluted vasculature. This overturns the perception that the lung of *Uca* has few, very restricted branchiostegal branches, forming a "smooth lung with limited branching" (Farrelly & Greenaway, 1993). Moreover, we found that the entire lung, composed of the branchiostegal lung and the thoracic wall, lines approximately two third of the cephalothorax, revealing its advanced role in the respiratory physiology of this species.

The presence of such an efficient lung in mangrove crabs generated new hypotheses about their respiration during high tide, corroborated by the behaviour of many *Uca* spp., which plug their burrows just before tidal flooding (see Supplementary Materials, Movie S1), suggesting that air could be trapped within burrows to maintain aerial respiration.

We therefore measured the air trapped during high tide in the burrows of two species dominating the sea- and landward belt of East African mangroves, *U. urvillei* and *U. annulipes*, and assessed the oxygen content of air within these burrows during high and low tide, to derive estimates of oxygen consumption by the crabs. Air and oxygen surveys were carried out in a Kenyan (tropical) and a South African locality [e.g. see supplementary materials]. In both localities, mean densities/m^2 of fiddler crabs were measured in sea- and landward mangrove belts using standard techniques (Skov et al., 2002: Cannicci et al., 2009). Mean air volume trapped in burrows was quantified by piercing the burrows during high tide and trapping the air in graduated beakers. Mean air [O_2] was measured with a PSt3 PreSens oxygen sensor connected to a single channel oxygen transmitter Fibox 3. Statistical differences among sexes and species were analysed using Permutational Analysis of Variance (PERMANOVA), using PRIMER software. Both species trapped an average of more than 70 ml of air per burrow, with no significant differences between species (Pseudo-F_1= 0.04; P= 0.83). Males of *U. annulipes* trap higher volumes than females (Pair Wise test, t= 6.11; P= 0.0001) and *U. urvillei* males (Pair

Wise test, t= 3.52; P= 0.0012), while *U. annulipes* females trap less air than *U. urvillei* females (Fig. 2). The O_2 concentration in trapped air decreased during high tide (Pseudo-F_1= 21.57; P= 0.0001), with the larger *U. urvillei* showing faster uptake rates than *U. annulipes* for both sexes (Pair Wise Tests, P < 0.03 in all cases).

Nevertheless, the oxygen content of air in burrows did not decrease steeply during high tide, retaining a mean concentration of 0.012 ± 0.0002 mmol ml^{-1} when the burrows were re-opened at low tide. Given the amount of air trapped in each burrow and the density of burrows, this indicates a considerable volume of O_2 is stored in the soil throughout the entire high tide period. To evaluate the ecological relevance of this air-storing behaviour on the whole mangrove system, we combined these figures with data on density and sex ratios of four *Uca* species, surveyed in sandy and muddy substrata of a tropical Kenyan, and a subtropical South African mangrove. Air volume and O_2 content per square meter of soil were estimated to be consistently higher than 2 litres of air and around 30 mmol of $O_2 m^{-2}$ at all sampled sites, values being especially high in the muddy substratum of the Kenyan mangrove (9778.36 ± 1873.67 ml of air m^{-2} and 128.59 ± 24.02 mmol of $O_2 m^{-2}$; Fig. 3). Thus, considering an average air-filled soil porosity of 20% [values considered optimal for root growth (Zou et al., 2001)], and assuming an average burrow depth of 40 cm (Kristensen, 2008) then the air estimated to be trapped in the crab burrows is at least one order of magnitude larger than the average air concentrations in agricultural soil (Cook et al., 2013). To project across larger geographical scales, we applied the estimates of air and oxygen content at our study sites using data on crab densities reported for other East African localities from Kenya, Zanzibar and Mozambique (Skov et al., 2002: Cannicci et al., 2009). Our estimates of O_2 availability due to *Uca* activity show that the mangrove soils are neither anoxic nor even hypoxic during high tide as previously believed (Supplementary Materials, Fig. S1).

To date, no physiological adaptation of mangrove trees could explain their high productivity under the hyper-saline, hypoxic soil conditions they experience when waterlogged. Here we present data supporting the novel hypothesis that their root system can rely on a continuous supply of oxygen through the air stored in crab burrows. Our estimates of the volumes of highly oxygenated crab-trapped air in every vegetation belt studied in a variety of East African mangrove forests indicate far higher amounts of readily diffused oxygen in the soil than expected. These estimates are sufficient to address the oxygen requirements of mangroves throughout the tidal cycle, offering an explanation for their huge productivity and their important role in global carbon cycling (Lovelock et al., 2006; Donato et al., 2011; Alongi, 2014; Andreetta et al., 2014). Indeed, based on mangrove root respiration rates ranging from 0.000625 to 0.0075 mmol O_2 kg^{-1} s^{-1} (Cannicci et al., 2008) and a fine root biomass accumulation of 8 kg ha^{-1} yr^{-1} measured in a mangrove forest in Gazi Bay, Kenya (Githaiga, 2013), then mangrove root oxygen

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requirements would oscillate between 0.0018 to 0.0216mmol O_2m^{-2} h^{-1}, well below the values we estimated for the crab burrows.

The presence of massive air reservoirs in the soil is the result of the evolutionary adaptation of fiddler crabs to air breathing, which is a fundamental step towards the terrestrial invasion performed by various crab families (Burggren & McMahon, 1988). It is important that the families of both extant terrestrial and semi-terrestrial crabs and of mangrove trees originated on Indo-Pacific equatorial coasts, and that their present distribution and evolutionary history largely overlap in time and space (Ellison et al., 1999; Giomi et al., 2014). We believe that this explains the evolution of the mangrove ecosystem. Our evolutionary hypothesis is that a strong link exists between the appearance of terrestrial crabs and the conquest of the intertidal by mangrove trees, resulting in mutually beneficial co-evolution. The significance of this is on a par with the co-evolution of angiosperms, humus and earthworms. We believe we can now respond to Darwin, who, amazed by the role of earthworms as natural ploughs for terrestrial soil, wondered "whether there are many other animals which have played so important a part in the history of the world, as have these lowly organized creatures" (Darwin, 1881). Considering the invaluable ecological functions and services provided by mangrove systems to human kind, we suggest that the crabs that sustain mangrove growth and production through their burrowing activities are another animal which has played a critical part in the history of the world.

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Supplementary Materials

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Materials and Methods

Fig. S1

Movie S1

experimental design, together with Fa.P., analysed the data on air storage, with I.O., and wrote the manuscript with major input from C.M. and from N.B., I.O. and S.M. F.P. and C.W. carried out the branchiostegal casts, the SEM examinations, the MicroCT analyses. M.F., C.L.B. and Fa.P. superintended and carried out the field work in Kenya and South Africa, performing preliminary data analyses. N.B., M.F., Fa.P., C.M. and S.M. interpreted and discussed the results and improved the paper.
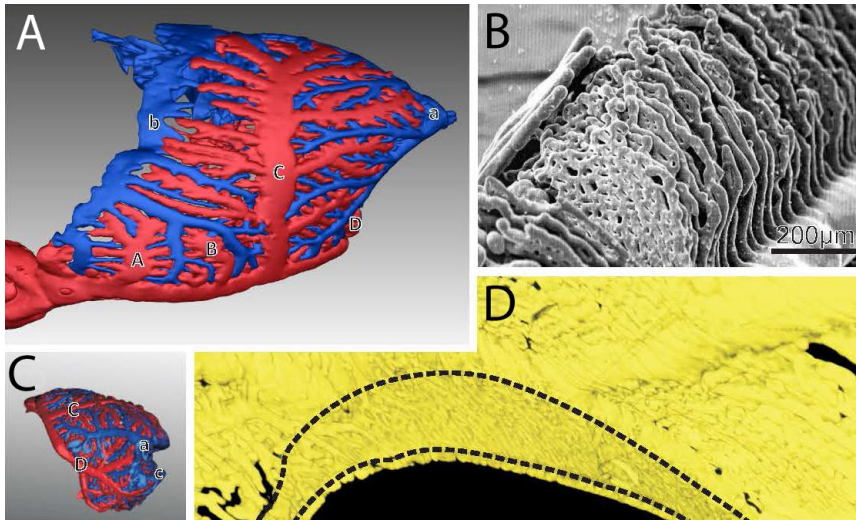

Fig. 1.The morphology of the branchiostegal lining and the gills in *U. vocans* shows a strong air-breathing adaptation. The PDF version of this article contains interactive 3D content. Open the file with Adobe Reader and click on figure a to activate the content and then use the mouse to rotate the objects. Use the menu in the activated figure to use further functions. a + b, Surface rendering of the branchiostegal lining. Afferent vessels in blue, efferent vessels in red. (**A**), Dorsal aspect. (**B**), Lateral aspect. (**C**), Corrosion cast of the sinuses in gill 6. (**D**), Volume rendering of the branchiostegal shelf (bordered by broken lines). Note density of sinuses.
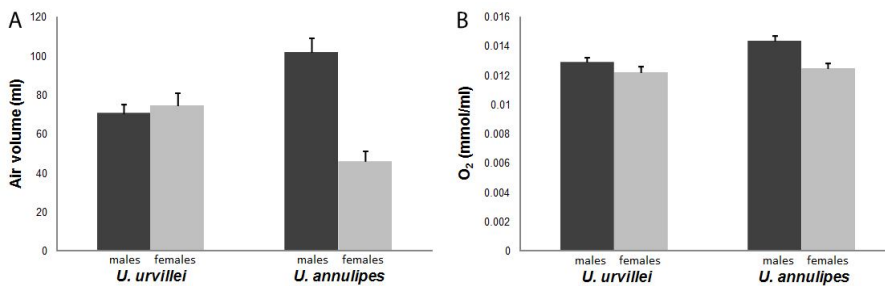
Fig. 2. The two *Uca* species dominating the landward and seaward mangrove belts of East Africa store a considerable amount of oxygenated air in their burrows at high tide. (**A**), amount of air and, (**B**), of oxygen recorded at high tide within the burrows of males and females of *U. urvillei* and *U. annulipes*, the most abundant fiddler crabs inhabiting, respectively, the

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seaward and landward mangrove belts of East Africa (means ± standard errors, n = 128 and n = 80 burrows for air and oxygen content, respectively).
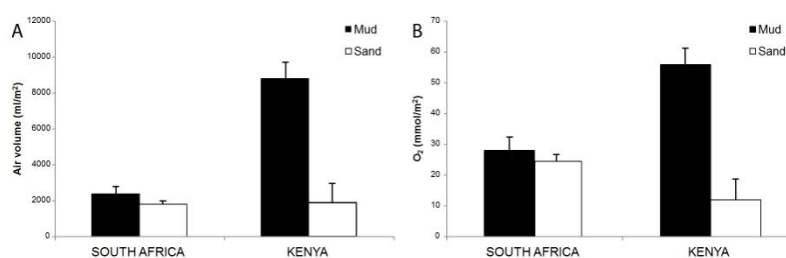
Fig. 3.A large reservoir of oxygenated air is stored under the soil of Kenyan and South African mangrove systems. (**A**), estimation of average amount of air and, (**B**), of oxygen stored per unit area and calculated for the mangrove forest of Gazi Bay and Mgazana. The estimations were calculated multiplying on the data collected per burrow, showed in Fig. 2, by the average number of burrows recorded through field surveys carried out in the seaward and landward belts of the same forests (means ± standard errors, n = 10 plots for each

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Supplementary Materials for

Oxygen Storage by Air-Breathing Crabs drives Mangrove Productivity

</div>

Stefano Cannicci, Nadia Bazizinha, Marco Fusi, Carmelo La Barba, Irene Ortolani, Francesco Paoli, Francesca Porri, Christian Wirkner, Christopher McQuaid & Stefano Mancuso

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This PDF file includes:

>   Materials and Methods
>   Fig.S1
>   Caption for Movies S1

Other Supplementary Materials for this manuscript includes the following:

Movie S1

Materials and Methods

Casts of branchiostegal circulatory system of *Uca vocans* were obtained by perfusion of the polyurethane based casting resin Pu4ii (VasQtec, Zürich, Switzerland) into the heart or infrabranchial sinus of specimens collected at Gazi Bay, Kenya. Soft tissues were then macerated in 7.5% KOH, followed by

decalcification of cuticle with sodium hypochlorite, each for 24 h at 70° C. Some casts were rinsed in water, freeze dried, mounted on aluminium stubs, coated with gold using a Balzer Med 010 and then examined with a Philips XL20 scanning electron microscope (SEM) operating at 10kV.

Other samples injected with Pu4ii were analyzed through Micro Computed Tomography (MicroCT) after fixation in Bouin's fixative for 24 hours. The X-ray beam production was performed by a Nanotom MicroCT system (Phoenix X-Ray, GE Sensing and Inspection, Wunstorf, Germany) set at 35 Volts and 280 Ampère with a voxel resolution of 7.5 µm, using molybdenum for the target. The 3D reconstruction of the image stacks produced by MicroCT was created using the software Imaris 6.4. (Bitplane, Switzerland).

Air and oxygen surveys were carried out in two localities of eastern Africa at different latitudes: tropical Shirazi (Kenya) and sub-tropical Mgazana (South Africa). In both localities, mean density of mangrove crabs were measured in both sand substratum areas (*U. annulipes* and *U. inversa*) and mud substratum areas (*U. chlorophthalmus* and *U. urvillei*). The relative abundances of species and sexes were estimated by counting the individuals in three subsamples (50×50 cm) in every transect. The burrows were counted in ten transects (1×1 m) for each substratum type in each locality. Burrows were attributed to species and sex accordingly to the recorded relative abundances to determine crab abundances/m^2.

Mean air volume trapped in the burrows was quantified by piercing the burrows during high tide and trapping the air in graduated beakers. The burrows were previously marked (during low tide) to attribute the air volume to the proper species and sex (32 males and 32 females per species). Statistical differences among sexes and species were analysed using Permutational Analysis of Variance (PERMANOVA), using the PERMANOVA+ routine for PRIMER® software (Anderson et al, 2008).

Mean [O_2] in the burrow air was measured from samples taken at the end of tidal flow from burrows that had previously been marked (20 males and 20 females per species). An oxygen sensor (Sensor Type PSt3 PreSens, Regensburg, Germany), glued to the inside wall of the sampling vials and connected to a single channel oxygen transmitter Fibox 3 (PreSens, Regensburg, Germany) through optical SMA fiber with was utilized.

Total soil air volume (ml m^{-2}) and O_2 availability (mmol m^{-2}) were calculated for both substrata at both localities on the basis of recorded crab densities.

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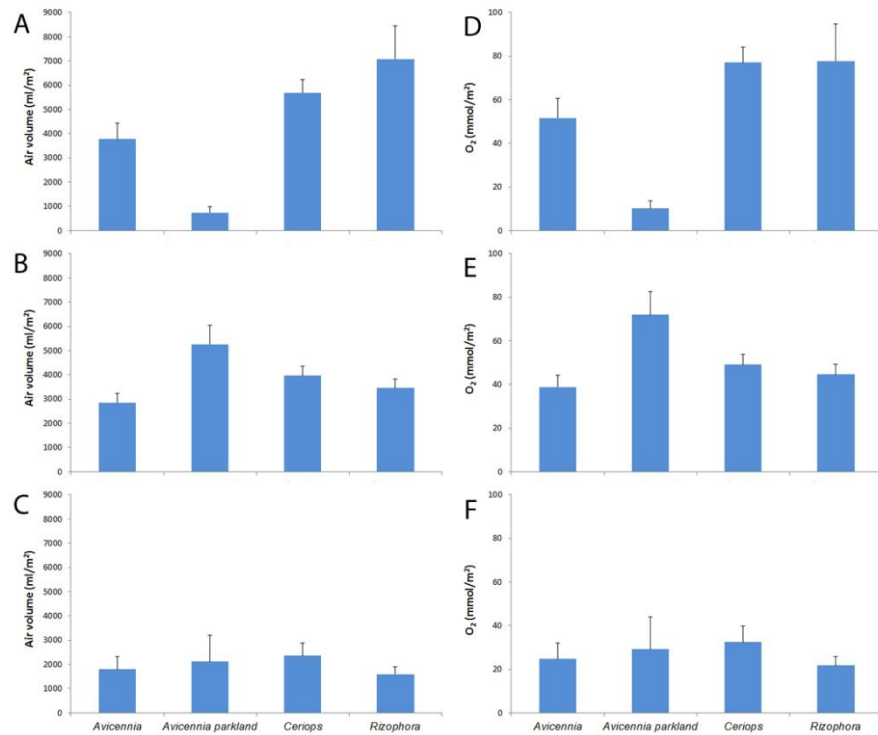
Fig. S1. Estimates of amounts of oxygenated air stored in the soil at high tide by fiddler crabs for East African mangrove systems. (A-C), estimation of average amount of air and, (D-F), of oxygen stored per unit area and calculated for the mangrove forests of Mida Creek (Kenya), Saco da Inhaca (Mozambique) and Kisakasaka (Zanzibar, Tanzania). The estimations were calculated multiplying on the average amounts of air and oxygen recorded per burrow in the present study by the average number of burrows recorded through field surveys carried out in previous studies (Cannicci et al. 2009; Bartolini et al., 2011; Githaiga, 2013) in the different belts typically found in East African mangrove forests (from land to the sea: *Avicennia = Avicennia marina* dominated belt; *Avicennia* parkland = sandy dominated belt with few *A. marina* specimens; *Ceriops = Ceriops tagal* dominated belt; and *Rhizophora = Rhizophora mucronata* dominated belts, (means ± standard errors).

Movie S1

A male of *Uca urvillei* plugs its burrow before flooding tide using a highly stereotyped behaviour.

Chapter 4: Fiddler crabs as a model of transition from water to land: degree of terrestrialization in two species of the genus *Uca*

In preparation

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Abstract

Mangrove forests represent an ideal ecological bridge between sea and land, a natural laboratory in which many taxa, such as the fiddler crabs (*Uca* spp.), show behavioural and physiological adaptations to terrestrial life. Within this frame, the family Ocypodidae has been considered a paradigmatic taxon for studying the evolutionary transition from water to land. In this study, we investigated the degree of terrestrialization of both sexes of two species of fiddler crabs (*Uca urvillei* and *U. annulipes*) from both a behavioural and physiological point of view.

We conducted a series of physiological tests, recording the level of oxygen consumption in aquatic and subaerial environment. Experiment showed higher oxygen consumption rate in *U. urvillei* with respect to *U. annulipes*. Generally, females showed a higher oxygen consumption rate with respect to males. We also conducted a series of behavioural assays, recording the level of restlessness during forced submersion, the effect of such submersion on the escape reaction, and the crabs' habitat preference when both immersed and emerged areas were available. We recorded a higher level of restlessness in males with respect to females in both species. The escape reaction was affected by forced submersion: in *U. urvillei*, individuals forced to submersion showed slower escaping reaction with respect to animals with access to the air. Males of both species and females of *U. urvillei* showed a preference for the emerged habitat respect to water.

4.1 Introduction

Land crabs have been studied for many years to understand the evolutionary transitions that semi-terrestrial and terrestrial crabs have experienced as they emerged from ancestor aquatic environments (Burggren & McMahon, 1988; Farrelly & Greenway, 1993). During the evolution of land crabs, such as the Ocypodidae, intertidal environments, as mangroves forests, have played the role of ecological bridges able to connect the sea to the land (Little, 1990).

Extant species of fiddler crabs (*Uca* spp.) are indeed ubiquitous in mangrove forests and their activity is concentrated during low tide (Barnwell, 1966, 1968; Burggren & McMahon, 1988; Booksmythe et al., 2010) .Notwithstanding such terrestrial behaviour, they have been considered for long time as simple amphibious crabs, because their gill chambers were found barely vascularised and articulated as lungs (Farrelly & Greenway, 1993). Currently that definition is outdated by recent anatomical and physiological data. In fact, physiological studies (Fusi et al., 2014) revealed that *Uca* spp. have a good oxygen intake, linked with a high dissolved oxygen concentration in the arterial haemolymph, in aerial medium. So, they can be considered true air-breathing organisms (Fusi et al., 2014). Moreover, they present peculiar adaptation to the terrestrial life revealing the need for air-storing behaviour as a highly vascularised branchial chamber, functioning as a well-developed lung (Chapter 3, present thesis). They also dig burrows used as refuges during high tide, trapping an air-bubble inside (Chapter 2, present thesis). In fact *Uca urvillei* and *Uca annulipes* species (Chapter 2, 3 present thesis) store an average volume of about 70 ml of air during the high tide phase with *U. annulipes* presenting a clear between sex (males trap a double volume in respect to females, Chapter 2, present thesis). At the end of the high tide, the level of oxygen saturation is elevated. In fact it is almost 70 % for *U. urvillei* and at 79 % for *U. annulipes*.

However, fiddler crabs are not totally independent from water, because of their life cycle. In fact the female fiddler crab carries her eggs attached to the abdominal appendages, beneath the carapace. She remains in her burrow during a two week gestational period, after which she ventures out to release her larvae into the ebbing tide. The larvae remain planktonic for a further two weeks (Anger et al., 1990; Borgianini et al., 2005; Brodie et al., 2007).

Starting on these premises, we wanted to investigate, using both a physiological and a behavioural approach, the degree of terrestrialization of both sexes of *U. urvillei* and *U. annulipes,* two different species inhabiting two different mangrove areas, characterised by different substrates. We firstly focused on the amount of oxygen consumption in subaerial and aquatic condition. We then tested the effect of submersion on the level of restlessness and on the quality of the escape reaction. Finally, we tested the possible habitat preference under laboratory conditions.

4.2 Study species, period of research and sampling area

We studied two species of fiddler crabs, both belonging to genus *Uca* of Ocypodidae family: *U. urvillei* (H. Milne-Edwards, 1852)*, and *U. annulipes* (H. Milne-Edwards, 1852). Our study was conducted from November 2013 to April 2014 andthe sampling area was the mangrove forest in the Umngazana Estuary, South Africa (Chapter 1, present thesis).

4.3 Materials and Methods

4.3.1 Sampling, transport and rearing

The specimens were collected during low tide and stored in plastic containers with some mud and sea water. After the transport to Grahamstown, they were allocated in thermostatic rooms at the constant temperature of 20 °C. The day and night cycle was 12 hours with light from 07:00 AM to 07:00 PM. After the transport to the laboratories and before starting experiments, acclimation period was of 48 hours. For our experiments we tested a total of 150 *U.urvillei* males, 150 *U. urvillei* females, 120 *Uca annulipes* males and 120 *U. annulipes* females.

4.3.2 Bimodal breathing test

We wanted to investigate the characteristics of the bimodal breathing performed by the fiddler crabs, recording oxygen intake of both sexes of *Uca urvillei* and *Uca annulipes*. Specimens were maintained for 2 days before the experimentation in big plastic tanks (50×70×35 cm) with fresh mud and aerated seawater (35‰ salinity) at 20 ± 1 °C, and exposed to an artificial 12 h light cycle.

The rates of oxygen consumption (MO_2 thereafter) were measured in both air and water media. For each medium, a total of 60 individuals per species were tested. Considering the size difference, individuals of *U. urvillei* were tested using darkened Perspex chambers with a volume of 450 ml, while the individuals of *U. annulipes* were tested using darkened chambers with a volume of 48 ml. For each trial eleven chambers, ten of which occupied by crabs, were placed in a temperature controlled water bath (20-22 °C), for a total of 6 trials per medium per species. An oxygen sensor (Sensor Type PSt3 PreSens, Regensburg, Germany), glued to the inside wall of each chamber and connected to a single channel oxygen transmitter Fibox 4 (PreSens, Regensburg, Germany) through optical SMA fiber, was used to measure the partial pressure of oxygen in both air and water trials. Data were recorded using the PreSens Datamanager software.

Prior to measurements, sensors were calibrated in air-equilibrated seawater (100% saturation) and in sodium thiocyanate saturated solution in seawater (0%) and every crab was kept for 8 hours (overnight) of acclimation in the chamber. To test the oxygen consumption in air, we reduced the volume of chambers using glass marbles with a known volume. For the air-breathing tests, each chamber was connected to the external atmosphere by a couple of rubber pipes to keep a high level of oxygen during the acclimation time. For the water test instead, during the acclimation time the chambers were connected to the water bath by water pumps. An empty chamber was run in each trial as control, to account for background oxygen depletion, which was less than 2% of the crabs' consumption in water, and negligible in air. Following each trial, each animal was weighed and its volume calculated by immersing it in a graduated cylinder and measuring the volume of water displaced.

After 8 hours of acclimation we sealed each chambers to create an isolated environment. In this way it was possible to observe and to record the decreasing of oxygen saturation level due to crabs breathing.

We recorded the oxygen consumption measuring the oxygen level once sealed the chambers (T0), after 2 hours and 30 minutes (T1) and after 5 hours (T2). Every measurement lasted 1 minute, with a sampling rate of 5 seconds.

4.3.3 Submersion tolerance and escape reaction tests

With this experiment we wanted to analyze the effects of both partial and forced submersion in water of *U. uvillei* and *U. annulipes*, during a period of time of five hours, to simulate a high tide phase during the afternoon (from 1:00 PM to 6:00 PM).

For partial submersion, we tested *U. urvillei* (30 males, 30 females) and *U. annulipes* (30 males, 30 females), releasing each individual in a plastic bucket (diameter 30 cm). Each crab was covered by 500 ml and 200 ml of sea water *U. urvillei* and *U. annulipes*, respectively. In this way, crabs were able to reach the water surface. Each hour we recorded if the individual showed behavioural restlessness (i.e. attempts to escape long the bucket side or to position the mouth parts out of the water).

For total submersion, we tested also 30 males and 30 females of *U. urvillei*, keeping them for five hours in total submersion: the water level did not allow them to reach the water surface to breathe air. We could not test *U. annulipes* in that condition because of the limited number of specimens sampled in the field.

After five hours of submersion we tested the reactivity of each crab, recording the time needed to capsize itself and the type of reaction once released in an empty bucket (Immobility, Slow Escape, Rapid Escape). About the escape response, we consider the condition of rapid escape as the natural

reaction to an external disturb. In fact, fiddler crabs are characterized by high sensibility to movement and rapid escape reaction.

4.3.4 Habitat preference test

Our third experiment consisted in a habitat choice test, aimed at studying their preference for aquatic or subaeral condition. We tested the two species *U. urvillei* (30 males and 30 females) and *U. annulipes* (30 males and 30 females) releasing each crab in a plastic bucket for a time of 5 hours, from 1:00 PM to 6:00 PM, to simulate a high tide phase. Each bucket was equipped with some sea water and some stones to permit to crabs to emerge. Every hour we recorded the position of the crabs (In or Out of the water).

4.3.5 Statistical analysis

For all experiments we calculated average and standard error for the considered parameters. We also performed a 4-way PERMANOVA Test to investigate the role of factors Species, Sex, Medium and Interval on level of oxygen consumption while, about behavioural observation (reaction to submersion, habitat preference), we applied the Chi Square Test to detected differences among tested groups. Finally, the influence of the factors Species and Sex on subaerial enviroment preference has been investigated by a full factorial 2-way PERMANOVA.

All these analyses were performed using PRIMER 6.1 and the PERMANOVA+ for PRIMER routines (Anderson et al., 2008).

4.4 Results

4.4.1 Bimodal breathing test

The full factorial 4-way PERMANOVA test showed strong differences between first and second test interval; moreover, it revealed significant differences between Species, Sex, Medium and among their interaction (Tab. 1).

Table 1 Results of the full factorial 4-way PERMANOVA test on oxygen consumption. Degree of freedom (df), mean square values (MS) and Pseudo-F values are reported. Probability level of Pseudo-F are indicated as **: p<0.01.

Source	df	MS	Pseudo-F	
Time	1	2.5549	14.131	**
Species	1	7.2536	40.118	**
Sex	1	6.1318	33.913	**
Medium	1	0.78095	4.3192	**
Spe×Se×Me	1	2.3046	12.746	**
Residuals	224	0.18081		
Total	239			

As showed in tables 2 and 3, we recorded a higher level of oxygen consumption during the first interval with respect to the second interval. That could be due to the stress for movements and vibrations caused by the starting sealing of chambers; this stress could likely induce an initial higher metabolic rate.

Table 2 Breathing in water: average oxygen consumption rates of *U. urvillei* and *U. annulipes* expressed in $\mu molO_2g^{-1}h^{-1}$; standard errors are reported.

Group in water	I Interval	II Interval
U. urvillei **Males**	2.28 ± 0.2	1.37 ± **0.1**
U. urvillei **Females**	**2.**61± 0.4	1.38 ± **0.2**
U. annulipes **Males**	1.16 ± 0.1	0.69 ± **0.1**
U. annulipes **Females**	1.64 ± 0.1	1.38 ± **0.1**

Table 3 Breathing in air: average oxygen consumption expressed in $\mu molO_2g^{-1}h^{-1}$; and standard error are reported.

Group in air	I Interval	II Interval
U. urvillei **Males**	1.76 ± 0.4	1.4 ± **0.3**
U. urvillei **Females**	5.46 ± 1	4.32 ± **0.8**
U. annulipes **Males**	2.61 ± 0.6	1.64 ± **0.4**
U. annulipes **Females**	2.61 ± 0.4	1.38 ± **0.2**

We conducted the Pair Wise test for different levels of species, sexes and

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media. Considering the two species, only *U. uvillei* showed differences between sexes: in both air (t=5,35, P=0.0001) (Fig.1 A-C)and water (t=3.20, P=0.0013) (Fig 1 B-D), females showed a higher breathing rate with respect to the males.

In *U. annulipes*, instead, a difference was found between sexes only in water medium, in which females showed a higher breathing rate with respect to the males (t=3.34 , P=0.0021) (Fig 2 F-H).

Considering the two sexes, the Pair Wise test individuated differences between species. In males, the breathing rate in water of *U.urvillei* was higher with respect to *U. annulipes* (t=8.29 , P=0.0001) (Fig.1 C, Fig.2 F). No differences have been found in air medium (Fig. 1 A, Fig. 2 E).

Considering females, we found instead a higher breathing rate of *U. urvillei* respect *U. annulipes* in both air (t=4.32 , P=0.0002) (Fig. 1 C, Fig. 2 G) and water (t=5.46 , P=0.0001) (Fig. 1 D, Fig. 2 H).

Finally, considering the two media, the Pair Wise test showed a difference between sexes in *U. urvillei*. In fact males had a higher breathing rate in water respect to the air (t=3.04 , P=0.0027) (Fig. 1 A-B), while females showed an opposite result, with a higher breathing rate in air than in water (t=2.17 , P=0.0316) (Fig. 1 C-D). *U. annulipes* males, differently from *U.urvillei* males, showed a higher breathing rate in air than in water (t=2.92 , P=0.0051) (Fig. 2 E-F). No difference between media has been found for females of *U. annulipes* (Fig. 2 G-H).
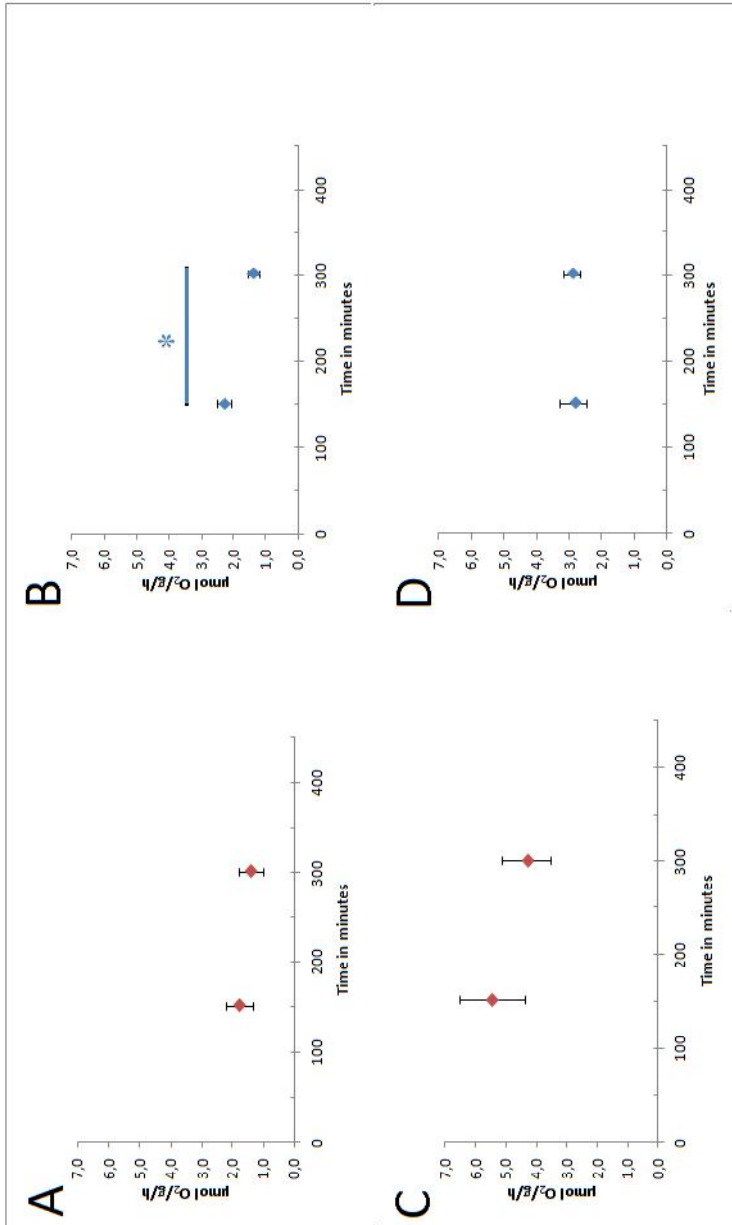
Figure 1 Level of Oxygen Consumption in *U. urvillei* **recorded at the two intervals of 2h30'
and 5h. A)** *U. urvillei* **males in Air, B)** *U. urvillei* **males in Water C)** *U.* **urvillei females in
Air, D)** *U. urvillei* **females in Water. Standard errors are reported. Probability level of
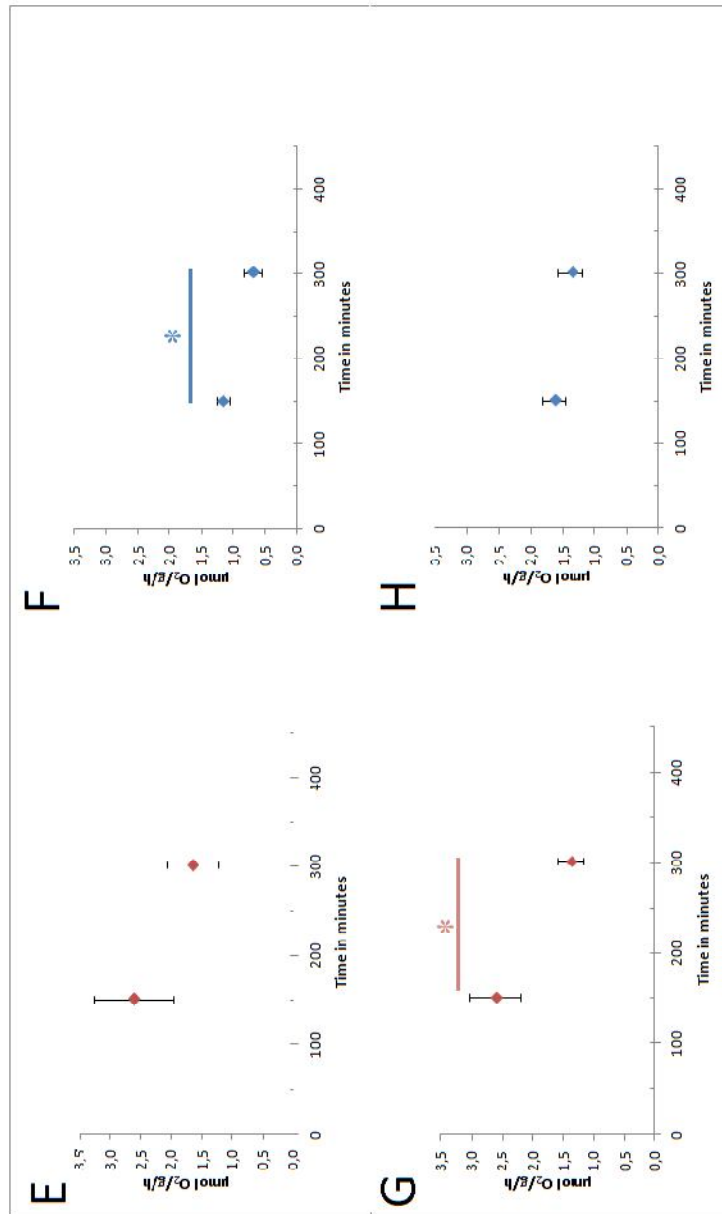Pseudo-F are indicated as *: p<0.05**

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Figure 2 Level of Oxygen Consumption in *U. annulipes* recorded at the two intervals of 2h30' and 5h. E) *U. annulipes* males in Air, F) *U. annulipes* males in Water G) *U. annulipes* females in Air, H) *U. annulipes* females in Water. Standard errors are reported. Probability level of Pseudo-F are indicated as *: p<0.05

4.4.2 Submersion tolerance and escape reaction

As showed in fig.3, *U. urvillei* males seem to be particularly restless during the five hours period. In fact, the average number of restless animals is 16.2 ± 0.8. that is the more than half of total tested crabs.
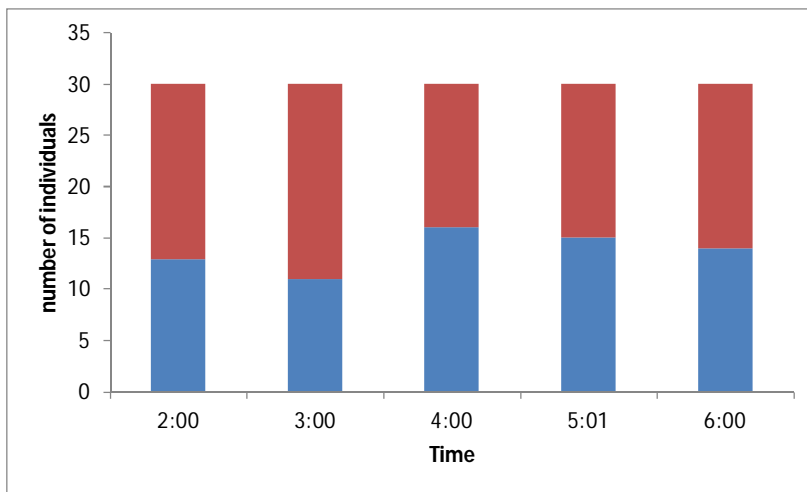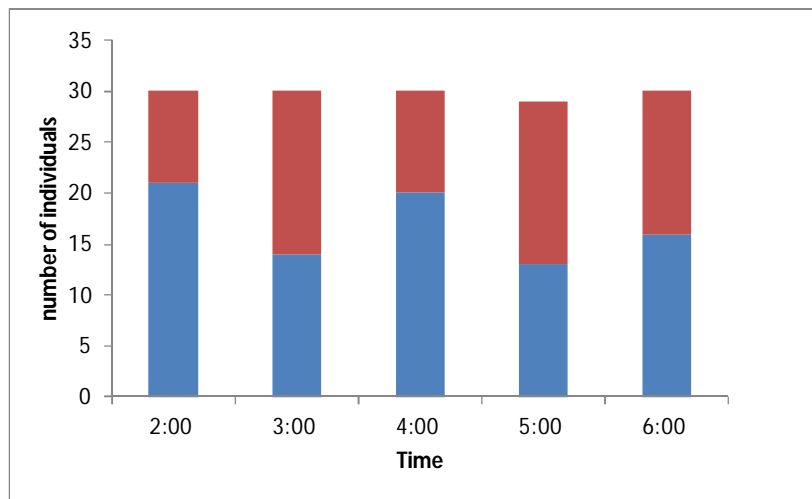

Figure 3 Ratio between restless (in red) and quiet (in blue) *U. urvillei* males recorded during five hours of partial submersion

A different result was showed by *U. urvillei* females. The number of quiet submerged crabs is significantly higher than restless crabs (Fig. 4). In this case, the average number of restless animals is only 5.2 ± 0.2.
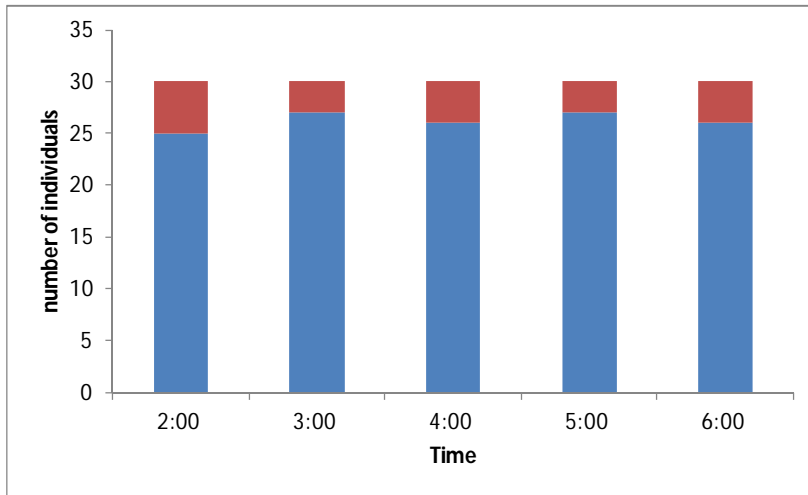
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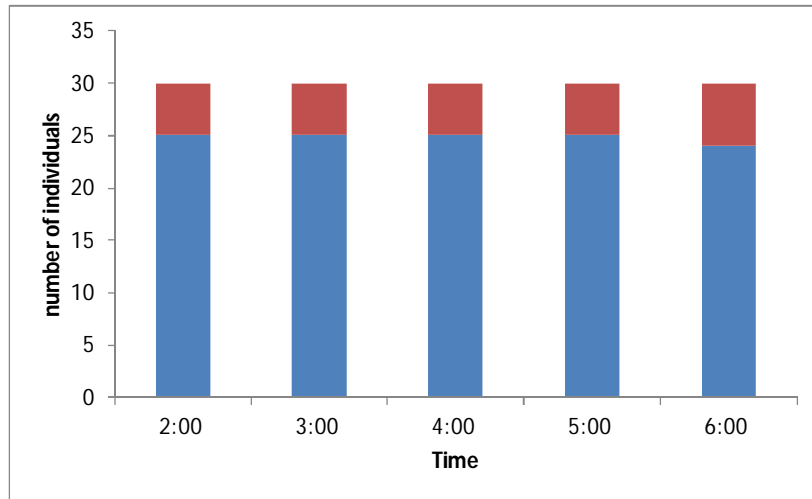
Figure 4 Ratio between restless (in red) and quiet (in blue) *U. urvillei* females recorded during five hours of partial submersion

U. annulipes shows a pattern similar to *U. urvillei*. Also in this case males (Fig. 5) showed a remarkable rate of restlessness, with an average number of restless individuals of 13±1.4, while almost all the females (Fig. 6) stayed quiet during all the observation time, with an average number of restless individuals equal to 3± 0.3.

Figure 5 Ratio between restless (in red) and quiet (in blue) *U. annulipes* males recorded during five hours of partial submersion

Figure 6 Ratio between restless (in red) and quiet (in blue) *U. annulipes* females recorded during five hours of partial submersion

As showed in table 4, only a small number of crabs were unable to capsize itself, while for all groups the average time of capsizing was very rapid.

Table 4 Capsizing performances of *U. urvillei* and *U. annulipes* in different condition of submersion; the mean time of capsizing ±SE and the number of individuals unable to do it are reported.

Group	Submersion	Unable to capsize (n/tot)	Time of capsizing (s)
U. urvillei **Males**	Partial	5/30	3±0.3
U. urvillei **Females**	Partial	5/30	2.2±0.3
U. urvillei **Males**	Total	6/30	2.4±0.4
U. urvillei **Females**	Total	0/30	1.7±0.1
U. annulipes **Males**	Partial	1/30	2±0.1
U. annulipes **Females**	Partial	1/30	1.7±0.1

As showed in fig. 7 and 8, most part of *U. urvillei* males was motionless or only able to escape slowly, both in partial and total submersion both. In fact, the two groups of males showed only the 30% and 15% of rapid response, respectively.
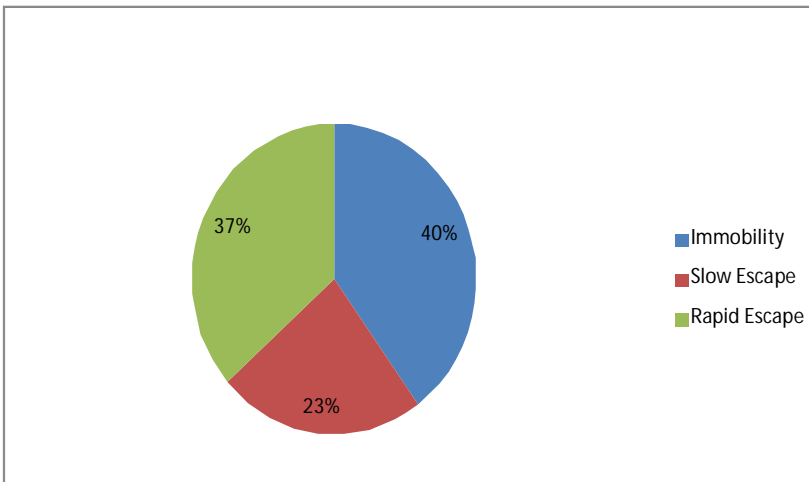
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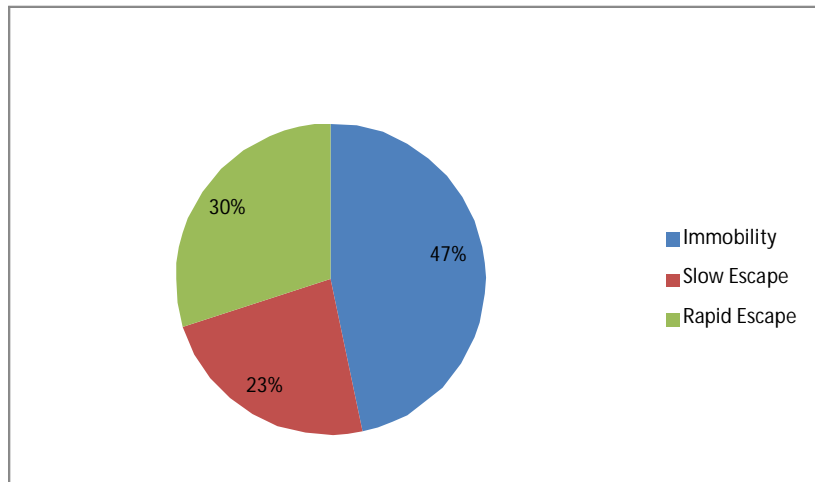
Figure 7 Disturbance response in *U. urvillei* males after partial submersion. The graph shows the percentages of individual that performed the three observed reactions
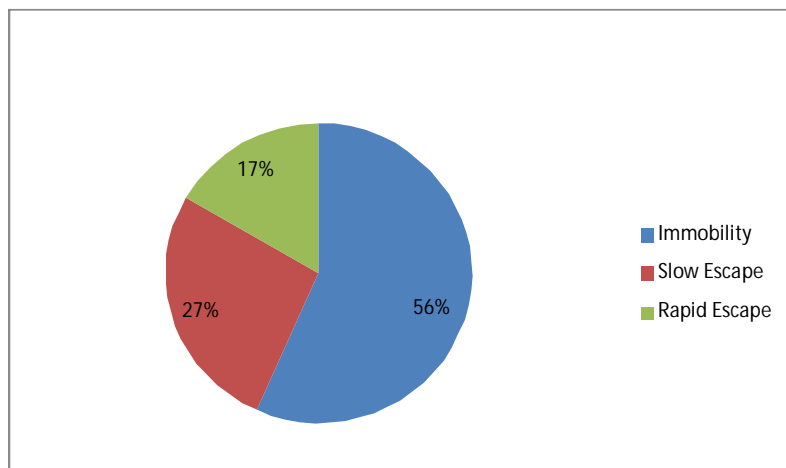
Figure 8 Disturbance response in *U. urvillei* females after total submersion. The graph shows the percentages of individual that performed the three observed reactions

As found for the males, also *U. urvillei* females showed a scarce tendence to rapidly escape. In fact, after partial (Fig.9) and total (Fig.10) submersion only the 37 % and 20% respectively of released individuals escaped in a rapid way.

Figure 9 Disturbance response in *U. urvillei* females after partial submersion. The graph shows the percentages of individual that performed the three observed reactions.
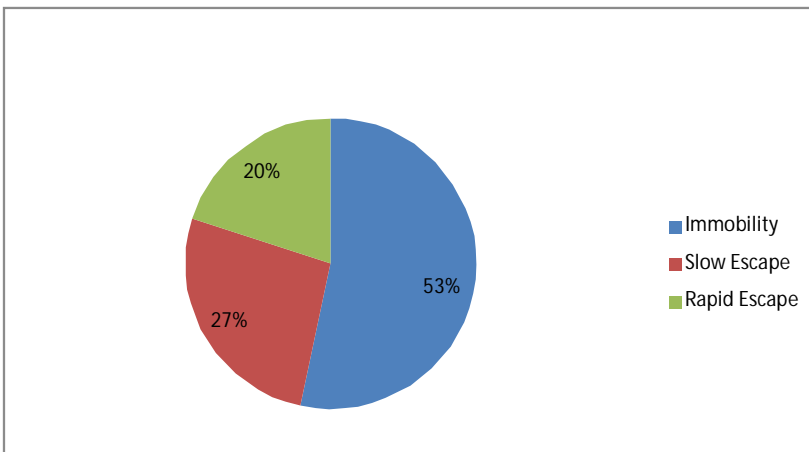

Figure 10 Disturbance response in *U. urvillei* females after total submersion. The graph shows the percentages of individual that performed the three observed reactions.

About the disturbance response, the Chi^2 Test found a significant difference (df= 2, χ^2= 9.09, p< 0.05) between *U. urvillei* individuals in total submersion and those in partial submersion, that showed a higher level of rapid escape. In both partial and total submersion conditions, no difference between sexes has been found by Chi^2 Test in the disturbance response of *U. urvillei*.

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We found a high level of inability to escape also in *U. annulipes* after partial submersion. Both sex showed a low level of rapid escape: males (Fig. 11) escaped rapidly only in 33% of trials, while females (Fig. 12) only in 37 % of trials. No difference in the disturbance response between sexes has been found by Chi^2 Test.

Figure 11 Disturbance response in *U. annulipes* males after partial submersion. The graph shows the percentages of individual that performed the three observed reactions.

Figure 12 Disturbance response in *U. annulipes* females after partial submersion. The graph shows the percentages of individual that performed the three observed reactions.
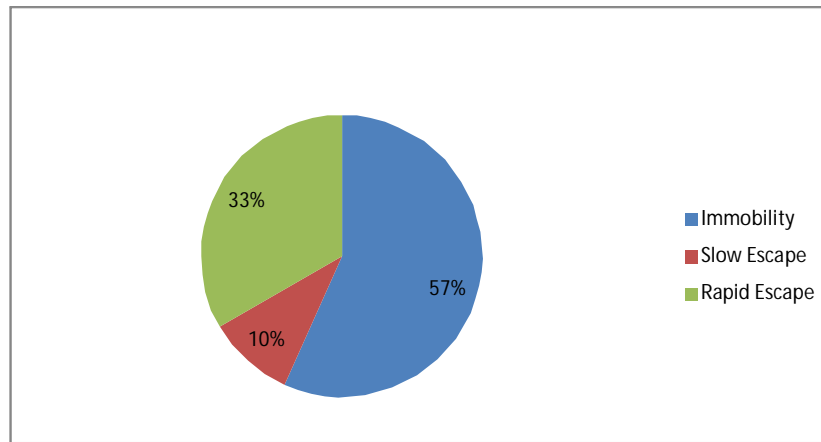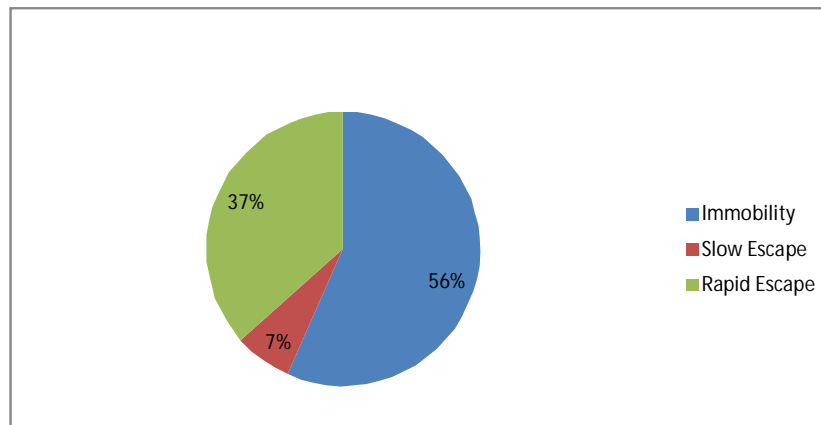
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4.4.3 Habitat preference test

As showed in Figure 14, in males of *U. urvillei*, the percentage of crabs out of the water showed an increasing trend with the time (Fig. 13). At the beginning, only the 30% of males prefered to stay out of the water. The percentage increased until 50% at the end of the trial.
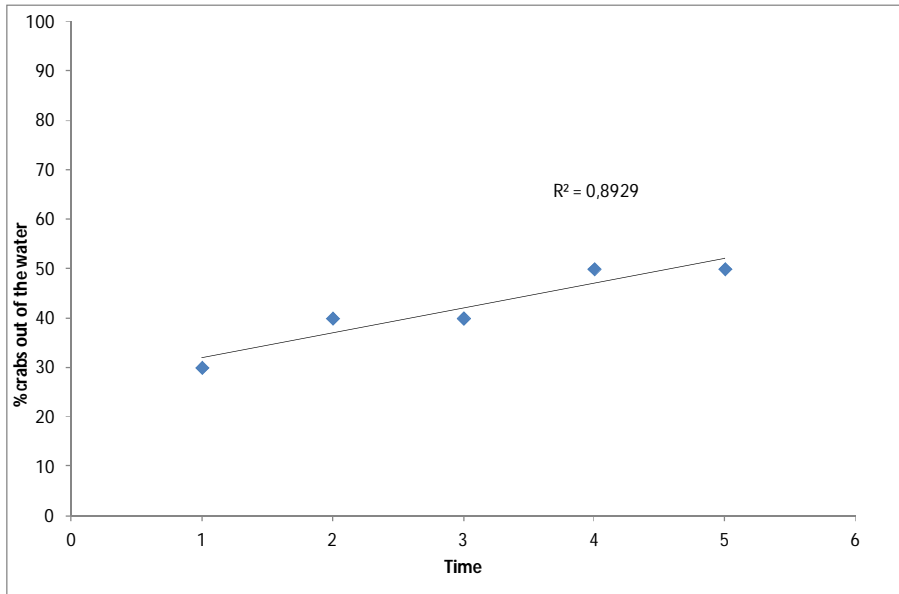
Figure 13 Percentage of *U. urvillei* males observed out of the water during five hours

The females of *U. urvillei*, instead, showed a weaker correlation between time and percentage of crabs out of the water (Fig 14).

52
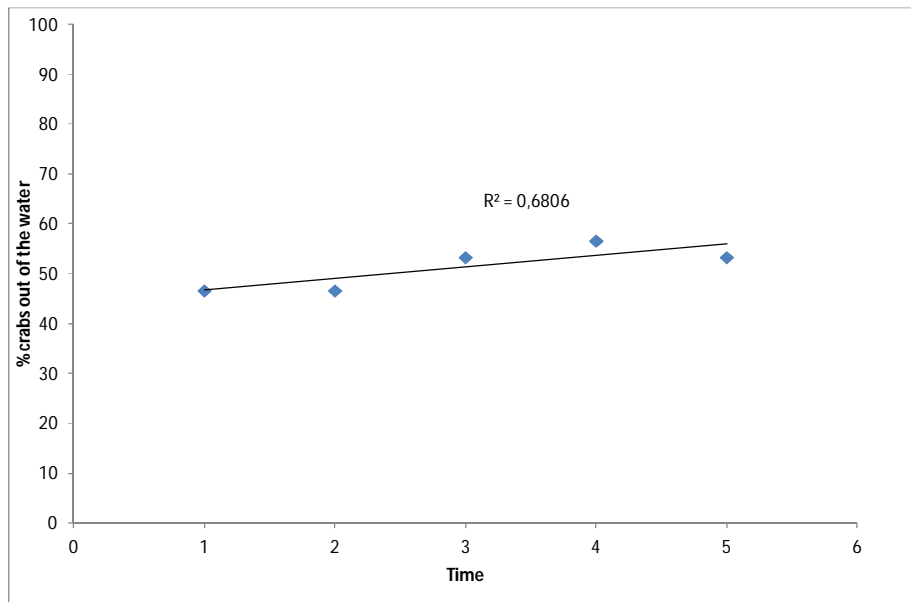
Figure 44 Stress response in *U. urvillei* females after partial submersion time

In this case, however, we observed a higher percentage of females out of the water already at the beginning of the trial (50%). *U. annulipes* specimens showed a strong preference for staying out of the water, the 50% of males showed a preference for land since the begininng of the trial, and this percentage increased consistenly during the trial until around 80% of crabs out of the water at the end (Fig. 15). Females appeared instead to have a weaker initial preference for emersion. In fact only the 35 % of animals chose the stay out of the water. Moreover the percentage never increases over 40% (Fig.16).

Figure 15 Stress response in _U. annulipes_ males after partial submersion time

Figure 16 Stress response in _U. annulipes_ females after partial submersion time

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We also analyzed the number of observations in subaerial condition for each crab by mean of a, full factorial 2-way PERMANOVA test that showed a significant differences for the interaction between species and sex factors (Tab. 5).
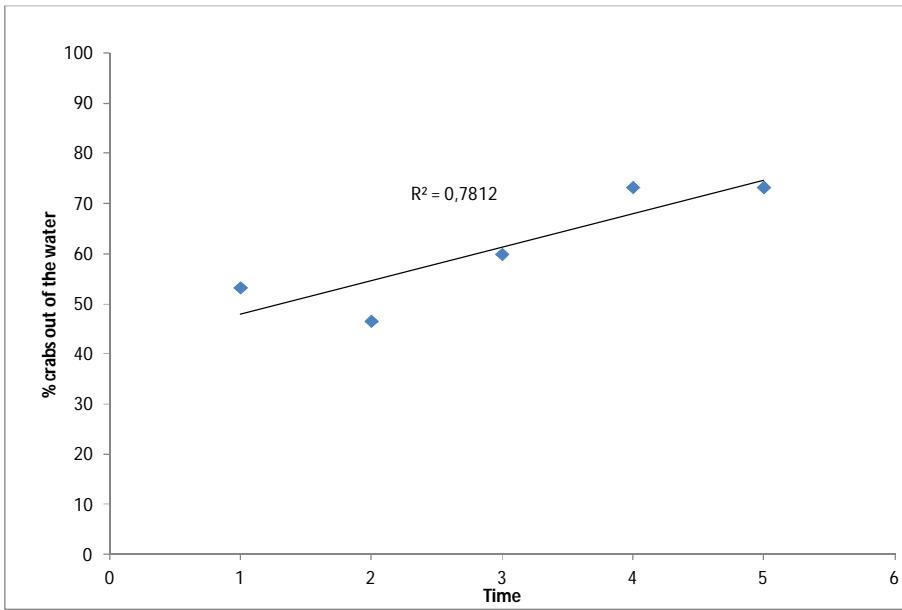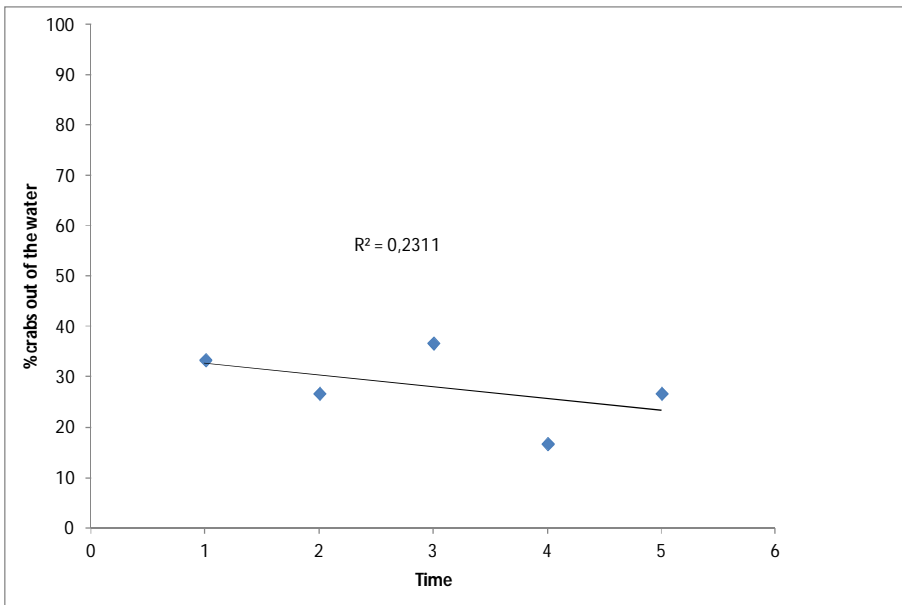
Table 5 Results of the full factorial 2-way PERMANOVA test on the time spent out of water. Probability level of Pseudo-F are indicated as **: p<0.05

Source	Df	MS	Pseudo-F	
Species	1	7.2536	9.228e2	
Sex	1	6.1318	2.956	
Spe×Sex	1	2.3046	9.508	**
Residuals	116	0.3233		
Total	119			

We conducted the Pair Wise test for the interaction between species and sex factors. Considering the two species, only *U. annulipes* showed differences between sexes (t=3.66 , P=0.0001): males resulted more frequently observed out of the water with respect to females. *U. urvillei* didn't show any difference between sex.

4.5 Discussion

Oxygen consumption

Generally, our result confirmerd the idea of fiddler crabs as good air-breather, evidenced in previous study on *U. urvillei* species (Fusi et al., 2014; Giomi et al., 2014).The comparison between species showed a clear difference in the oxygen consumption rate. *U. urvillei* species showed higher metabolic rate with respect to *U. annulipes*. The reason of that could be found in the body size and in the body mass percentage due to chitin and calcium-carbonate: in smaller animals (*U. annulipes*) the exoskeleton could represent a high percentage of the total volume, as well as of the total weight of the individual

Also the comparison between sexes showed a clear difference in the oxygen consumption rate. Generally females of both species showed a higher metabolic rate respect to males. That could be explained considering the physiological status of females. Oogenis (Naylor et al., 1997; Fernandez et al., 2000; Brante et al., 2003), in fact, require a strong physiological effort, and that could upholding the difference of metabolic rate between males and females

(Weissburg, 1993; Negro et al, 2013). Unlike previous comparisons between species and sexes, that between medium didn't show a homogeneous framework.

For *U. urvillei* the pair wise test showed an opposite result in males and females. Males showed a higher metabolic rate in water with respect to air, accordingly to our prevision about a semiterrestrial species. Instead, females of *U. urvillei* showed a higher metabolic rate in air respect to the water.

Mangale Vilas and Kulkarni Balasaheb (2014) found in *U. annulipes* species that oxygen consumption increased and salinity tolerance levels decreased in dilute seawater, but they investigated neither the air-breathing nor the role of sex in oxygen consumption. In our study, for *U. annulipes,* the pair wise test showed a significant difference between medium only in males. In fact males showed a higher metabolic rate in air respect to the water. That is reasonable if we consider that fiddler crabs accomplish their activities mainly in subaeral environment, that is during low tide phase (Barnwell, 1966, 1968; Burggren & McMahon, 1988; Booksmythe et al., 2010) because being active determines a higher metabolic rate (Wallace, 1972). No difference between medium has been found for females of *U. annulipes*. In general, considering the values of oxygen consumption, *U. annulipes* does not show clear difference in aquatic and subaeral condition. In both condition, the species is able to satisfy their metabolic necessity. In addition, about subaerial conditions, a possible explanation could be found in the natural history of the two species. In fact, while *U. annulipes* is a species inhabiting the higher zone of intertidal environment, *U. urvillei* inhabits the seaward zone (Vannini & Valmori, 1981). That means that *U. annulipes* deals with longer low tide phases with respect to *U. urvillei*. That implies for *U. annulipes* a longer time of activity in subaerial condition. So being a good air-breather (lower oxygen consumption rate) could represent a key adaptation to upper intertidal zone. Nevertheless, this hypothesis is supported only by results about females.

So the question is why males of *U. annulipes* species in subaerial condition present higher oxygen consumption with respect to males of *U. urvillei* in same condition? Perhaps that is due to the difference in density of assemblages. In fact *U. annulipes* species is characterized by high density in which males strongly compete for space and females (deRivera & Vehrencamp, 2001) while *U. urvillei* assemblages show low density and low level of completion for space (Chapter 5, present thesis). So a higher metabolism could represent the response to the effort caused by social competition.

Water tolerance and habitat preference

Behavioural observation during partial submersion showed a high level of restlessness (almost 50%) in males of both *U. urvillei* and *U. annulipes*: many crabs tried to partially emerge out of the water, to float on the water

surface or to climb the bucket walls. Females of both , instead, showed to be quiet in the water medium. The forced or partial submersion seems affecting the reaction of escape in both species and in both sexes. In fact, after partial or total submersion, most of the crabs showed immobility or slow reaction of escape. So the forced immersion in water medium for a prolonged period seems to negatively influence their reaction of escape. The capsizing time does not seem to be influenced by the medium, in fact almost all crabs have been able to capsize   in   few   seconds. About the habitat preference we observed a clear difference between males and females in both species. Considering fiddler crabs as semiterrestrial animals, the behaviour of males satisfied our prevision to find an increasing preference for the emerged substrates during experimental time. At the beginning of trials, *U. annulipes* showed a higher number of individuals preferring the emerged substrate in respect to *U. urvillei*. Most part of males in fact used the way of escape that was available. On the other hand, females did not totally satisfy our prevision. In fact not over 50% of them show a preference for an emerged condition and looked to be at ease in the water. Moreover the number of females preferring emerged substrates did not increase during the trials time. That could be explained considering their different physiological status. Fiddler crabs females in oogenesis could be less reactive than males, and because of that they would spend more time in water than males. In fact, it is well known that ovigerous fiddler crabs (*Uca* spp.) spend most of their time in burrows; where actity is limited; than on the substrate (Edney, 1961). Females fiddler crabs rarely leave their burrows, where, for instance, they can control and limit embryo desiccation (Crane, 1975; Wolcott 1988).

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Acknowledgements

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Chapter 5: Fiddler crabs as Dear Enemy: spatial analysis of neighbouring behaviour in the species *Uca urvillei* (H. Milne-Edwards, 1852)

In preparation
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Abstract

The Ocypodidae family has been considered as paradigmatic taxon for many behavioural studies. In this study, we investigated the spatial behaviour of couples of males considerable as neighbour. We focused our study on the species *Uca urvillei* (H. Milne-Edwards, 1852).

We conducted a series of behavioural observations in nature, at the Umgazana mangrove forest, South Africa, measuring time and position around burrows. Results showed a strong trend to occupy own territory around the burrow, limiting strongly the exploration of new area and the sharing of a same sector. High level of tolerance between neighbours has been found as well as defensive behaviour with respect to intruders.

We found a significant relationship between the individual time passed by each crab in the boundary zone between burrows and the time of copresence of both neighbours in the same area.

Generally, results show a low occurrence of defensive behaviour by residents towards neighbours. About 25% of time was spent into the burrow and about 70% of time in the area around their own burrow. Residents showed also a low level of copresencecopresence in restricted sectors. We observed that couples of residents showing neighbour defence behaviour spent much more time in the limit zone respect to couples without neighbour defence behaviour .

5.1 Introduction

In study on social behaviour, the 'dear enemy' effect describes the phenomenon whereby residents are less aggressive towards neighbouring territory owners than towards strangers (non neighbours) (Fisher, 1954; Booksmythe et al., 2010).

Territoriality represent the shared behavioural frame in which the dear enemy effect occurs in very different taxa as mammals (e.g. delBarco-Trillo et al. 2009), birds (e.g. Briefer et al. 2008), reptiles and amphibians (e.g. Jaeger 1981; Husak & Fox 2003), fish (e.g. Frostman & Sherman 2004) and invertebrates (e.g. Langen et al. 2000; Pratt & McLain 2006). The dear enemy

phenomenon can be explained by several hypotheses grouped into (1) those based on the different level of familiarity that a resident has with neighbours and strangers; and (2) those based on the difference in level of threat represented by intruders of each type (Temeles 1994, Booksmythe et al., 2010). Relative threat hypotheses require a mechanism by which residents can differentiate between neighbours and strangers and their level of threat. In many cases, familiarity with an individual is likely to be a contributing indicator of the level of threat to the resident (i.e. neighbours are familiar and pose a small threat). Fiddler crabs (*Uca* spp.) are ubiquitous in mangrove forests and their activity is concentrated during low tide. As is known, they also present peculiar adaptation to the terrestrial life as highly vascularised branchial chamber and air-storing behaviour during high tide (Chapter 3, present thesis) . The 'dear enemy' effect and the ability to recognize neighbours has been described for several species of fiddler crabs as *U. capricornis* (Detto et al., 2006), *U. mjobergi* (Booksmythe et al., 2010) and *U. pugilator* (Pratt & McLain, 2006). For example, in *U. capricornis* the distinct carapace colour patterns enable males to discriminate between their female neighbours and unfamiliar females while *U. mjobergi* residents use behavioural of another crab to determine the level of threat posed and distinguish between neighbours and strangers. Moreover *U. pugilator* residents can differentiate between resident and floating intruders without the need for individual recognition.

In this study, we wanted investigated the dear enemy effect analyzing relationship occurring between couples of resident males of species *U. urvillei,* sharing a same area. We tested if this relationship between residents can be influenced by temporal-spatial strategies.

5.2 Study species and study area

We studied a species of fiddler crabs, belonging to the Ocypodidae family: *Uca urvillei* (H. Milne-Edwards, 1852) (Chapter 1 present thesis). Our study was conducted from February to March 2013. The area of experimental animals sampling was the mangrove forest in the Umngazana Estuary, South Africa (Chapter 1, present thesis).

5.3 Materials and Methods

We observed the behaviour of a total of 26 couples of fiddler crab males, who were neighbour, defined as owners of burrows distanced from 10 to 50 cm between each other and with no other burrow comprised between the observed ones. Behaviour has been recorded by one observer, for about 20 minutes, by mean of a Panasonic HX-WA10 Camcorder, positioned at 50 cm above ground and at about 3 meters of distance by focused burrows.

We divided the space around the two burrows in four ideal sectors (S1, S2, S3, S4,), considering three imaginary boundary limits (Fig. 1).
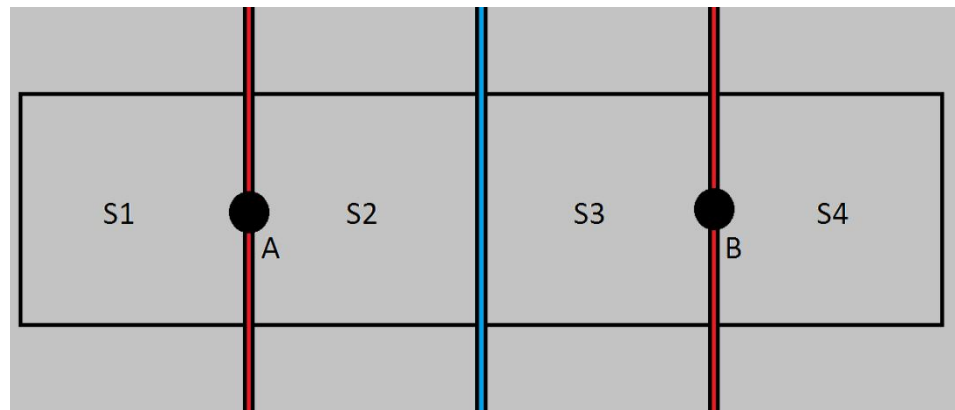
Figure 5 Imaginary division of space around neighbour's burrows (A and B) in four sectors (S1, S2, S3, S4) considering three imaginary boundary limits, two passing from the burrows openings (in red) and a median one (in blu) passing between the two burrows

We recorded the occurrence of "neighbour defence behaviour" (**ND**) showed by a resident in response to neighbour's presence. ND is defined as the occurrence during observations of one or more following behaviours: escaping into the burrow, waving or fighting (Crane, 1975). In addition, we recorded also if escaping into the burrow, waving and fighting were showed by residents in response to intruder's presence. We also assigned each couples a class of distance (DIS): Near (< 20 cm), Middle (20-30), Far (> 30 cm).

We used the VESPOSI software to record following parametes: as a first step, we calculated the time in which both residents were in activity (independently by copresence in various sectors) during our observations, to be sure the focal crabs were able to detect each other in the space.

As a second step we calculated other three different parameters: time passed into the burrow, time passed in the area around the burrow (S1+S2 and S3+S4) and time of copresence in a same sector (S1-S4). These three parameters are expressed as percentage of total observation time. Finally, we calculated the ratio between time of copresence in the limit zone (LZ) defined as the space included between residents' burrows (S2+S3) and total time of copresence (TS) (total time of copresence in each sector).

5.4 Statistical analysis

Using PRIMER 6.1 and the PERMANOVA+ for PRIMER routines (Anderson et al. 2008) and considering as factor a) distance between burrows (near if <20cm, middle if 20-30 cm, far if > 30cm) and b) occurrence of ND between residents (yes/no); we performed three full factorial 2-way PERMANOVA tests to detect a possible effect of our two factors on: 1) total time of copresence (sum of copresence times in any sector), 2) shared time only in the limit zone (S2+S3), 3) the ratio between time of copresence in the limit zone (LZ) (S2+S3) and total time of copresence (TS) (S1+S2+S3+S4).

5.5 Results

In only 9 of 26 couples of residents we observed ND. Instead in 19 couples of resident , we recorded at least once escaping into the burrow, waving or fighting showed by one of resident in response to intruder's presence (non residents) .The average time of in which both residents were in activity (independently by sector) resulted to be of 56%.

As showed in figure 2, during our observations, resident crabs spent 25% of time into the burrow and almost the 70% of time in the area around their own burrow. Only 6% of time was spent by residents in the neighbour's area.
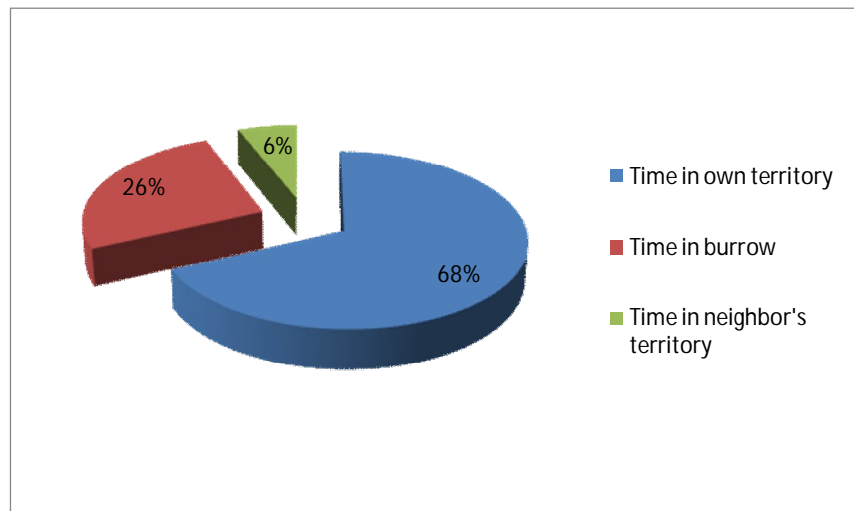
Figure 2 Average percentage of time spent in burrow, in own and in neighbour's territory

Considering all the total observation time, we recorded that only in the 5,6% of time occurred the copresence of the two neighbours in a same sector. If we consider only the time in which both residents were in activity, the time of copresence in a same sector increases to 8%.

The first full factorial 2-way PERMANOVA test did not show any significant result. The total time of spatial sharing (sum of copresence time in any sector))is not influenced by distance between burrows and occurrence of neighbour defence behaviour. Instead the second full factorial 2-way PERMANOVA test showed that shared time in limit zone (S2+S3) is significantly influenced by the factor ND but not by factor DIS.

Table 1 The full factorial 2-way PERMANOVA test showing significant difference for the factor ND but not for factor DIS . Degree of freedom (df), mean square values (MS) and Pseudo-F values are reported. Probability level of Pseudo-F are indicated as *: p<0.05.

Source	df	MS	Pseudo-F	
DIS	2	5.5842	5.6799	
ND	1	0.8222	0.836	*
DISxND	2	2.125	2.161	
Res	20	0.98316		
Total	25			

Couples of residents showing ND spent much more time in the limit zone respect to couples without ND .Moreover the third full factorial 2-way PERMANOVA test showed significant differences among considered groups. About the ratio LZ/TS, the full factorial 2-way PERMANOVA test showed a significant differences only for the factor ND and the interaction between DIS and ND (Tab. 2).

Table 2 The full factorial 2-way PERMANOVA test showing significant difference for the facto ND and for the interaction between DIS and ND factors. Degree of freedom (df), mean square values (MS) and Pseudo-F values are reported. Probability level of Pseudo-F are indicated as **: p<0.01 and as *: p<0.05.

Source	df	MS	Pseudo-F	
DIS	2	0.3072	2.7511	
ND	1	1.3819	12.376	**
DISxND	2	0.55124	4.9365	*
Res	20	0.11166		
Total	25			

We conducted the Pair Wise test for the factor ND and for its interaction with factor DIS.

Considering the factor ND, we found that couples showing this behaviour spend more time in the limit zone respect those do not perform it (t=3.51, p<0.01).

Considering the level of factor DIS of the interaction between factors ND and DIS , we found significant difference only at the level Middle Distance (20-30 cm), for the LZ /TS ratio between couples showing ND and those not showing it (t=5.22 , P<0.01). At the middle distance, in fact, it has been found a higher LZ/TS ratio in couples showing ND with respect to couples that did not perform it. No significant differences have been found at near and far distance levels.

Considering the level ND of the interaction between factors ND and DIS, we found significant difference for the considered ratio between crabs living at near distance (10 - 20 cm) and at middle distance (20 - 30 cm) (t=10.29 , P<0.01) and also between crabs living at near and far distance (40-50 cm) (t=6.67 , P<0.01) only when ND occurred. In this behavioural frame, crabs living at near distance showed a lower LZ/TS respect those living at middle and far distance. No difference has been found at any distance when ND doesn't occur.

We investigated also the relationship between the Individual Time passed in the Limit Zone (Sectors 2 and 3) singularly by each crab (ITLZ) and the Shared Time in Limit Zone (STLZ) (resulted summating time of

copresence in sectors 2 and 3). We applied the linear regression model to test the considered relationship.

The analysis of variance resulted to be statistically significant (dfF=1, F=19.5 **). So consequently we could test the significance of the relationship between ITLZ and STLZ.

The analysis of regression (Table 3) gave a significant result showing a slight trend due to the relationship between ITLZ and STLZ (R^2=0.28).

Table 3 Linear Regression Model for the relationship between ITLZ and STLZ

	Coefficient	*SE*	*Stat t*	*p*
Intercept	-0.08442	0.208134	-0.4056	0.686765715
ITLZ	0.116263	0.026311	4.418777	5.33804E-05

In detail, as showed in figure 3, the increasing of shared time in limit zone seems to be caused by an increasing of individual time spent in limit zone. That is, the more time each resident spend in the limit zone (S2+S3), for a longer time he will share a sector with the neighbour.
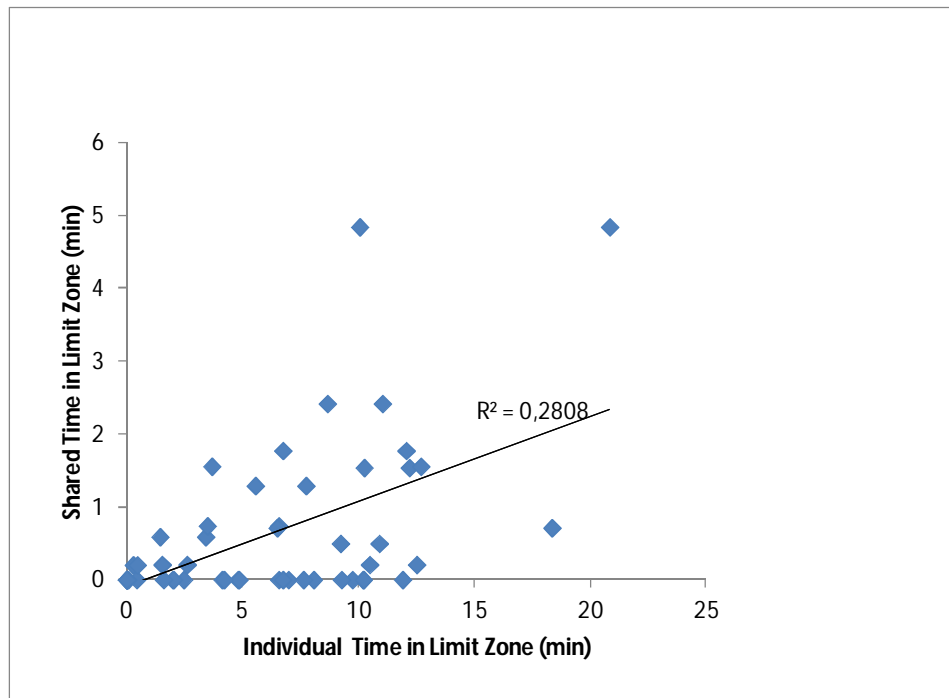
Figure 3 Linear Regression Model for the relationship between Individual Time in Limit Zone (ITLZ) and Shared Time in Limit Zone (STLZ) in neighbour couples of *U. urvillei*

If we apply the linear regression model considering separately the three range of distance (near, middle and far) we obtain a significant relationship between parameters for all three distance: short (R^2=0.62, df=1, F=21.05, p<0.01), middle (R^2=0.23, df=1, F=6.38, p<0.05) and far (R^2=0.41, df=1, F=7.83, p<0.05) . So generally, the presence of a resident in the limit zone is attractive for the other resident.

We applied the linear regression model also to test the relationship between time passed in the burrow by a resident and time passed in resident's area by the neighbour but no significant results have been found.

5.6 Discussion

On average, any observed crab was active simultaneously with other resident for more than 50 % of time. Considering this information with the high visual acuity characterizing fiddler crabs, we were sure that any resident was able to percept own neighbour (Zeil & Hofmann, 2001, Detto et al., 2006, Zeil & Hemmi, 2005, Detto, 2007).

We found in couples of *U. urvillei* males, a low level of defensive behaviour addressed to a neighbour by residents and a high level of defensive behaviour addressed by resident to intruders of both same and other species.

Our observations showed as resident crabs spent a quarter of time into the burrow. This could be explained by two elements: 1) the fiddler crabs' need to humidify their gill chamber, exploiting the water at the bottom of their burrow, 2) the need of maintenance of the burrow structure, digging and removing mud from the internal.

During their external activity , resident crabs spent most part of the time around their own burrow, strongly limiting the exploration of neighbour's area. In fact only the 6% of time has been spent around the neighbour's burrow. This result could be explained by: 1) the absence of need to research resources in the neighbour's area (i.e. microalgae), 2) the need to control the burrow to avoid to be evicted by another crab. Point 2 is supported by a study on *U. vomeris* showing as many of them responded to persistent dummy's presence by disappearing below ground (Hemni & Zeil, 2003a).

In addition, our results showed that residents crabs preferred not to occupy a sector already occupied by the neighbour. In fact resident crabs share a sector with the neighbour only for 5,6% of time. Merging the low level of defensive behaviour addressed by residents to a neighbour and the short time of spatial sharing, we suggest the *U. urvillei* males adopt a spatial strategy to properly avoid a direct encounter with a neighbour, also considering that previous study in *U. vomeris* showed that knowledge of their own distance from the burrow has an influence on their decision to respond (Hemni & Zeil, 2003b). If familiarity was the only factor regulating a relationship between neighbours, we would expect a major spatial sharing. Probably instead *U. urvillei* are able to value the relativity of the threat represented by the neighbour. A study about *U. mjobergi*

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species (Booksmythe et al., 2010) found that familiarity does not affect the intensity of fights under new circumstances, suggesting that residents use cues in the behaviour of an intruder to determine the level of threat posed and distinguish between neighbours and strangers. That is *U.urvillei* could recognize a neigbour not by physic caracteristics but by timing and presence in different area around its burrow.

Generally we observed that couples of residents showing neighbour defence behaviour spent much more time in the limit zone respect to couples without neighbour defence behaviour .

We found also that, when Neighbour Defence behaviour occurs, resident crabs living at short distance share mostly the space of the limit zone respect those living at middle and far distance. These results suggest that an obligated spatial sharing (short distance between burrows) causes the occurrence of Neighbour Defence behaviour, independently by the familiarity. In this case, limit zone is a resource mostly sought by the two residents considering the probable overlapping of the two home range. That could be an evidence of "dear enemy effect" occurrence in *U. urvillei* considering that also in *U. pugilator*, it has been found that proximity and orientation determine the ease with which a neighbour may be engaged (Pratt & McLain, 2006).
The above strategy, has effect also at a middle distance between burrows (20-30 cm). In fact, couples sharing the limit zone for more time are those showing more probably Neighbour Defence behaviour. Moving around the limit zone could be percept as a threat for own burrow. So in *U. urvillei* as *U. vomeris* (Hemni & Zeil, 2003a) resident crabs could evaluate the other crab's movements relative to their burrow and not relative to themselves.
Finally we found a cue suggesting that a resident's spatial strategy is not independent by the one of the neighbour. In fact, independently by the distance between burrows, the limit zone sharing time seems to be partially influenced by the presence of a resident. That is a resident could be attracted in the limit zone by the presence of the neighbour. In this way each resident could control own border and acquire information on the level of threat represented by the neighbour. Considering separately couples by distance (near, middle, far), we observed a similar relationship between the Individual Time passed in the Limit Zone by each crab (ITLZ) and the Shared Time in Limit Zone by both residents. So at any burrows distance, the presence of a resident in the limit zone is attractive for the own neighbour.

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Conclusions

Overall, the results of my research support the definition of fiddler crabs as true terrestrial organisms. The ability to trap large air volume in their burrows, during the high tide, suggests a preference for a subaerial environment. Also behavioural observations in laboratory support this hypothesis, considering that high level of restlessness in water and preference for subaerial environment have been recorded. Moreover my results contribute to define how important is the ecological role played by fiddler crabs with respect to oxygen requirement for mangrove ecosystems. I also showed how one of the focus species of fiddler crabs studied in this thesis is using behavioural characteristics, such as time budget and spatial strategy, to tell the difference between a known neighbor and an intruder, thus to apply a "dear enemy" strategy to avoid conflicts.

About burrowing behavior in fiddler crabs (Chapter 2-3), my results show as the three species of fiddler crabs investigated do not store different air volumes in their burrow, independently of substrate (sand vs mud) and body size. All species, in fact, store an average volume of about 70 ml of air during high tide. Nevertheless, my analyses revealed significant differences among sexes, depending upon the species. Among three studied species, only *U. annulipes* showed differences between sexes, with the males trap a volume almost double with respect to the females. In *U. urvillei* and *U. chlorophthalmus*, males and females store the same amount of air. Furthermore, the volume of air trapped by *U. annulipes* males is greater than the volume trapped by other two species. An opposite trend is recorded among females of the three species. In fact, both *U. urvillei* and *U. chlorophthalmus* females show a higher volume of trapped air with respect to *U. annulipes* females.

Previous comparative study on mate searching in fiddler crabs highlighted that female searching is associated with high burrow density, small body size, large soil size, display complexity and short eyestalk (de Rivera & Vehrencamp, 2001).. All these factors characterized the species *U. annulipes*. In detail, high density and large soil size lead females to mate and incubate in male's burrow. After oviposition, the male leaves the burrow which is then used by the mated female (Christy 1987; Christy & Salmon 1991). This frame could explain the difference between sexes recorded in *U. annulipes*. In fact, for hosting a female, a male should dig a greater burrow in order to trap a volume of air adequate for females with eggs and to provide a better thermal environment for incubation, as found also in U. beebei (Christy, 1987) and in *U. mjoebergi* (Reaney & Backwell, 2007). , Backwell and Passmore (1996) suggested that in *U. annulipes* females, mate choice is a two-stage process. First, females select males based on male size. They then decide whether or not to mate with a male based on burrow features. So burrow volume could represent one of features valued by females.

The previous frame is not the case of *U. chlorophthalmus* and *U. urvillei*. In fact, bigger species with lower density and living on finer-grained soil as mud are characterized by male searching (Ribeiro et al., 2010). In this case, females do not mate and incubate inmale burrow and that could explain why there is no difference between sexes in trapped air volume of both *U. urvillei* and *U. chlorophthalmus.*

I also showed as the air trapped in the burrows presents high levels of oxygen concentration during high tide (Chapter 2). The average level of oxygen saturation is in fact over 80% (~ 0,015 mmol ml^{-1}) for both *U. urvillei* and *U. annulipes* at the beginning of the high tide. At the end of the high tide, the oxygen saturation decreases at almost 70 % for *U. urvillei* and at 79 % for *U. annulipes*. The oxygen concentration in male burrows of *U. annulipes* resulted to be higher than in the burrows of females of the same species. I did not individuate significant differences between females of the two species, nor between sexes in *U. urvillei.*Regardless of species and sex, the volume of air trapped into the burrows represents a remarkable source of atmospheric oxygen for fiddler crabs, a stock able to avoid hypoxic conditions during the high tide (Chapter 3). Volumes of trapped air are several orders of magnitude greater than air concentrations in agricultural soil (Cook et al., 2013), providing an enormous, and yet undescribed,, source of underground oxygen to mangrove roots. Corrosion casting techniques and Micro Computed Tomography confirmed the presence of a well-developed lung in the gill chamber of *U.* spp., confirming the need for air-storing behaviour. In South African and Kenyan forests It has been observed that fiddler crabs seal their burrows as the tide rises, trapping an average of 2213±174 ml m^{-2} and 6110±1308 ml m^{-2}, respectively, of well oxygenated air during high tide. Due to the high densities of crabs in these systems, the total amount of oxygenated air available to mangrove roots can reach up to 9778±1874 ml m^{-2}, more than enough to fulfil mangrove requirements. The present data show for the first time that the adaptations of fiddler crabs for air-breathing result in the presence of a large reservoir of oxygenated air within sediments, previously thought to be highly anoxic. This new finding drastically changes our understanding of oxygen availability and fluxes in mangrove sediments, their evolutionary success and, ultimately, their productivity and carbon sink functions.

My results support the novel hypothesis that mangrove root system can rely on a continuous supply of oxygen through the air stored in crab burrows. My estimates of the volumes of highly oxygenated crab-trapped air in every vegetation belt studied in a variety of East African mangrove forests indicate far higher amounts of readily diffused oxygen in the soil than expected. These estimates are sufficient to address the oxygen requirements of mangroves throughout the tidal cycle, offering an explanation for their huge productivity and their important role in global carbon cycling (Lovelock et al., 2006; Donato et al., 2011; Alongi, 2014; Andreetta et al., 2014). Indeed, based on mangrove

root respiration rates ranging from 0.000625 to 0.0075 mmol O_2 kg^{-1} s^{-1} (Cannicci et al., 2008) and a fine root biomass accumulation of 8 kg ha^{-1} yr^{-1} measured in a mangrove forest in Gazi Bay, Kenya, (Githaiga, 2013), then mangrove root oxygen requirements would oscillate between 0.0018 to 0.0216 mmol O_2 m^{-2} h^1, well below the values we estimated for the crab burrows.

About the level of terrestriality in fiddler crabs, the comparison between *U. urvillei* and *U. annulipes* species showed a clear difference in the oxygen consumption rate (Chapter 4).

U. urvillei species showed higher metabolic rate with respect to *U. annulipes*. The reason of that could be found in the body size and in the body mass percentage due to chitin and calcium-carbonate: in smaller animals (*U. annulipes*) the exoskeleton could represent a high percentage of the total volume, as well as of the total weight of the individual.

Also the comparison between sexes showed a clear difference in the oxygen consumption rate. Generally females of both species showed a higher metabolic rate respect to males. That could be explained considering the physiological status of females. Oogenis (Naylor et al., 1997,; Fernandez et al., 2000; Brante et al., 2003), in fact, require a strong physiological effort, and that could upholding the difference of metabolic rate between males and females (Weissburg, 1993; Negro et al, 2013). Unlike previous comparisons between species and sexes, that between medium didn't show a homogeneous framework.

For *U. urvillei* the pair wise test showed an opposite result in males and females. Males showed a higher metabolic rate in water with respect to air, accordingly to our prevision about a semiterrestrial species. Instead, females of *U. urvillei* showed a higher metabolic rate in air respect to the water.

Mangale Vilas and Kulkarni Balasaheb (2014) found in *U. annulipes* species that oxygen consumption increased and salinity tolerance levels decreased in dilute seawater, but they investigated neither the air-breathing nor the role of sex in oxygen consumption. In our study, for *U. annulipes,* the pair wise test showed a significant difference between medium only in males. In fact males showed a higher metabolic rate in air respect to the water. That is reasonable if we consider that fiddler crabs accomplish their activities mainly in subaeral environment, that is during low tide phase (Barnwell, 1966, 1968; Burggren & McMahon, 1988; Booksmythe et al., 2010) because being active determines a higher metabolic rate (Wallace, 1972).

No difference between medium has been found for females of *U. annulipes*. In general, considering the values of oxygen consumption, *U. annulipes* does not show clear difference in aquatic and subaeral condition. In both condition, the species is able to satisfy their metabolic necessity. In addition, about subaerial conditions, a possible explanation could be found in the natural history of the two species. In fact, while *U. annulipes* is a species inhabiting the higher zone of intertidal environment, *U. urvillei* inhabits the seaward zone (Vannini &

Valmori, 1981). That means that *U. annulipes* deals with longer low tide phases with respect to *U. urvillei*. That implies for *U. annulipes* a longer time of activity in subaerial condition. So being a good air-breather (lower oxygen consumption rate) could represent a key adaptation to upper intertidal zone. Nevertheless, this hypothesis is supported only by results about females.

So the question is why males of *U. annulipes* species in subaerial condition present higher oxygen consumption with respect to males of *U. urvillei* in same condition? Perhaps that is due to the difference in density of assemblages. In fact *U. annulipes* species is characterized by high density in which males strongly compete for space and females (deRivera & Vehrencamp, 2001) while *U. urvillei* assemblages show low density and low level of completion for space (Chapter 5, present thesis). So a higher metabolism could represent the response to the effort caused by social competition.

My behavioural observation during partial submersion showed a high level of restlessness (almost 50%) in males of both *U. urvillei* and *U. annulipes*: many specimens tried to partially emerge out of the water, to float on the water surface or to climb the bucket walls. Females of both species instead showed to be quiet in the water medium. The forced or partial submersion seems affecting the escape reaction in both species and in both sexes. In fact, after partial or total submersion, most of the crabs showed immobility or slow escape reaction. Thus, the forced immersion in water for a prolonged period of time seems to negatively influence their escape reaction. The capsizing time does not seem to be influenced by the medium, in fact almost all crabs have been able to capsize in few seconds.

About the habitat preference I observed a clear difference between males and females in both species. Considering fiddler crabs as semiterrestrial animals, the behaviour of males satisfied my expectation to find an increasing preference for the emerged substrates along experimental time. At the beginning of the trials, a higher number of individuals of *U. annulipes* showed to prefer the emerged substrate, with respect to *U. urvillei*. The majority of males, in fact, used the available escape routes. On the other hand, females did not totally satisfy our prevision. In fact no more than 50% of them showed a preference for an emerged condition and showed to be at ease under water. Moreover the number of females preferring emerged substrates did not increase during the experimental time. That could be explained considering their different physiology. Females of fiddler crabs in oogenesis and egg brooding (Edney, 1961; Crane, 1975; Wolcott 1988) could be less reactive than males to concentrate their metabolic effort in egg production and caring. As previously seen, in fiddler crab oxygen consumption by females in water is equal or minor than that in air. Because of that, they would spend time in water without spending energetic resources to move out of the water, as instead seen in males.

About territoriality in couples of neighboring *U. urvillei* males (Chapter 4), on average, any observed crab was active simultaneously with other resident ones for more than 50% of time. Considering this information and the high

visual acuity characterizing fiddler crabs, I am confident in hypothesizing that every resident crab was able to percept its own neighbour (Zeil & Hofmann, 2001, Detto et al., 2006, Zeil & Hemmi, 2005, Detto, 2007). I found a low level of defensive behaviour occurrence addressed to a neighbour by residents.

Only the 6% of time has been spent around the neighbour's burrow. Moreover, my results showed that resident crabs share a sector with the neighbour only for 5.6% of activity time. Merging the low level of defence behaviour addressed by residents to a neighbour and the short time of spatial sharing, I suggest that *U. urvillei* males adopt a spatial strategy designed to properly avoid a direct encounter with a neighbour. If familiarity was the only factor regulating a relationship between neighbours, I would expect a major spatial sharing. Probably, instead, *U. urvillei* are able to value the relativity of the threat represented by the neighbour. So the characteristics of time budget and the relative position of individuals seem to be the behavioural cues used by *U. urvillei* to distinguish between neighbours and intruders. Generally I observed that couples of residents showing neighbour defence behaviour spent much more time in the limit zone respect to couples without neighbour defence behaviour .

I found also that, when neighbour defence behaviour occurs, resident crabs living at short distance share mostly the space of the limit zone with respect to those living at middle and far distance. That could represent an spatial sharing caused by the occurrence of neighbour defence behaviour, independently by the familiarity. Finally I found a cue suggesting that a resident's spatial strategy is not independent by the one of the neighbour. In fact, independently by the distance between burrows, the limit zone sharing time seems to be partially influenced by presence of a resident. In this way each resident could control own border and acquire information on the level of threat represented by the neighbour That is a resident could be attracted in the limit zone by the presence of the neighbor.Generally, low level of defence behavior and low level of spatial co-presence could be evidences of "dear enemy effect" occurrence (Fisher, 1954) in *U. urvillei*

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