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Connectivity patterns and early life history of the black-faced blenny *Tripterygion delaisi* (Cadenat and Blache, 1970)

FARAHNAZ NISSA SOLOMON

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Supervisors

Dr. Rita Borges

Professor Serge Planes

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Short Abstract

An understanding of population connectivity and the scale of larval dispersal is essential for designing management and conservation plans for meta-population dynamics, fisheries, and biodiversity reserves. In demersal reef fish that are relatively sedentary as adults, connectivity tends to occur via the pelagic larval phase. This pelagic larval phase, which is variable in duration, is an extremely critical stage in the life-cycle, and inherent characteristics such as early life history traits and larval behaviour usually interact with oceanographic processes and habitat characteristics to determine patterns of connectivity.

In this thesis, connectivity of the black-faced blenny, *Tripterygion delaisi* was investigated at a local scale indirectly through the use of population genetic structure. Various aspects such as larval spatial distribution, allometric growth and larval development, and early life history characteristics of the critical pelagic larval stage was then examined.

Local scale population genetic structure of this species showed that the three sample locations, Arrábida, Sines and Cascais were highly genetically connected over a small spatial scale of approximately 100 km. Within this scenario of high gene flow however, several pieces of evidence indicated that limited or restricted dispersal was occurring leading to some degree or extent of local scale genetic homogeneity. An examination of spatial and temporal distribution showed that *T. delaisi* larvae has a mainly inshore larval distribution and that all the different developmental stages occur in the nearshore environment where it might be completing its life-cycle. We provided estimates for a number of early life history parameters (size-at-hatch, size-at-settlement, pelagic larval duration, and larval and juvenile growth rates) of *T. delaisi*. In particular our PLD estimates (both mean the value and minimum – maximum values) are higher than those obtained from two previous studies conducted in the Mediterranean and may have consequences for connectivity. Overall, the early life history traits exhibited by *T. delasi* can explain a pattern of restricted limited/restricted dispersal

Long Abstract

An understanding of population connectivity and the scale of larval dispersal is important for designing management and conservation measures for meta-population dynamics, fisheries, and biodiversity reserves. In demersal reef fish, which are relatively sedentary as adults, connectivity tends to occur via the pelagic larval phase. This pelagic larval phase is an extremely critical stage in the life-cycle, and inherent characteristics such as early life history traits (ELHTs) and larval behaviour usually interact with oceanographic processes and habitat characteristics influencing the extent of larval dispersal and consequently patterns of connectivity.

In this thesis, connectivity of the black-faced blenny, *Tripterygion delaisi* at a local scale was examined indirectly through the use of population genetic structure. Additionally, various aspects of the critical pelagic larval stage such as larval spatial distribution, allometric growth and larval development, and early life history characteristics such as Pelagic Larval Duration (PLD) among others, were investigated. The main findings across chapters are discussed in terms of how the connectivity pattern observed in this species can be explained and influenced by these inherent "characteristics" of the pelagic larval stage.

An examination of local scale population genetic structure of *T. delaisi* utilizing 8 highly polymorphic microsatellite loci showed that all pairwise F_{ST} values between adult samples were low and non-significant, indicating genetic homogeneity throughout the study area. Within this scenario of high gene flow: (1) significant isolation by distance; (2) several genetic clusters identified by Structure; and (3) the clustering and separation of sites in multivariate analyses, all alluded to the possible occurrence of restricted dispersal and local scale genetic heterogeneity despite the absence of population structure. When larval and juvenile samples were included in the analyses, the occurrence of some significant pairwise F_{ST} values, the detection of several genetic clusters, and the separation of sites by the various methods, all indicated the occurrence of temporal genetic variability. Overall, our study detected restricted larval dispersal amidst high gene flow, illustrated the phenomenon of "chaotic genetic patchiness", and highlighted the importance of sampling at multiple spatial and temporal scales when attempting to decipher connectivity patterns.

The spatial distribution of *T. delaisi* larvae in the Arrábida Marine Park was investigated across different periods from samples caught via two gear types, light traps and plankton nets attached to underwater scooters, over three years. For the first time light traps were deployed inshore and offshore in the Arrábida Marine Park. The main findings were: (1) all the different larval ontogenetic stages of *T. delaisi* (preflexion, flexion and postflexion) were present in the nearshore environment; (2) the density of *T. delaisi* larvae was greater inshore compared to

offshore; (3) there were differences in the ontogenetic composition of *T. delaisi* caught in the scooter and light traps and (4) vertical differences in the ontogenetic structure of samples collected at distinct depth strata could be detected. The results support previous studies which document a mainly inshore larval distribution for species belonging to the Family Tripterygiidae. Comparison of gear types highlighted the importance of using multiple gear types when sampling to determine larval distribution patterns. Using just one gear type may not be sufficient to ensure that all the different developmental stages are represented.

In terms of larval development and allometric growth, growth coefficients generated from bivariate morphological relationships indicated that most of the body proportions of *T. delaisi* exhibited allometric growth during larval development. When inflexion points of growth were detected, growth was biphasic with the inflexion points occurring within a very narrow range of body length (L_B) = 8.7 to 8.9 mm. Considering allometric growth patterns and ontogenetic descriptions together, it is hypothesized that the 1st phase primarily relates to the development of the digestive system (coiling of the gut occurs during this stage), and the swimming abilities of the larvae (development of the myomeres in terms of length and width, and caudal fin development occurs during this stage), and the 2nd phase involves continued locomotory development via progressive fin formation and development, and the transition from larvae to juvenile. It is also hypothesized that the development of some of the essential sensory systems may have already been well developed on hatching. Distinctive morphometric and meristic characteristics which are central to identifying this species from related taxa were also described for the first time, in a complete description of larval development for this species.

Otolith microstructure analyses of the sagittal otoliths of larvae and early recruits was used to determine several early life-history parameters for *T. delaisi*. Apart from PLD studies previously conducted in the Mediterranean which utilized lapillar otoliths, there is no information available on life history parameters during the early life stages of this species. We examined larvae belonging to all three developmental stages (preflexion, flexion and postflexion of the notochord) and there was a good match between the age of the older larvae examined and the age at settlement, derived from back-calculations from the settlement mark. PLD estimates ranged between 29 and 34 with a mean of 31.75 ± 1.54. These values are higher than that obtained from previous studies conducted in the Mediterranean, and can indicate a larger potential for dispersal. Growth related traits proved to be variable, indicating possible plasticity of such early traits, ecologically relevant. Instantaneous growth rates, size-at-hatching and size-at-settlement were similar to those documented for other demersal reef fish.

This study provided evidence that *T. delaisi* can exhibit restricted dispersal amidst high gene flow along a 100 km stretch of coastline, on the west coast of Portugal. Several of the early life history characteristics for this species can promote this pattern of restricted dispersal where local retention as opposed larval dispersal may be the main ecological process occurring. These characteristics include, a mainly inshore larval distribution, the occurrence of all the different larval developmental stages inshore and the early development of sensory and swimming abilities. An understanding of both the connectivity patterns of *T. delaisi* in our study area, as well connectivity patterns in general can be enhance in future studies by: (1) A better understanding of the small-scale local processes occurring in the nearshore environment and the mechanisms which may be acting to promote larval retention (2) A better understanding of the Coastal Boundary Layer (3) Studies aimed at understanding sensory and swimming abilities (4) The more routine application of models to predict patterns and (5) The utilization of a combination of complimentary approaches.

RESUMO

A compreensão dos padrões de conectividade entre populações, assim como a escala a que a dispersão das larvas de organismos marinhos ocorre, são essenciais para o desenvolvimento de medidas de gestão e conservação adequadas, tendo em vista a dinâmica de metapopulações e medidas de gestão da pesca e reservas de biodiversidade. Nas espécies de peixes demersais, que são relativamente sedentários na fase adulta, é a fase larvar pelágica que contribui largamente para a conectividade entre populações. Esta fase, representa uma etapa crítica no ciclo de vida dos peixes; os padrões de conectividade dependem de mecanismos biofísicos que condicionam a distribuição larvar, como a interacção entre as características intrínsecas dos indivíduos nos períodos iniciais de vida, os aspectos sensoriais ou comportamentais das larvas, e os processos oceanográficos e características do habitat.

Nesta tese, foi examinada a conectividade enre populações do caboz-de-três-dorsais, *Tripterygion delaisi*, indirectamente através do uso da estrutura genética populacional, a uma escala local. Foram também investigados vários aspectos da fase larvar desta espécie, como a distribuição espacial de larvas, padrões de crescimento alométrico, o desenvolvimento larvar e algumas características relevantes dos períodos iniciais de vida. Os principais resultados obtidos são discutidos de modo a entender como o padrão de conectividade observado para esta espécie pode ser explicado e influenciado por estas características inerentes à fase larvar pelágica.

A análise da estrutura genética populacional, a uma escala local, foi feita recorrendo a 8 microssatélites altamente polimórficos. Os resultados demonstraram que todos os valores *pairwise Fst* obtidos entre amostras de adultos eram baixos e sem significância, apontando para homogeneidade genética na presente área de estudo. Apesar deste cenário de alto fluxo genético verificou-se (1) isolamento significativo pela distância; (2) vários agregados genéticos identificados através do software Structure; e (3) a agregação e separação de locais através de análises multivariadas. Estes resultados apontam para a possibilidade de ocorrência de dispersão larvar restrita e heterogeneidade genética a uma escala local. Após a inclusão das amostras dos estados larvar e juvenil, observou-se a ocorrência de valores significativos *pairwise Fst*, deteção de vários agregados genéticos e separação de locais através de vários métodos, indicando ocorrência de padrões temporais na variabilidade genética. Em perspectiva este estudo detectou uma dispersão larvar restrita apesar do fluxo genético, fenómeno de “chaotic genetic patchiness”, e salientou a

importância da amostragem a diferentes escalas espaciais e temporais na tentativa de decifrar os padrões de conectividade observados.

De modo a se investigar a possível reteção de larvas junto à costa, foie studada a distribuição espacial e temporal de larvas de *T. Delaisi* no Parque Marinho da Arrábida (PMA) a partir de amostras recolhidas através de duas diferentes metodologias, armadilhas de luz e arrastos de plâncton através de "scooters", ao longo de três anos. Pela primeira vez, foram colocadas armadilhas de luz muito próximo dos recifes e mais ao largo no PMA. Os principais resultados incluiram: (1) todas as fases ontogenéticas (preflexão, flexão e posflexão) das larvas de *T. delaisi* estavam presentes na área do "nearshore"; (2) a abundância de larvas de *T. delaisi* era maior "inshore" do que "offshore"; (3) verificaram-se diferenças na estrutura ontogenética das larvas de *T. delaisi* entre as amostras recolhidas pelos dois méodos e (4) diferenças verticais na estrutura ontogenética das amostras recolhidas com umdos métodos. Os resultados obtidos reforçam estudos anteriores para espécies da família Tripterygiidae, que demostram uma distribuição larvar maioritariamente muito próximo de costa, indicando a possibilidade de retençção larvar e de crescimento local. Os resultados realçam também a importância da utilização de vários tipos de metodologias para a determinação da ocorrência e dos padrões de distribuição larvar. A utilização de apenas uma metodologia ou equipamento poderia ser insuficiente para a recolha de todos os estados de desenvolvimento larvar.

Em termos de desenvolvimento larvar e crescimento alométrico, os coeficientes de crescimento obtidos através de relações morfológicas bivariadas, demonstram que a maior parte das proporções corporais de *T. delaisi*, exibiam crescimento alométrico durante o desenvolvimento larvar. Quando foram detetados pontos de inflexão de crescimento, este era bifásico com os pontos a ocorrer no intervalo de comprimento corporal (Lb) = 8.7 a 8.9 mm. Tendo em conta os padrões de crescimento alométrico juntamente com as descrições ontogenéticas, foi colocada a hipótese de a 1ª fase consistir primariamente no desenvolvimento do sistema digestivo e, possivelmente, das capacidades natatórias da larva (desenvolvimento dos miómeros em comprimento e largura, e desenvolvimento da barbatana caudal), e da 2ª fase no desenvolvimento das capacidades de locomoção através do progressivo desenvolvimento e formação das diferentes barbatanas e da subsequente transição de larva para juvenil. Foi ainda colocada a hipótese de que algumas estruturas sensoriais já estariam num estado avançado de desenvolvimento à eclosão. É apresentada pela também, pela primeira vez, uma descrição completa do desenvolvimento larvar desta espécie, essencial na identificação da mesma e para a correcta interpretação de padrões ecológicos.

Com o objectivo de determinar vários parâmetros dos estádios inciais do ciclo de vida de *T. delaisi,* procedeu-se à análise da microestutura dos otólitos *sagittae* de larvas e recrutas. Com a excepção dos estudos acerca de da duração da fase larvar (PLD) realizados no Mediterrâneo em que foram utilizados *lapilli*, não existia informação disponível acerca dos parâmetros das etapas iniciais de vida desta espécie. Foram examinadas larvas pertencentes aos três estados de desenvolvimento establecidos (preflexão, flexão e posflexão) e através de leituras de anéis diários a partir para a marca de assentamento, estimou-se a idade dos indivíudos, tendo-se verificado uma correspondência positiva entre a idade da larva mais velha analisada e a idade ao assentamento. O PLD estimado neste estudo para esta espécie situou-se no intervalo de 29 a 34 dias com uma média de 31.75 ± 1.54. Estes valores são mais elevados do que os obtidos em estudos anteriores para o Mediterrâneo. Por sua vez, as taxas de crescimento, comprimento à eclosão e comprimento no assentamento foram similares aos documentados para outros peixes demersais de recife.

Este estudo demonstrou que a espécie *T. delaisi* exibe um padrão de dispersão restrita em simultâneo com alto fluxo genético ao longo de 100 km da linha costeira na costa oeste de Portugal. Várias das características do ciclo inicial de vida desta espécie podem contribuir para este padrão de dispersão restrita, onde a retenção local é o processo ecológico dominante em oposição à dispersão larvar. Estas características incluem, distribuição mais restrita e a ocorrência de todas as fases de desenvolvimento larvar larvar maioritariamente junto à costa, e o desenvolvimento de capacidades locomotoras e possivelmente sensoriais nas fases iniciais de vida. A compreensão dos padrões de conectividade para *T. delaisi* na área de estudo, assim como, os padrões de conectividade em geral podem ser melhorados no futuro através de (1) um maior aprofundamento do conhecimento dos processos locais de pequena escala que ocorrem no "nearshore" e dos mecanismos que podem promover retenção larvar (2) um estudo das características físicas do local que podem favorecer a rentenção "Coastal Boundary Layer" (3) Estudos que tenham como objectivo o estudo das capacidades sensoriais e locomotoras nas primeiras fases de vida desta espécie e (4) o desenvolvimento e aplicação de modelos para prever padrões e (5) idealmente, uma combinação de abordagens complementares a estes temas.

RESUMÉ

Une compréhension de la connectivité des populations et de l'échelle de dispersion larvaire est importante pour établir des mesures de gestion et de conservation pour les dynamiques méta-populationnelles, les pêches et les réserves de biodiversité. Chez les poisons récifaux démersaux qui sont relativement sédentaires au stade adulte, la connectivité se produit au moment de la phase larvaire pélagique. Cette phase larvaire pélagique est une étape extrêmement critique au cours du cycle de vie, et les caractéristiques inhérentes telles que les traits d'histoire de vie précoces et le comportement larvaire interagissent habituellement avec les processus océanographiques et les caractéristiques de l'habitat pour déterminer les modèles de connectivité.

Dans cette thèse, la connectivité du triptérygion à bec jaune, *Tripterygion delaisi* à une échelle locale était examinée indirectement par l'utilisation de structure génétique populationnelle. Plusieurs aspects de l'étape larvaire pélagique critique tels que, la distribution spatiale, la croissance allométrique et le développement larvaire, et les traits d'histoire de vie précoces étaient ensuite examinés. Les principaux résultats sont discutés en tenant compte de la manière dont le modèle de connectivité chez cette espèce peut être expliqué et influencé par ces "caractéristiques" inhérentes à l'étape larvaire pélagique.

L'analyse de la structure génétique populationnelle à l'échelle locale de *T. delaisi* en utilisant 8 loci microsatellites hautement polymorphes montrait que toutes les valeurs de F_{ST} par paire des échantillons adultes étaient faibles et non significatives, indiquant une homogénéité génétique de la région étudiée. Dans ce scénario de flux génique élevé: (1) l'isolement significatif par la distance; (2) l'identification de plusieurs groupes génétiques par Structure; et (3) le regroupement et la séparation des sites dans les analyses multivariées, suggèrent la présence possible d'une dispersion restreinte et d'une hétérogénéité génétique à l'échelle locale. Quand les échantillons de larves et de juvéniles étaient inclus dans l'analyse, une variabilité génétique temporelle était suggérée par la présence de valeurs de F_{ST} par paires significatives, la découverte de plusieurs groupements génétiques, et par la séparation des sites par les différentes méthodes utilisées. Globalement, notre étude a montré une dispersion larvaire restreinte au milieu d'un flux génétique élevé, a illustré le phénomène de "répartition génétique chaotique", et a souligné l'importance d'échantillonnage à différentes échelles spatiales et temporelles pour déchiffrer les modèles de connectivité.

Les distributions spatiales et temporelles des larves de *T. delaisi* dans le Parc Marin d'Arrábida étaient étudiées à partir d'échantillons collectés par deux types d'équipements, les pièges lumineux et les filets à plancton attachés aux scooters sous-marins, sur une période de trois

ans. Pour la première fois, des pièges lumineux étaient utilisés près des côtes (inshore) et aux larges des côtes (offshore) dans les AMP. Nos principales découvertes étaient: (1) tous les différents stades ontogénétiques larvaires de *T. delaisi* (préflexion, flexion et postflexion) étaient présents dans les environnements près de la côté (neashore); (2) l'abondance des larves de *T. delaisi* était plus grande près des côtes (inshore) qu'aux larges des côtes (offshore); (3) des différences dans la composition ontogénétique de *T. delaisi* étaient observées entre les échantillons collectés avec le scooter et les pièges lumineux et (4) des différences verticales dans la structure ontogénétique des échantillons collectés à différentes profondeurs ont pu être détectées. Nos résultats corroborent les études précédentes qui rapportent une distribution larvaire principalement près des côtes (inshore) pour les espèces appartenant à la famille des Tripterygiidae. La comparaison des types d'équipement était aussi en accord avec les études précédentes et a souligné l'importance de l'utilisation de différents équipements d'échantillonnage pour déterminer les modèles de distribution larvaire. L'utilisation d'un seul type d'équipement ne peut être suffisante pour s'assurer que tous les différents stades de développement soient représentés.

En ce qui concerne le développement larvaire et la croissance allométrique, les coefficients de croissance générés à partir des relations morphologiques bivariées indiquaient que la majorité des proportions du corps de *T. delaisi* montrait une croissance allométrique durant le développement larvaire. Quand les points d'inflexion de croissance étaient détectés, la croissance était biphasique avec les points d'inflexion se situant dans une gamme très étroite de longueur du corps (L_B) = 8.7 to 8.9 mm. En tenant compte des modèles de croissance allométrique et des descriptions ontogénétiques, nous suggérons que la 1ère phase se rapporte essentiellement au développement du système digestif (l'enroulement du tube digestif se produit durant ce stade), et aux capacités de nage des larves (le développement des myomères en termes de longueur et de largeur, et du développement de la nageoire caudale se produisent durant ce stade), et que la 2^{ième} phase implique le développement locomoteur continu via le développement et la formation progressive des nageoires, ainsi que la transition des larves aux juvéniles. Nous suggérons que le développement de certains systèmes sensoriels essentiels peut déjà être bien développé au moment de l'éclosion. Les caractéristiques morphométriques et méristiques essentielles à l'identification de cette espèce à partir de taxons apparentés ont été décrites pour la première fois.

Les analyses de microstructure d'otolithes des otolithes sagittales des larves et des jeunes recrues étaient utilisées pour déterminer les paramètres de traits de vie précoces de *T. delaisi*. A l'exception des études sur la durée de vie pélagique larvaire (PLD) précédemment menées en Méditerranée en utilisant des otolithes lapillaires, il n'y a aucune information sur les paramètres des

traits de vie durant les étapes de vie précoces de cette espèce. Nous avons examinés des larves appartenant aux trois stades de développement (préflexion, flexion et postflexion) et il y avait une bonne correspondance entre l'âge des plus vieilles larves examinées et l'âge à l'établissement, provenant des calculs à rebours de la marque d'établissement. Les estimations de PLD variaient entre 29 et 34 avec une moyenne de 31.75 ± 1.54. Ces valeurs sont plus grandes que celles obtenues dans les études précédentes menées en Méditerranée. Les taux de croissance instantanés, la taille à l'éclosion et la taille à l'établissement étaient similaires à ceux décrits pour d'autres poissons récifaux démersaux.

Cette étude a montré que *T. delaisi* présentait une dispersion restreinte au milieu d'un flux génique élevé le long d'un tronçon de 100 km de côte, sur la côte ouest du Portugal. Plusieurs des caractéristiques de traits de vie précoces subséquemment dérivés pour cette espèce peuvent promouvoir ce modèle de dispersion restreinte où la rétention locale à l'opposé de la dispersion larvaire est le principal processus écologique se produisant. Ces caractéristiques incluent, une distribution larvaire principalement près de la côté (inshore), la présence de tous les différents stades de développement larvaire près de la côté (inshore) et le développement précoce des capacités sensorielles et natatoires. Une compréhension à la fois des modèles de connectivité de *T. delaisi* dans notre site d'étude, ainsi que des modèles de connectivité en général peut être améliorée par: (1) une meilleure compréhension des processus à petite échelle locale se produisant dans les environnements près de la côte (nearshore) ainsi que des mécanismes pouvant promouvoir la rétention larvaire, (2) une meilleure compréhension des couches limites côtières, (3) des études visant à la compréhension des capacités sensorielles et natatoires, (4) l'application plus systématique des modèles pour prédire des modèles et (5) l'utilisation d'une combinaison d'approches complémentaires.

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CHAPTER 1: GENERAL INTRODUCTION

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GENERAL INTRODUCTION
PART A

1.1 The bipartite life cycle

The bipartite lifecycle

Most demersal reef fish have a bipartite (two phase) lifecycle involving relatively sedentary adults on the reef, and a pelagic larval phase hatched from demersal eggs (Leis, 1991). These larvae, which develop in the water column until they are ready to settle in the benthos as juveniles, subsequently grow and mature into adults that reproduce to complete the life-cycle. The duration of the planktonic stage is species specific and may vary from days to weeks to months (Victor, 1986; Wellington & Victor, 1989; Macpherson & Raventós 2006; Beldade *et al*., 2007). This is a critical stage in the lifecycle in which important transitions occur, and it is often characterized by high mortality rates due to predation and starvation (Bailey & Houde, 1989; Houde & Zastrow, 1993) as the larvae undergo several ontogenetic developmental stages (i.e. yolk sac, preflexion, flexion and postflexion) until they are competent to settle. At this stage, it is crucial to locate suitable habitats for settlement. If such habitats are not present, some larvae may delay settlement until conditions are appropriate (Cowen, 1991). Metamorphosis from the pelagic larva to the benthic juvenile or adult, usually involves significant changes in morphology, pigmentation patterns, physiology and behaviour (McCormick *et al*., 2002; Cunha *et al*., 2013). Young fish settle onto the reef and will contribute to a process known as recruitment—the addition of new individuals to a population. Recruitment can be affected by larval supply as well as by post-settlement processes, and can be highly variable both spatially and temporally (Caley *et al*., 1996; Sale *et al*., 2005b; Sponaugle & Grorud-Colvert, 2006; Sponaugle *et al*., 2006; Shima & Swearer, 2009). It has important implications for local population dynamics (Caley *et al*., 1996). By growing and developing in the pelagic environment, larvae can easily disperse with currents. The extent to which larvae disperse will affect local recruitments patterns and population connectivity, particularly in species

The pelagic larval phase

Recruitment

having sedentary adults that live attached to demersal habitats.

1.2 Connectivity in the marine environment

Definition of connectivity

In marine ecosystems, connectivity can be broadly defined as "the extent to which discrete populations or sub-populations of individuals are linked by dispersal" (Palumbi, 2003). It can occur via the dispersal of larvae, juveniles, or adults, can be viewed as a continuum ranging from no connectivity (closed populations) to high connectivity (open populations), and can be regarded as either evolutionary (genetic) or demographic (ecological). Evolutionary connectivity is concerned with how gene flow affects populations over a timescale of many generations whilst demographic connectivity focuses on the exchange of individuals among local populations, and its effects on population demographics which can be detected on a contemporary time-scale (Lowe & Allendorf, 2010; Sale *et al*., 2010; Leis *et al*., 2011). In most demersal reef fish, adults are relatively site attached and most of the connectivity tends to occur via the pelagic larval phase (Doherty *et al*., 1995; Cowen *et al*., 2007; Cowen & Sponaugle, 2009).

Evolutionary vs demographic connectivity

1.3 Open vs. closed populations

A temperate setting

In a temperate setting, much of the early work on larval fish was conducted on non-reef species belonging to the orders Clupeiformes, Gadiformes and Pleuronectiformes (Leis & McCormick, 2002). Due to this bias, much of the information on the larval biology of temperate fishes was based on species belonging to these orders, which were either pelagic or occupied soft bottoms as adults. Larval characteristics of these species were often erroneously extended to include all temperate fish larvae including those of the rocky reef environment, dominated by species belonging to the order Perciformes (Leis & McCormick, 2002). These characteristics, which included poor swimming abilities and poorly developed sensory systems in the early stages, contributed to the development of the traditional view where fish larvae were seen as passive, and expected to have a large dispersal potential, promoting open and

connected populations (Caley *et al*., 1996). Under such circumstances, it could be expected that the recruitment of fish populations including reef fish, would come mainly from external sources i.e. most of the juveniles settling on a reef would not be the product of eggs spawned on that reef but would be derived from eggs produced elsewhere.

Tropical vs temperate

Early attempts at tropical vs. temperate larval comparisons were based on comparisons between temperate clupeiform fish and tropical perciform fish (Leis & McCormick, 2002). It was subsequently recognized that there were fundamental differences in terms of life history traits between these two groups and that such comparisons without considering taxonomical differences could be misleading. A more appropriate temperate vs. tropical comparison would be between temperate reef and coral reef species belonging to similar groups, and sharing similar larval life history traits, many of which differ from those of pelagic Clupeiformes and to those traits which form the basis of the "open population" paradigm (Leis & McCormick, 2002). Two of the main differences between species spawning pelagic eggs (such as Clupeiforms) and demersal eggs (e.g. Perciforms and other families that are abundant in reef systems), with implications for larval dispersal and connectivity include: (1) The extent or degree of development at a given size: at a given size, reef fish larvae spawned from demersal eggs tend to be more developed in terms of fin development and in terms of development of the feeding and sensory apparatus compared to larvae hatching from pelagic eggs (Leis & McCormick, 2002). (2) Swimming abilities: reef fish larvae exhibit better swimming abilities both in terms of swimming speed and endurance, compared to larvae from pelagic eggs (Leis & Carson-Ewart, 1997, 2003; Fisher *et al*., 2005). Both these differences indicate that unlike temperate clupeiform larvae, temperate reef fish larvae may not be passive dispersers but are be able to exert some control over their movement and position in the water column.

Clupeiformes vs. Perciformes

From as early as the 1980s and 1990s, patterns of larval spatial distributions have alluded to the fact that dispersal and connectivity maybe

Larval spatial distribution

more restricted than previously thought in some fish species (Leis *et al*., 2011). In both temperate and tropical environments, the larvae of some species exhibit restricted dispersal by remaining within a few hundred metres of their spawning site (Barnett *et al*., 1984; Brogan, 1994). Vertical and horizontal sampling of the nearshore environment have also found restricted inshore larval distributions for several species belonging to the families Gobiesocidae and Tripterygiidae (Kingsford & Choat, 1989; Sabatés *et al*., 2003). The occurrence of all the different larval developmental stages of a single species in the nearshore environment, which suggests that some degree of local retention and development may be occurring, has also been documented (Leis *et al*., 1998; Leis *et al*., 2003; Borges *et al*., 2007b; Borges *et al*., 2009). Overall, though useful for providing general insights into connectivity patterns, and supporting evidence for potentially limited dispersal, larval spatial distribution cannot definitively exclude the occurrence of more longshore/offshore dispersal. Conclusions drawn from these studies must be treated cautiously and preferably substantiated by other more definitive methods (Leis *et al*., 2011).

The "open population" paradigm

Providing support against the paradigm of "open populations", several lines of evidence have since shown that reef fish larvae may exhibit behaviour that can actively influence their dispersal thus dispelling the prevailing belief that they are passive dispersers. Vertical migration exhibited by many larvae can alter their horizontal position in the water and may serve to increase retention near natal reefs (Paris & Cowen, 2004; Leis *et al*., 2007b). Both *in situ* and laboratory based experiments show that the larvae of many reef fish species have swimming capabilities (swimming speed, swimming endurance and oriented swimming) which allow them to actively influence their dispersal (Leis & Carson-Ewart, 1997; Stobutzki & Bellwood, 1997; Fisher *et al*., 2000; Fisher, 2005; Leis *et al*., 2007a). Some larvae are also capable of detecting olfactory (Atema *et al*., 2002; Gerlach *et al*., 2007; Paris *et al*., 2013) and aural (Tolimieri *et al*., 2000; Simpson *et al*., 2004; Tolimieri *et al*., 2004; Montgomery *et al*., 2006) cues which may assist them in detecting, navigating, and actively

Larval behaviour

selecting habitats.

Local processes

Several local processes (i.e. surface gravity waves, tides, boundary effects, internal waves and bores) operate in the nearshore environment resulting in flows that are more complex than in the open ocean (Largier, 2003; Pineda & Hare, 2007; Morgan et al., 2009). Even in the absence of larval behaviour, these nearshore systems can promote local retention due to inherent characteristics such as shallow depths, shoreline proximity and rocky bottom topography (Pineda, 2000; Largier, 2003). The interaction between dominant tidal currents that run parallel to the shoreline and shallow underwater rocky habitats that extend only a short distance offshore has been shown to create multidirectional layers of flow which reduce water flow near the epibenthic boundary, ultimately favouring larval retention in that area (Pineda & Hare, 2007). In shallow environments "the coastal boundary layer" may cause friction at the bottom, reducing the effects of wind currents and favouring larval retention (Largier, 2003). Additionally, internal tidal bores (sharp surges of cold water associated with thermoclines) can promote the shoreward transport of planktonic organisms (Pineda, 1991; Pineda & Hare, 2007). The interaction of alongshore coastal currents with headlands can also facilitate planktonic retention via the formation of headland eddies or upwelling shadows in their lee (Largier, 2003; Roughan, 2005).

Self-recruitment

More definitive findings contradictory to the "open population" paradigm were obtained by Jones *et al*. (1999) who used tetracycline to tag approximately 10 million embryos of a damselfish on the Great Barrier Reef over a three-year period. An analysis of the otoliths from re-captured larvae yielded 15 tagged individuals, and on the basis of an estimate of the proportion of embryos marked, it was calculated that between 15%–60% of recruits to the study area were locally produced (self-recruitment). These figures suggested that self-recruitment was occurring at a much larger extent than was previously thought. Since then, numerous studies using a variety of novel techniques have yielded similar results for various species of clownfish belonging to the genus *Amphiprion* (Jones *et al*., 2005; Almany *et al*., 2007; Planes *et al*., 2009; Berumen *et al*., 2012; Madduppa

et al., 2014); the bluehead wrasse, *Thalassoma bifasciatum* (Swearer *et al*., 1999); the Tripterygiid, *Tripterygion delaisi* (Carreras-Carbonell *et al*., 2007a) and the damselfish, *Stegastes partitus* in the Bahamas (Christie *et al*., 2010a) and in the western Caribbean (Hogan *et al*., 2012).

A changing paradigm

Overall, these various lines of evidence have led to a change from the classical view of predominantly open populations with demographically significant movement of individuals to a more conservative one characterized by a range or continuum of connectivity, facilitated by non-passive larval dispersal. This serves to highlight the complexity of connectivity patterns in the marine environment and has led to an increase in the number of studies aimed at estimating larval dispersal and connectivity given its importance to both evolutionary and ecological processes in populations.

1.4 Why study connectivity: MPA Design

Metapopulations

In the marine environment, populations of organisms can be viewed as metapopulations consisting of numerous spatially separated local populations connected by varying levels of larval dispersal (Kritzer & Sale, 2004). Whilst the internal dynamics of each local population is mostly self-dependent, demographic influence from other local populations arise through connectivity. This connectivity ultimately determines the spatial scales over which populations operate; hence, it has major evolutionary and ecological consequences. More specifically, it has important implications for population dynamics (Caley *et al*., 1996), population genetic structure (Pelc *et al*., 2009), biogeography and endemism, population resilience to natural and anthropogenic disturbances (Hughes *et al*., 2010), and the application of marine conservation measures (van der Meer *et al*., 2012).

Much of our present day interest in connectivity, however, as reflected by a review of the published literature, stems from its importance in the design and implementation of marine protected areas (MPAs) (Wood *et al*., 2008), or more recently, networks of MPAs – a collection of individual but inter-connected MPAs. MPAs are widely advocated as a tool

MPAs

to conserve and manage highly threatened marine ecosystems such as rocky reefs, coral reefs and mangroves (Halpern *et al*., 2007), by supporting the objectives of both fisheries management and biodiversity conservation (Jones *et al*., 2007; Gaines *et al*., 2010). A comprehensive knowledge of larval dispersal and connectivity for a wide range of organisms is important for the effective design and implementation of these MPAs (Palumbi, 2003; Sale *et al*., 2005a; Almany *et al*., 2009; McCook *et al*., 2009; Chittaro & Hogan, 2013; Green *et al*., 2014). For example, this knowledge may be utilized to determine the correct size, spacing and placement of no-take reserves so as to not only ensure persistence of target populations (via self-recruitment and dispersal), but to also benefit fisheries management by protecting stocks within, and enhancing juvenile and adult fish outside the area through spillover. An additional process leading to population replenishment and fisheries enhancement in the outer areas is the so-called "recruitment subsidy"—the export of eggs and larvae from MPAs to outside the protected area (Sale *et al*., 2005a).

Despite its importance, population connectivity remains very difficult to quantify directly and is often a limiting factor in the design and implementation of MPAs (Burgess *et al*., 2014). In the absence of the required empirical information, most managers of MPAs adopt general guidelines or "best guesses" which they hope will maintain connectivity for a range of species when determining size, location and spacing (McCook *et al*., 2009).

1.5 Estimating larval dispersal and connectivity in the marine environment

Estimating larval dispersal

In demersal reef fish, connectivity is largely dependent upon the scale or extent of dispersal during the pelagic larval phase/stage (Cowen *et al*., 2007). Larval dispersal, however, is very difficult to estimate because it involves tracking very minute organisms in a vast medium that is the ocean, for prolonged periods of time. Given the difficulties of tracking larvae via direct observations, a number of indirect methods and tools have

been developed to determine or infer patterns of larval dispersal and connectivity (Thorrold *et al*., 2002; Levin, 2006). A brief overview of the more common methods is given below.

1.5.1 Physical and biophysical modelling

Modelling

Numerous mathematical models have been used to study larval dispersal. Early models were mainly physical—depicting larvae as passive particles depending solely on ocean currents for dispersal (Roberts, 1997). Many present-day models are more complex and incorporate biological factors (i.e. vertical movement, larval mortality, pelagic larval duration; swimming capabilities) which are known to influence larval dispersal (Hare *et al*., 1999; Cowen *et al*., 2000; James *et al*., 2002; Cowen *et al*., 2006; Paris *et al*., 2007; Andrello *et al*., 2013). Compared to other methods, whilst modelling allows for the tracking of virtual individuals over longer spatial and temporal scales, the numerous physical and biological parameters which are required for them to be accurate or representative are often not available and outputs cannot be validated in the field. They are however particularly useful for developing scenarios and testing hypotheses, and are best used with complementary approaches (e.g. genetics) that provide validation or support (Galindo *et al*., 2010).

Nearshore environments

Additionally, modelling larvae in the nearshore environment continues to be a challenge as most models utilize large grid sizes (tens of metres to kilometres) which are unable to model small-scale hydrodynamic processes (e.g. micro-scale turbulence, surface waves and internal bores) which commonly occur in coastal environments. Since many larvae originate from and return to the nearshore environment to complete their life-cycle, important behaviours at these vital stages can be missed (Pineda *et al*., 2009).

Partial or complete reviews of the use of physical and biophysical models in connectivity studies are provided by Werner *et al*. (2007), Leis *et al*. (2011), and Staaterman and Paris (2013).

1.5.2 The application of artificial and natural tags

Artificial tags – fluorescent compounds and radioactive isotopes

Some of the early studies documenting self-recruitment and local retention of larvae in the marine environment utilized artificial tags such as fluorescent compounds and radioactive isotopes to mark the otoliths of embryonic or larval stages (Jones *et al*., 1999, 2005; Almany *et al*., 2007). The work of Jones *et al*. (1999) is outlined in Section 1.3 above. In a subsequent study, barium isotope tagging of larvae via the transgenerational marking of embryonic otoliths (Thorrold *et al*., 2006) applied to two coral reef fish species (*Amphiprion percula* and *Chaetodon vagabundus*) around Kimbe Island, Papua New Guinea, showed that both species exhibited significant self-recruitment (approximately 60%) despite differences in the their reproductive strategies (Almany *et al*., 2007). Both of these studies were extremely successful providing direct estimates of connectivity. However, in both instances, tagging was labour intensive and the recovery rate for artificially marked individuals was low (i.e. in Jones *et al*. (1999), 10 million embryos were tagged, and only 15 out of the 5000 juveniles recovered were marked)—two major limitations of traditional capture, mark and re-capture studies.

Natural tags – otolith chemistry

The use of natural tags that mark all the individuals in a population can eliminate the limitation of low recapture rates that plague the use of artificial tags (Thorrold *et al*., 2002). One method that is increasing in popularity in fishes is "otolith chemistry"—an analysis of the elemental signature of the otolith or ear stone found in fishes. These elemental signatures function as a natural "tag" with the underlying basis being that the chemical characteristics of the surrounding aquatic environment are incorporated into the calcium carbonate matrix of the otolith during growth, resulting in signatures that could be identified spatially. Substantial variation in the elemental composition of the tags among sites of interest is very important for this method to be successful (Thorrold *et al*., 2002; Thorrold *et al*., 2007) and for this reason, this method was initially applied mainly to studies in coastal temperate waters, especially on estuarine species (Thorrold *et al*., 1998; Thorrold *et al*., 2001). Since then,

technological advancements in trace element quantification, improvements in the techniques of examining distinct regions of the otolith (e.g. core vs. edge) and a better understanding of the chemical signatures themselves, have resulted in an increase in the number of studies applying this method to investigations of larval dispersal and connectivity (Leis *et al*., 2011). These studies have been conducted on a range of species with different life-history characteristics, in varying habitats, and on varying spatial and temporal scales (Patterson *et al*., 2005; Patterson & Swearer, 2007; Ben-Tzvi *et al*., 2008; Ashford *et al*., 2010; Ashford *et al*., 2011; Chittaro & Hogan, 2013).

Studies utilizing this technique have shown that demersal reef fish exhibit a broad range of connectivity patterns ranging from low connectivity characterized by high levels of self-recruitment and local retention (Swearer *et al*., 1999; Patterson *et al*., 2005; Chittaro & Hogan, 2013), to a combination of even dispersal and retention (Patterson *et al*., 2005; Patterson & Swearer, 2007) to high connectivity characterized by the dominance of larval dispersal (Ben-Tzvi *et al*., 2008).

1.5.3 Genetics

Genetic methods are often used to assess population connectivity in the marine environment. Studies have traditionally focused on evolutionary connectivity (historical gene flow) through the use of markers such as allozymes and mtDNA which exhibit slow mutation rates allowing the signature of events in the distant past to be detected (Selkoe & Toonen, 2006). MPA managers and ecologists, however, are more concerned with demographic connectivity on contemporary time-scales, and it was not until the advent of microsatellites or simple sequence repeats (SSRs) that studies addressing this type of connectivity became more prevalent (Selkoe & Toonen, 2006). Microsatellites, which are tandem repeats of 1–6 nucleotides occurring at high frequencies in most nuclear genomes, are better suited for assessing contemporary (recent past) processes because of their high mutation rates (Ellegren, 2004). These high mutation rates also allow microsatellites to generate high levels of allelic diversity (high

Microsatellites

polymorphism) making them more informative and better suited to the application of many present-day statistical analyses. These numerous advantages, coupled with the fast and large-scale cost-effective genotyping of samples via the use of PCR technology, have made microsatellites the marker of choice for population genetic studies (Sunnucks, 2000; Zhang & Hewitt, 2003; Selkoe & Toonen, 2006). Connectivity estimates are inferred from microsatellite markers via 2 main types of methods:

(1) Indirect methods based on the extent or degree of genetic differentiation between populations and

(2) Direct methods which focus on assigning individuals to either their population of origin (assignment tests) or to their parents (parentage analysis).

Indirect methods: Since genetic structuring ultimately reflects the number of alleles exchanged between populations, the most traditional approaches to assessing population genetic structure are based on comparing differences in allelic or genotypic frequencies to detect patterns of kinship or spatial genetic structure among *a priori* defined populations (i.e. locations). The underlying premise is: low connectivity between populations results in restricted gene flow, and due mainly to random genetic drift, lead to genotypic differentiation and substantial structure (Bohonak, 1999; Hedgecock *et al*., 2007). In contrast, high connectivity facilitates regular gene flow which limits the build-up of genetic differences and results in little or no genetic differentiation or structure. These approaches commonly employ Wright's *F*-statistics (Wright, 1965) where F_{ST} or several of its derivatives (i.e. R_{ST}), and population genetic distances such as Nei's *D* are the most commonly reported measures or indices of genetic differentiation.

Indirect methods

The most commonly used measure, F_{ST}, is derived from the Island Model (Wright, 1943) which is based on several formatted assumptions including genetic equilibrium unlikely to be realized, and must thus be interpreted with caution, especially in scenarios characterized by high gene flow (Lowe & Allendorf, 2010). Another limitation of these traditional approaches is the *a priori* grouping of individuals—the placing of

F_{ST}

individuals into groups before differentiation can be assessed. Pre-groupings are usually based on morphological differences, habitat characteristics or sampling locations. These criteria, however, are not always a good reflection of the genetic structure and may result in unwanted biases and circular reasoning as individuals collected from specific locations may have come from elsewhere (Mank & Avise, 2004). In these circumstances, admixed or hybrid individuals may not be detected. Fortunately, many of the inherent limitations of these population models as well as *a priori* biases can be eliminated by the use of Bayesian clustering algorithms which are now widely used in population genetic studies (Beaumont & Rannala, 2004; Mank & Avise, 2004).

Isolation by distance

Additionally, more appropriate models such as the isolation by distance model (IBD), which describes the increase in genetic differentiation at neutral loci with increasing geographic distance, is growing in popularity (Purcell *et al*., 2009; Puebla *et al*., 2012). Despite these improvements however, indirect methods ultimately characterize evolutionary connectivity and are unable to differentiate between historical and contemporary gene flow. When focusing on ecological connectivity, direct genetic approaches such as parentage analysis and assignment or exclusion tests are more appropriate (Hedgecock *et al*., 2007).

Direct methods

Direct methods, which seek to match up parent-offspring pairs (parentage analysis) or to assign individuals to their population of origin (assignment tests), are the most reliable genetic methods that can be used to obtain quantitative estimates of ecological connectivity. Parentage analysis was used to provide the first direct estimates of connectivity for a marine fish in a proposed network of marine reserves. In that study, which was conducted at Kimbe Bay, Papua New Guinea, 40% of the orange clownfish (*Amphiprion percula*) settling into anemones in an island MPA were derived from parents resident in the reserve. Juveniles spawned by individuals resident on Kimbe Island were detected as far as 35 km away and accounted for up to 10% of the recruitment in the adjacent MPAs (Planes *et al*., 2009). Larval dispersal distances using microsatellite DNA parentage analysis have subsequently been determined for *Amphiprion*

Parentage analysis

ocellaris and *Amphiprion perideraion* on Indonesian reefs (Madduppa *et al*., 2014), *Amphiprion polymnus* in Papua New Guinea (Saenz-Agudelo *et al*., 2009), *Zebrasoma flavescens* in Hawaii (Christie *et al*., 2010b), *Stegastes partitus* in the Bahamas (Christie *et al*., 2010a) and *Tripterygion delasi* in the Mediterranean (Schunter *et al*., 2014). A major limitation of this method, however, is that a large proportion of the parental population must be sampled and genetically characterized. This type of extensive sampling is difficult for most reef fish species and thus, this method is often applied only to small-scale studies or to species that exhibit limited/specialized habitat requirements where sampling a large part of the parent population is possible (Kane & King, 2009; Planes *et al*., 2009; Saenz-Agudelo *et al*., 2009; Leis *et al*., 2011).

Assignment tests

Requiring less intensive sampling than parentage analysis are assignment tests involving the assignment of larvae or recruits to a source population (Manel *et al*., 2005) based on shared genotypic profiles between the larvae and the parental population. Early assignment tests utilized maximum likelihood analyses; however, present day assignment tests utilize Baysian analyses (Rannala & Mountain, 1997; Paetkau *et al*., 2004; Piry *et al*., 2004).

Parentage analysis vs assignment tests

In high gene flow scenarios parentage analysis appears to be more accurate than assignment tests at estimating connectivity (Saenz-Agudelo et al., 2009). For most marine species which tend to exhibit low FST values indicative of high connectivity, parentage analysis, despite its limitations, might be the only suitable method.

SNPs – single nucleotide polymorphisms

Despite being the marker of choice for population studies within the last decade, microsatellites possess some characteristics that confound their use in many of the commonly used analyses such as fixation indices (F_{ST}) and Bayesian clustering (Putman & Carbone, 2014). Some of their inherent limitations include: complex and variable mutation patterns, high genotyping error rates and low genomic density (DeFaveri *et al*., 2013). In recent years, SNPs—single nucleotide polymorphisms—have been increasing in popularity as a marker in many applications of molecular ecology, including population genetics. Highly desirable properties of

SNPs include: high abundance throughout the genome, simplicity (i.e. they are bi-allelic) which allows for high-throughput screening, low genotyping error, low mutation rates, and high transferability between laboratories in that data analysis and interpretation can be automated and standardized (Liu *et al*., 2005; Anderson & Garza, 2006). The application of SNPs to population genetics in the marine environment is still relatively new and thus for now, the general consensus is that both markers (SNPs and microsatellites) have strengths and weaknesses; hence, determining which one is most suitable needs to be assessed on a study-by-study basis taking into consideration characteristics of the populations being studied, as well as logistics such as cost. Given the general advantages of SNPs however, and the fact that studies have shown that the biallelic nature of SNPs which may limit their resolving power in some applications (i.e. parentage and kinship analyses) in comparison to multiallelic microsatellites can be overcome by larger marker sets (Hauser *et al*., 2011), the use of SNPs is expected to increase in the future (Putman & Carbone, 2014). With the focus on investigating fine scale patterns of larval dispersal, SNPs have been applied to kinship analyses of *T. delaisi* in the Mediterranean (Schunter *et al.*, 2014). More details of this study are given in Section 1.9.5

Genetic reviews

Technical and/or methodological reviews of one or more of the methods described in this section are given by the following authors: Campana (1999), Hellberg *et al*. (2002), Thorrold *et al*. (2002), Irisson *et al*. (2004), Thorrold *et al*., (2006), Waples and Gaggiotti (2006), Hedgecock *et al*. (2007), Thorrold *et al*. (2007), Werner *et al*. (2007), Selkoe *et al*. (2008), Saenz-Agudelo *et al*. (2009), Jones *et al*. (2010), Lowe and Allendorf (2010) and Leis *et al*. (2011).

1.5.4 Combined approaches

Combined approaches

Given the complexity of marine connectivity, the best approach would be to use two or more methods in combination (Leis *et al*., 2011) so that the results of one method may be validated by another. This approach is especially useful when attempting to decipher patterns of demographic connectivity using population genetics, since the exchange of a few

individuals per generation can homogenize genetic structure but be of no consequence to demographic connectivity (Waples & Gaggiotti, 2006). The few studies that have utilized a combination of approaches have found it to be advantageous, providing a more comprehensive description of connectivity. For example, Jones *et al*. (2005) used both otolith tagging and DNA parentage analysis to examine connectivity patterns of the clownfish *Amphiprion polymnus* off Papua New Guinea. Although there were some discrepancies in the actual self-recruitment rates obtained by both methods, in general, the results of both methods were in agreement and the use of these two methods in combination served to provide important insights into some of the ecological processes that might have been affecting connectivity. These processes would have not been detected otherwise. Similarly, matching results were obtained for connectivity studies of another clownfish, *Amphiprion percula* of Papua New Guinea, using transgenerational otolith marking with stable isotope enrichment (Almany *et al*., 2007) and DNA parentage analysis (Planes *et al*., 2009).

Other combinations of methods that have been utilized include otolith chemistry and population genetics (Bradbury *et al*., 2008; Liu *et al*., 2010; Papetti *et al*., 2013; McKeown *et al*., 2015), and physical modelling and genetics (Gerlach *et al*., 2007).

1.5.5 Summary and future approaches

Future approaches

A number of direct and indirect methods/techniques are available to assess connectivity in the marine environment. Each method has its own pros and cons, hence some methods are more popular than others. It is important to know exactly what scale is being measured, what each method measures, and its limitations. Given the complexity of marine connectivity, it is foreseen that the use of multiple methods in combination will become more prevalent (Hedgecock *et al*., 2007; Leis *et al*., 2011).

1.6 Population structure of temperate reef fish

In general, the use of genetic methods to assess population connectivity patterns of Perciform reef fish have been more widely applied

to tropical coral reef fish than to temperate rocky reef species. Apart from the studies conducted on *Tripterygion delaisi*, which are discussed in detail in the latter parts of this Chapter (Section 1.9.5) some of the studies using microsatellites to investigate population connectivity in temperate rocky reef species and the main conclusions drawn are listed below:

Population genetics in a temperate environment

(1) **Curley and Gillings (2007)** – Population genetic structure of the damselfish, *Parma microlepis* was investigated across multiple spatial scales along a 400 km stretch of coast off New South Wales (NSW), Australia, using seven microsatellites This species has short PLD of 2–4 weeks, the production of benthic eggs and low adult mobility, and thus was expected to exhibit spatial genetic structure and significant isolation by distance. Sampling involved the collection of 336 adults using a hierarchical design of regions across multiple spatial scales (from regions 70–80 km apart to max resolution 1–2 km between sites) (3 per study area; 70–80 km apart), locations (4 per region; 10–15 km apart) and sites (2 per location; 1–2 km apart). Across all spatial scales, results indicated that there was widespread genetic homogeneity and an absence of isolation by distance. It was hypothesized that factors influencing pre-settlement dispersal i.e. oceanographic processes in the form of the EAC (East Australian current), and habitat continuity, could be the drivers of significant gene flow in this species.

Parma microlepis

(2) **Siegle *et al*. (2013)** – The genetic structure of the yelloweye rockfish (*Sebastes ruberrimus*) was examined in the northeast Pacific to determine whether oceanographic features were functioning as barriers to dispersal. Previous studies on rockfishes have shown that despite an extended PLD, many species exhibit population structure over regional scales which can be attributed to a number of factors including the absence of appropriate habitat for settlement, and oceanographic features/processes. In this study, individuals were sampled from 13 sample locations during the period 1998–2006, and screened at 9 microsatellite loci. Results indicated that there was subtle genetic structure between the Strait of Georgia and the "outer coast"; thus, the Juan de Fuca Strait was acting as a barrier, limiting dispersal. Isolation by distance was not detected along the

Sebastes ruberrimus

"outer coast", and the Bowie Seamount sample was not genetically differentiated from the "outer coast" locations which were panmictic. These results have implications for the management and conservation of this species as the restricted dispersal means that it is unlikely that the outer coast locations would function as substantial larval sources for the inshore populations.

(3) **Knutsen *et al.* (2013)** – The spatial genetic structure of the Corkwing Wrasse (*Symphodus melops*), was investigated over its entire geographic distribution which presently range from Portugal to Norway. This species is suspected of experiencing a northward shift in its range, characterized by near extinction in the Mediterranean (the southern end of its distribution), and an increase in abundance in the northern areas of its distribution. Adults collected along the coasts of Norway, Sweden, the UK, Spain and Portugal were screened using nine microsatellite loci. Findings support a major genetic discontinuity/break between the Scandinavian and the Atlantic (i.e. southern) samples indicating a lack of present gene flow across the North Sea. This lack of gene flow was attributed to habitat discontinuity, and the life history characteristic of a short pelagic larval phase which limits dispersal by ocean currents.

Symphodus melops

(4) **González-Wanguermert *et al.* (2010)** – In this study, two markers, mt DNA and microsatellites, were used to examine the role of oceanographic processes in determining connectivity between continental and insular populations of the white seabream *Diplodus sargus* in the North East Atlantic (NEA) and Mediterranean. Samples were collected over the entire study area. DNA extracted from tissue was screened across eight microsatellite loci. Results showed that the Azores population was genetically differentiated from the rest of the samples. Apart from historical factors, this genetic differentiation was attributed to the breakdown of genetic exchange due to the occurrence of deep water, and isolating currents (hydrodynamic processes) acting as a barrier to dispersal. The lack of genetic differentiation between the Mediterranean and continental samples was attributed to hydrodynamic processes promoting maximal larval dispersal between these two areas resulting in high gene

Diplodus sargus

flow.

1.7 Factors affecting larval dispersal and connectivity

Larval dispersal is an extremely complex biophysical process—it is influenced by multiple abiotic and biotic factors which function and interact in different ways on multiple temporal and spatial scales. How these different factors interact to influence connectivity is still relatively unknown. However, given the need for connectivity estimates, several factors have been identified as possible predictors of dispersal and connectivity. Factors commonly cited include:

(1) **Oceanographic processes** such as circulation patterns, upwelling, downwelling, upwelling shadows, wind, tides, currents, internal waves, fonts and eddies (Largier, 2003; Pineda & Hare, 2007; Galarza *et al*., 2009; Selkoe *et al*., 2010; White *et al*., 2010).

Oceanographic processes

(2) **Habitat characteristics** such as bottom topography, depth, distance from shore, degree of isolation and coastline orientation (Largier, 2003; Roughan, 2005; Pineda & Hare, 2007).

Habitat characteristics

(3) **Life history traits** such as migration patterns, temporal and spatial spawning patterns, reproductive strategies, egg type, type of spawning, fecundity, and maternal condition (Bradbury *et al*., 2008; Riginos *et al*., 2011)

Life history traits

(4) **Larval behaviour** such as vertical migration, swimming and orientation capabilities, visual, olfactory, and aural sensory abilities to detect and differentiate cues as well as to react to them (Tolimieri *et al*., 2000; Atema *et al*., 2002; Clark *et al*., 2005; Gerlach *et al*., 2007; Leis *et al*., 2007b; Paris *et al*., 2013).

Larval behaviour

(5) **Early life history traits** (ELHTs) such as size and degree of development at hatching and/or settlement, pelagic larval duration, growth rates, larval condition and mortality rates (Shima & Findlay, 2002; Macpherson & Raventós, 2005; Galarza *et al*., 2009; Shima & Swearer, 2010). Early life –history traits will be examined in this thesis hence more details are given below.

Early life history traits (ELHTs)

In organisms with multiple life-stages such as benthic fish with a

pelagic larval stage, early life history traits (ELHTs), particularly growth related traits, may influence dispersal and connectivity via their effects on larval survival as well as settlement and post settlement processes (Shima & Findlay, 2002; Macpherson & Raventós 2006; Gagliano *et al*., 2007; Hamilton *et al*., 2008; Shima & Swearer, 2010; Sponaugle, 2010). The relationship between these growth related traits, and settlement or recruitment success can be broadly summarized by the "growth-mortality" hypothesis, which predicts a survival advantage for larger and faster growing individuals (Anderson, 1988). In terms of specific traits, larger size-at-hatching, faster larval growth, shorter stage duration and larger size-at-settlement generally enhance post-settlement fitness and survival (Searcy & Sponaugle, 2001; Vigliola & Meekan, 2002; Macpherson & Raventós, 2005). The pelagic larval duration which is often used as a proxy for larval dispersal and connectivity is described in more detail.

Pelagic larval duration (PLD)

The time that pelagic larvae spend in the water column before settling onto the reef is referred to as the pelagic larval duration (PLD). PLD is taxon specific and highly variable even within species, ranging from days to weeks to months (Victor, 1986; Raventós & Macpherson, 2001; Beldade *et al*., 2007). Due to the early belief that marine larvae were passive dispersers and that PLD could potentially be a good proxy for realized dispersal, the relationship between PLD and connectivity has been investigated extensively, either directly through correlations with dispersal distances (Shanks *et al*., 2003; Shanks, 2009) or indirectly by correlations with genetic structure (Doherty *et al*., 1995; Riginos & Victor, 2001; Bay *et al*., 2006; Bradbury *et al*., 2008; Galarza *et al*., 2009; Weersing & Toonen, 2009). Under the traditional view of passive dispersal and demographically open populations, the expected relationship is that as PLD increases, dispersal distance should increase facilitating greater gene flow (and connectivity) and hence less genetic structure. Studies to date, however, have yielded variable and in some cases contrasting results. Some of these studies are outlined below.

Two meta-analyses investigating the relationship between pelagic duration and observed dispersal distance for the larvae and plant propagules of species belonging to a range of taxa, from both tropical and temperate marine environments, found that although there were exceptions, PLD could indeed be a crude indicator of dispersal distance. Overall, however, realized dispersal distances were generally shorter than those predicted from passive dispersal as well as predictions made from modelling, and based on patterns observed it was concluded that larval behaviour could also be influencing dispersal distances (Shanks *et al*., 2003; Shanks, 2009). In terms of correlation with genetic structure, in a comparison of three blennioids with varying life history characteristics including a PLD range of 28–50 days, it was found that PLD may be a good predictor of genetic structure (Riginos & Victor, 2001). Numerous studies, which show that marine species that exhibit direct development as opposed to planktonic larval stages display high levels of genetic structure (Doherty *et al*., 1994; Arndt & Smith, 1998; Hoffman *et al*., 2005), also indirectly lend support to this negative relationship between PLD and connectivity.

PLD

Contrasting results are also well documented, as a number of population genetic studies have reported a poor correlation between PLD and genetic structure (Shulman & Bermingham, 1995; Bay *et al*., 2006; Galarza *et al*., 2009). A meta-analysis specifically aimed at assessing the correlation between PLD and population genetic estimates of connectivity in marine taxa revealed a low but significant negative correlation ($r^2 < 0.1$) between average PLD and genetic structure represented by F_{ST} values. When non-pelagic species (species exhibiting direct development) were removed from the analyses, however, the relationship became non-significant ($p = 0.053$) (Weersing & Toonen, 2009). A similar finding was obtained when the relationship between PLD and genetic structure was examined for 8 species of fish belonging to the family Pomacentridae. A relationship between PLD and genetic structure was detected; however, when the single species exhibiting direct development was excluded this relationship was lost, even though PLD varied between 11–28 days among

the 7 remaining species (Bay *et al*., 2006).

In summary, although PLD continues to be widely regarded as a proxy for connectivity in the absence of direct estimates, this relationship is not as straight forward as originally thought and must be treated with caution as other factors, both abiotic and biotic, may come into play.

1.8 Tropical vs. temperate comparison of larval dispersal and connectivity

The scale of larval dispersal and connectivity can vary between temperate and tropical locations, as factors such as PLD, spawning mode, larval behaviour, and physical oceanographic processes can exhibit latitudinal differences (Bradbury *et al*., 2008; Leis *et al*., 2013). Temperature is often regarded as one of the main drivers of this variability, and theoretical assumptions together with a few influential studies with limited empirical data have led to the general conclusion that the scale of larval dispersal is wider at higher latitudes (Houde, 1989; O'Connor *et al*., 2007; Bradbury *et al*., 2008). Such a general conclusion is questionable as comparisons are often confounded by a paucity of empirical data, sampling biases, taxonomic differences, and the interaction between factors. Leis *et al*. (2013) reviewed some of the commonly proposed hypotheses of latitudinal differences in fish larval dispersal, their underlying assumptions, and supporting evidence. Guided by this review, presented below is a summary of some of the common factors that may influence fish larval dispersal, how these factors may vary between temperate and tropical regions, and their possible influence on the scale of larval dispersal and connectivity.

Latitudinal differences in connectivity

(1) **Pelagic Larval Duration**: PLD, is often regarded as a proxy for larval dispersal distance as explained above, i.e. as PLD increases larval dispersal distance should increase. With respect to differences between tropical and temperate areas, the expectation is—the larvae of tropical species will have a lower dispersal potential due to the effects of warmer temperatures on physiological processes leading to faster development and shorter PLDs (Houde, 1989; O'Connor *et al*., 2007). Although there is

PLD

some evidence indicating that the orders and sub-orders that are dominant in warm waters have shorter mean PLDs than the dominant cold water taxa, drawing definitive conclusions are difficult due to biases in the data (i.e. limited numbers of taxa sampled in both warm and cold waters, habitat type sampled in tropics dominated by shallow reefs) (Leis *et al*., 2013). Additionally, an examination of PLD data for differences between latitudes shows that, in some cases, regional variation in PLD was greater than differences between warm temperate and tropical locations for nearshore demersal species (Leis *et al*., 2013).

(2) **Spawning mode/egg type**: The type of egg spawned (i.e. pelagic vs. demersal) may influence dispersal distance (Bradbury *et al*., 2008; Riginos *et al*., 2011). In pelagic eggs, dispersal in the marine environment during the pre-hatching period can be many weeks long resulting in a high dispersal potential (Pauly & Pullin, 1988). The larvae from taxa that spawn demersal eggs have been shown to hatch at larger sizes and in a more developed state than those hatched from pelagic eggs (Thresher, 1984; Blaxter, 1986; Miller *et al*., 1988). These more developed larvae may possess sensory and swimming abilities that might allow them to actively influence their dispersal rather than exhibit passive dispersal, thus resulting in shorter realized dispersal distances (Leis, 2006). Additionally, larvae hatching from demersal eggs tend to spend less time in the pelagic stage and exhibit smaller scales of genetic connectivity, two factors which are commonly regarded as proxies for dispersal distances.

Spawning mode

There are marked latitudinal differences in spawning mode among taxa. The percentage of demersal nearshore species that spawn pelagic eggs is much greater (60–80%) in warmer areas compared to colder areas (15–27%) (Leis *et al*., 2013). Spawning mode is highly taxon specific with most species within a family exhibiting similar modes; when there are exceptions, the trend is for taxa from colder waters to move away from pelagic broadcast spawning. The implication for the scale of larval dispersal and connectivity is that the average scale of larval dispersal at higher latitudes (greater % demersal spawners) will be shorter compared to the lower latitudes (greater % broadcast spawners) (Leis *et al*., 2013).

(3) **Physical oceanographic** differences can exist between tropical and temperate environments and several hypotheses (listed below) were developed by Leis *et al*. (2013) with regards to their potential effects on larval dispersal and connectivity.

Oceanographic processes

Water movement varies with latitude, partly due to variability in Coriolis force and as a result it can be hypothesized that Ekman coastal upwellings should have least importance at low latitudes leading to less upwelling retention in the tropics. In contrast, more energetic eddies should form in colder waters, and these can either transport larvae away from their source, or retain them locally, resulting in more variable larval dispersal patterns. The mixed layer depth (MLD) can also influence dispersal: shallow MLDs may allow larvae to vertically migrate to slower waters below and thus avoid or retard dispersal. This is more probable in colder waters which exhibit seasonal variability in the depth of these layers leading to differences in dispersal between seasons.

Overall, there is a general paucity of information in the published literature to adequately assess the hypotheses proposed above. What the little available information indicates is that although variables that drive coastal circulation exhibit clear latitudinal differences, local factors (topography, coastal morphology, tidal regimes, and riverine influences) may result in significant regional and local differences which may confound or obscure these larger latitudinal differences (Leis *et al*., 2013).

(4) **Larval behaviour**: It is now widely accepted that larval behaviour, including swimming, can influence the scale of larval dispersal and connectivity (Leis, 2010). Numerous studies have shown that many larvae: (1) exhibit vertical migration which may allow them to alter their horizontal position in the water and hence ultimately influence their dispersal (Paris & Cowen, 2004; Leis *et al*., 2007b) and (2) possess swimming capabilities (speed, endurance and orientation) which are more than sufficient to actively influence their dispersal (Leis & Carson-Ewart, 1997; Stobutzki & Bellwood, 1997; Fisher *et al*., 2000; Fisher, 2005; Leis *et al*., 2007a).

Larval behaviour

With regards to latitude, temperature differences may result in

hydrodynamic and physiological influences with warmer waters ultimately supporting faster swimming (Fuiman & Batty, 1997). Small larvae may have to expend greater swimming effort in colder waters which have a higher viscosity compared to warmer waters. Whilst this effect will be less on larger larvae, there may still be some effects in the form of reduced metabolic rates and the inhibition of motor activity associated with swimming in colder waters (Hunt von Herbing, 2002).

There is some evidence in support of these hypotheses as some laboratory studies have shown that the larvae of some species swim faster at higher temperatures. Laboratory studies measuring critical speed at ambient temperatures provide much of the empirical data for latitudinal comparisons of swimming speed (Leis, 2010). These studies show that, regardless of size: (1) there is little difference in critical speed between tropical and warm temperate species, (2) cold water species exhibit speeds that are only 20–50% that of warm water species and (3) ontogenetic increases in speed are slower for cold water species compared to warm water species. This is in contrast to *in situ* studies which show that regardless of size, warm temperate larvae are slower than tropical species (Leis, 2010).

Overall, the main supported latitudinal pattern in terms of fish larval swimming speed is that once size biases are corrected, species belonging to tropical warm water and warm temperate species have similar critical speeds which are greater those of cold temperate species (Leis *et al*., 2013). Limited evidence also indicates that larvae in warm water environments not only swim faster, but also develop this ability at an earlier stage in ontogenetic development compared to cold water environments. If these larvae use this ability to restrict dispersal or to favor retention then this would lead to a decrease in the spatial scale of dispersal in warmer waters (Leis *et al*., 2013). However, genetic data are not showing more structures in warmer waters suggesting that habitat fragmentation may play a key role Nevertheless, more studies are needed in order to better understand these differences, in particular with species from similar taxonomic groups and considering life history aspects.

(5) **Habitat fragmentation**: Demersal fishes usually exhibit some level of habitat association and since larvae cannot settle where suitable habitat is unavailable, habitat continuity/discontinuity may influence the scale of connectivity (Pinsky *et al*., 2012). With regards to temperate vs tropical comparisons, islands more than 5 km apart are two to three times more abundant in the tropics than in higher latitudes (Leis *et al*., 2013). As opposed to continental margins which provide large areas of continuous habitat for nearshore demersal fishes, islands may provide some degree of isolation or discontinuity depending on the distance to nearby islands or continents (Mora *et al*., 2012) resulting in more fragmented populations and shorter successful dispersal distances.

Habitat fragmentation

Based on a review of the existing literature, Leis *et al*. (2013) found that the percentage of larvae settling in their spawning location (self-recruitment) was higher along continental coastlines compared to islands. When biases caused by the spatial extent of the study were excluded, species in patchy habitats were found to disperse approximately 60–100 km in contrast to species in continuous habitats which dispersed approximately 900 km.

Given the proportion of island habitats in the tropics compared to the temperate areas it is expected that the scales of dispersal and connectivity will be greater in temperate areas compared to tropical areas. This generalization must be treated with caution, however, as other factors such as oceanographic processes and larval behaviour may affect the influence of habitat fragmentation.

In summary, drawing definitive conclusions with regards to tropical vs. temperate comparisons of the scale of larval dispersal and connectivity, is difficult if not impossible, based on the literature published to date. Meaningful comparisons are hindered by a lack of data, sampling biases, taxonomic differences, and the interaction of numerous factors. Theoretical predictions, though plausible, are only partially supported by empirical data; thus, there is an overall need for more studies across latitudinal ranges, with biophysical modelling assuming a greater role given the interaction of numerous factors (Leis *et al*., 2013). Understanding

Summary and recommendations

latitudinal variations in connectivity patterns has assumed particular importance within recent times given the need to predict climate driven changes to marine ecosystems.

T. delaisi is distributed in both the Mediteranean and Atlantic. Temperature differences between these two areas may drive differences in early life history traits which may influence connectivity patterns. Thus, studies on *T. delaisi* may assist in understanding latitudinal differences in connectivity by providing information on temperature effects on early life history traits.

PART B – MODEL SPECIES

1.9 *Tripterygion delaisi* (Cadenat & Blache, 1970)

Tripterygion delaisi - A model species

Classified as a temperate reef species, *Tripterygion delaisi* (*T. delaisi*) is distributed in both the Atlantic and Mediterranean, being very abundant in nearshore reef habitats. These two areas exhibit subtle differences in water temperature throughout the year and thus variability in some early life traits can be expected. Additionally, this species is already considered a model species for connectivity studies in general due to several life history characteristics such as the production of benthic eggs, an inshore larval distribution, limited adult mobility and a short PLD, which suggest that it has low dispersal capabilities. A detailed description of this species in terms of distribution, ecology, behaviour, general life-history characteristics and previous studies is given in the following sections.

1.9.1 Classification and distribution

Classification

Tripterygion delaisi (*T. delaisi*), (Cadenat & Blache, 1970) belongs to the blennioid family Tripterygiidae which comprises some 171 species belonging to 29 genera (Froese & Pauly, 2014). The genus *Tripterygion* (Risso, 1826) itself comprises just 4 species distributed only in the eastern Atlantic and Mediterranean (Zander, 1986; Wirtz, 1990; Carreras-Carbonell *et al.*, 2007b). *Tripterygion melanurus* (*T. melanurus*),

Tripterygion tripteronotus (*T. tripteronotus*), and *Tripterygion tartessicum* (*T. tartessicum*) (Carreras-Carbonell *et al*., 2007b) are endemic to the Mediterranean (Wirtz, 1980; Zander, 1986). Geographically, the distribution of *T. delaisi* appears to be disjointed occurring in a southern area from western Africa north to Senegal and the Macaronesia islands, and a northern area which includes the Mediterranean Sea and the adjacent north-eastern Atlantic waters south to Morocco, and north to the British Isles (Zander, 1986).

Distribution

Initially, three *Tripterygion* species were described for the Mediterranean (*T. tripteronotus*, *Tripterygion xanthosoma* and *T. melanurus*), and two for the eastern Atlantic (*T. delaisi* and *Tripterygion atlanticus*). The genus was subsequently revised by Wirtz (1980) to the three recognized species, *T. tripteronotus*, *T. melanurus* and *T. delaisi*. More recently, Carreras-Carbonell *et al*. (2005, 2007b) found that *T. tripteronotus* exhibited two well differentiated clades which pointed to the existence of two different species which were disjunct in their geographical distribution. *T. tripteronotus* is found only in the northern Mediterranean basin, whilst *T. tartessicum* is distributed in the south (Carreras-Carbonell *et al*., 2007b). It is hypothesized that the low larval and adult dispersal capabilities of *Tripterygion* species (Heymer, 1977; Wirtz, 1980; Sabatés *et al*., 2003; Carreras-Carbonell *et al*., 2006), in addition to the circulation regime separating the northern and southern basins, may be responsible for maintaining the disjunct distribution of the two species in this area.

Speciation in the Mediterranean

T. delaisi sub-species

Two subspecies of *T. delaisi* have been validated, *T. delaisi xanthosoma* and *T. delaisi delaisi* (Carreras-Carbonell *et al*., 2005), with the phylogeographical break between subspecies most likely occurring between the Atlantic islands and the Atlantic coast of Europe and the Mediterranean (Carreras-Carbonell *et al*., 2005; Domingues *et al*., 2007). Morphological differences between subspecies are difficult to detect but possible when a large number of individuals are examined (Wirtz, 1980). Differentiation is also possible during courtship as *T. delaisi delaisi* males swim upward towards the surface in the form of a “figure-of-eight” while *T. delaisi xanthosoma* do this only on the bottom (Zander, 1986).

1.9.2 Ecology and behaviour

T. delaisi is a short-lived (maximum 3 years old) species, reaching sexual maturity at the end of its first year (Wirtz, 1978). Adults feed mainly on small crustaceans such as amphipods and copepods (De Jonge & Videler, 1989; Velasco *et al*., 2010). Throughout its range, *T. delaisi* is abundant in nearshore rocky reef habitats (La Mesa *et al*., 2006; Bertoncini *et al*., 2010). In the Mediterranean where it is sympatric with the three other *Tripterygion* species, it exhibits niche differentiation occupying biotopes with reduced light at depths of 3–40 m. The three other species are found at shallower depths (0–18 m) (Wirtz, 1978; Carreras-Carbonell *et al*., 2007b). Adults, which are highly territorial with high levels of homing behaviour, are incapable of swimming even short distances (tens of metres) in open water or on sandy bottoms (Heymer, 1977; Wirtz, 1978).

Longevity

Feeding

In the Mediterranean, spawning activity has been recorded during the period March to June (De Jonge & Videler, 1989). In the Atlantic the spawning season seems to be more prolonged (April–September) as evident by the occurrence of reproductive males and preflexion larvae (*pers. observation*). During the reproductive season, territorial males, which have a characteristic black head and yellow body, exhibit parental care of the benthic eggs in a nest surrounded by algae and sponges (De Jonge & Videler, 1989; Gonçalves & Almada, 1998). A detailed description of reproductive biology and spawning behaviour is provided by de Jonge and Videler, 1989. However, no studies on egg and larval development exist.

Habitat

Adult mobility

Spawning activity

Throughout its range, the larvae of *T. delaisi* are found almost exclusively in coastal waters usually between the coastline and 100 m offshore (Sabatés *et al*., 2003; Beldade *et al*., 2006; Borges *et al*., 2007b). Several studies focusing on the composition and distribution (vertical and horizontal) of larval fish assemblages in the study area, the Arrábida Marine Park, indicate that *T. delaisi* larvae are abundant exclusively in the nearshore environment, where all the developmental stages (preflexion, flexion and postflexion) can occur (Beldade *et al*., 2006; Borges *et al*., 2007a; Borges *et al*., 2007b; Borges *et al*., 2009).

Larval abundance and distribution

1.9.3 Otolith structure and morphology

Otolith microstructure

In assessing the otoliths of *T. delaisi* for microstructure analysis, Raventós and Macpherson (2001) found the lapillar otoliths on juveniles to be more suitable for age estimations as they exhibited better increment clarity and definition and had less change in the plane of growth compared to the sagittae. No sub-daily increments were identified on these lapillar otoliths and the settlement mark was characterized as Type Ia according to the classification scheme of Wilson and McCormick (1999) (Raventós and Macpherson, 2001).

1.9.4 Pelagic larval duration

PLD estimates

Pelagic larval duration (PLD) estimates for this species have been documented in two studies focusing on littoral species in the Mediterranean. In one study Raventós and Macpherson (2001) estimated the mean PLD of *T. delaisi* to be 17.7 days with a range of 17–18 days. Estimates were based on back-calculations from the settlement-mark on the lapillar otoliths of three post-settled individuals (< 43 mm in standard length). In a subsequent study (Macpherson & Raventós, 2006) which examined a greater number of individuals (N = 25), although the mean PLD was similar, there was an increase in the min-max range to 16–21 days. Similar mean PLDs and ranges were also obtained for the other two *Tripterygion* spp. examined.

1.9.5 Connectivity studies

Connectivity studies

Several connectivity studies using a variety of genetic methods including assignment tests, sibship analysis, and parentage analysis have been conducted for this species, mainly in Mediterranean waters. A brief overview of each of these studies is given below.

Population structure

(1) Carreras-Carbonell *et al*. (2006) examined the population structure of *T. delaisi* from 8 Mediterranean, and 2 Atlantic localities (Canary Islands and Azores Islands), using 10 highly polymorphic microsatellite loci. Atlantic specimens were characterized as belonging to the sub-species *T.*

delaisi delaisi, whilst Mediterranean specimens were assigned to *T. delaisi xanthosoma*. Significant findings in this study were:

- *T. delaisi* exhibited high genetic variability both in terms of (1) extensive polymorphism per population and locus and (2) high expected and observed heterozygosities.
- Using a Bayesian approach the two subspecies were clearly identified as two different evolutionary significant units.
- In *T. delaisi xanthosoma*, isolation by distance was detected between the eight analyzed populations, and six genetically homogeneous clusters were inferred by Bayesian analyses.
- Significant genetic differentiation was detected between two pairs of sites on a relatively small spatial scale. One pair, 144 km apart, was separated by deep water and the other pair, 163 km apart, was separated by a large stretch of sand. The authors hypothesized that these two features created habitat discontinuity acting as barriers to dispersal, resulting in restricted gene flow and population structure.

Isolation by distance

Significant genetic differentiation

High self-recruitment

(2) High self-recruitment rates have been observed for this species. Carreras-Carbonell *et al*. (2007) estimated self-recruitment in a population of *T. delaisi* in the North West Mediterranean over a 3-year period, using ten highly polymorphic microsatellite loci. New recruits of each year from the locality of Blanes, were compared with adult specimens previously collected from the same locality and from seven adjacent localities varying in distances between tens to hundreds of kilometres away. Results indicated that a mean of 66.4 ± 1.4% of the recruits settled in their natal population. When refined to a more local scale, 40.6 ± 8.9% of individuals self-recruiting to the Costa Brava population, which comprised the localities of Cap de Creus, Tossa and Blanes, self-recruited to Blanes. These results suggested that a high proportion of the larvae of *T. delaisi* remained close to, or never leave, their natal spawning area.

Parentage analysis

(3) More recently, Schunter *et al*. (2014) utilized two techniques, parentage analysis and sibship reconstruction analysis to investigate fine scale patterns of larval dispersal in this species along a 50 km stretch of coast in the Mediterranean. They found self-recruitment rates of 6.5%, and both

Bayesian analyses and pairwise F_{ST} values indicated that there was no population structure over this small spatial scale. The two techniques, however, revealed contrasting patterns of larval dispersal and connectivity. Sibship analyses detected recruitment at locations far away from natal sites while parentage analysis indicated that dispersal was more restricted (1.2 km) as larvae moved only a short distance along the coast.

Sibship analysis

Summary

Overall, these connectivity studies conducted on *T. delaisi* in the Mediterranean have yielded evidence of limited dispersal and connectivity in the form of high self-recruitment levels, genetic differentiation between populations, and isolation by distance (Carreras-Carbonell *et al*., 2006; Carreras-Carbonell *et al*., 2007; Schunter *et al*., 2014). The spatial scales examined vary from large (1500 km) to small (90 km stretch of coast), variable genetic markers were used (microsatellites and SNPs), and genetic techniques applied included assignment tests, sibship analysis and parentage analysis.

1.10 Study area

Our main study area is the Arrábida Marine Park (AMP) which is located on the west coast of Portugal between Sesimbra and Portinho da Arrábida—roughly between 9°00'15"–9°03'48"W and 38°26'–38°27'N [Figure 1.1]. The area is positioned in a southerly direction and is thus protected from the north and north-west winds by the adjacent mountain chain of Arrábida. Relatively calm sea conditions persist for much of the year allowing sampling of the very nearshore environment. Local oceanographic processes are dominated by tidal currents that run parallel to the shore, and the underwater rocky substratum created by boulders originating from the Arrábida mountain chain extend offshore for only about some tens of metres (and approximately 13 m in depth). The AMP is separated from the two nearest rocky reefs areas to the north and south, Cascais and Sines respectively, by sand. As such it can be considered as a continental island with rocky habitat. Together these three locations span approximately 100 km.

The Arrábida Marine Park

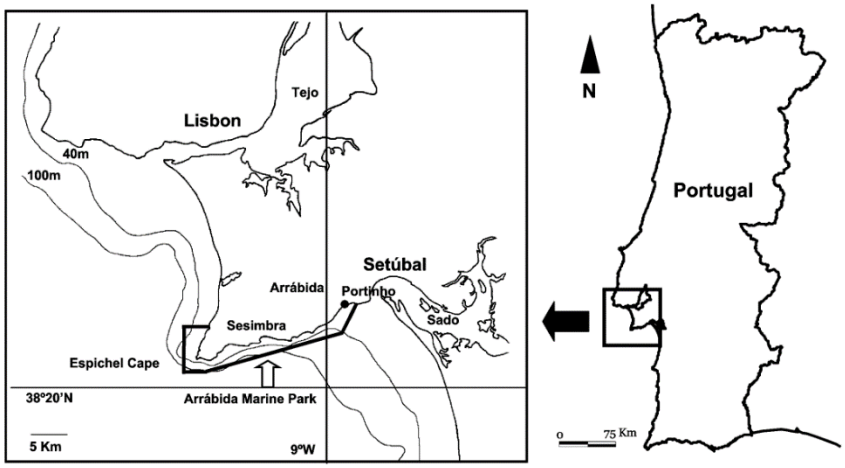

Figure 1.1 Map showing the location of the study area (Adopted from Borges *et al*., 2007).

PART C

DISSERTATION SYNOPSIS AND OUTLINE

1.11 Dissertation goals

The main goal of this dissertation is to provide insights into the connectivity patterns of the temperate reef fish, *Tripterygion delaisi*, along the west coast of Portugal. *T. delaisi* possesses several life-history characteristics (the production of benthic eggs, an inshore larval distribution, limited adult mobility and a short PLD) which suggest that it has low dispersal capabilities, and hence it is often used as a model species for connectivity studies. Previous connectivity studies conducted on this species in the Mediterranean have yielded evidence of limited dispersal and connectivity in the form of high self-recruitment levels, genetic differentiation between populations, and isolation by distance (Carreras-Carbonell *et al*., 2006; Carreras-Carbonell *et al*., 2007; Schunter *et al*., 2014). With the exception of one study which included two Atlantic island localities (Canary Islands and Azores Islands) (Carreras- Carbonell *et al*., 2006), the focus to date has been on the Mediterranean. There are no connectivity studies on this species in the Eastern Atlantic where its distribution spans the entire length of the Portuguese west coast.

High self-recruitment, IBD and genetic differentiation

The Arrábida Marine Park with its two nearby but discontinuous rocky shore habitats of Cascais and Sines, provides an ideal location to

study connectivity patterns of this species in Atlantic waters. In general, studies conducted on temperate rocky reef species are not as prevalent as studies on tropical coral reef species due to the inherent difficulties in sampling temperate nearshore rocky reef environments. In this area however, *T. delaisi* larvae belonging to all developmental stages are abundant in the nearshore environment (Beldade *et al*., 2006; Borges *et al*., 2007b) where relatively calm conditions allow larval sampling. Juveniles and adults are also abundant and accessible for both capture and *in situ* observations via SCUBA.

Tropical vs. temperate comparisons

Compared to the previous studies conducted in the Mediterranean, our connectivity study will differ in terms of spatial scale. Overall, the studies by Carreras-Carbonell *et al*. (2006, 2007) were conducted on a much larger scale (1500 km) whilst that by Schunter *et al*. (2014) was conducted on a much smaller scale (50 km). Our study area of 100 km, is similar to the distances between some of the localities sampled in Carreras-Carbonell *et al*. (2006), and the distance between Cascais and Arrábida and Arrábida and Sines (45 km and 70 km) is similar to the stretch of coast examined by Schunter *et al*. (2014). These similarities will allow for some comparisons between studies despite the differences in the overall spatial scale.

Connectivity patterns and the spatial scale of larval dispersal and connectivity will be inferred from (1) an investigation of population genetic structure between the AMP and the two nearest rocky reefs to the north and south, Cascais and Sines respectively (**Chapter 2**) and (2) larval spatial distribution patterns in the AMP (**Chapter 3**). Factors known to affect connectivity such as ontogenetic larval development (**Chapter 4**) and early life history traits such as PLD and larval growth (**Chapter 5**) will be examined. The results are discussed in terms of the potential impacts for dispersal and in relation to the connectivity patterns inferred from larval spatial distribution patterns and population genetic structure.

1.12 List of chapters

This dissertation comprises **6 chapters**:

Chapter 1: General introduction

In **Chapter 1** of this dissertation, a general introduction into the subject matter (**Part A**), a literature review of the model species *T. delaisi* (**Part B**), and a dissertation synopsis and outline (**Part C**) were presented above. *Chapter 1*

Chapter 2: Evidence of isolation by distance at micro-geographical scales in a temperate rocky reef fish, *Tripterygion delaisi*

An analysis of population genetic structure is often used to indirectly infer the extent larval dispersal and connectivity. In **Chapter 2** we examine the population structure of *T. delaisi* at a local scale (approximately 100 km) using 8 highly polymorphic microsatellite loci. Our sample sites are the Arrábida Marine Park and the two nearest rocky reef habitats to the north and south, Cascais and Sines respectively. In our study, a hierarchical sampling technique will allow us to look at population genetic structure at a local scale of about 100 km (the distance between the two furthest sites) and at a fine scale of between 2–5 km (the distance between two sites with a location). Unlike previous studies it will include all the different developmental stages (adults, juveniles and larvae) and will be conducted solely in Atlantic waters. Our results will be compared with previous studies and discussed in light of the numerous factors (physical and biological) that could be influencing genetic structure and connectivity in this species within the study area. *Chapter 2*

Chapter 3: Spatial and temporal distribution of *Tripterygion delaisi* larvae in the Arrábida Marine Park, Portugal

This chapter describes the spatial and temporal distribution of *T. delaisi* larvae at the AMP, our main sampling site. The distribution of reef fish larvae has been used to infer the scale of ecological connectivity in both temperate and tropical environments. Whilst some studies indicate *Chapter 3*

that the larvae of *T. delaisi* as well as other Tripterygiids may be restricted to an inshore larval distribution, distribution may be site specific as it can be influenced by environmental conditions as well as the physical characteristics of the study area

Chapter 4: Larval development and allometric growth of the black-faced blenny, *Tripterygion delaisi*

A knowledge of ontogenetic larval development may provide insights into the dispersal abilities of this species as it is linked to the development of early traits. The occurrence of all the larval developmental stages (yolk sac to postflexion) in the relatively calm nearshore environment of the AMP provides a unique opportunity to describe larval development for this species in its entirety. There are no previous descriptions of larval stages for this species, so a detailed description will also allow for the accurate identification of the larval stages of this species which is a key prerequisite for many ecological studies aimed at understanding early life history traits and the processes (such as connectivity) they influence.

Chapter 4

Chapter 5: Early life history characteristics of the black-faced blenny, *Tripterygion delaisi* inferred from otolith microstructure analyses

In organisms with multiple life-stages such as benthic fish with a pelagic larval stage, early life history traits (ELHTs), particularly growth related traits, may influence dispersal and connectivity via their effects on larval survival as well as settlement and post settlement processes. In this chapter, early life history characteristics of the black-faced blenny *Tripterygion delaisi* will be investigated from larval and early recruit samples. Microstructure analyses of the sagittal otoliths are used to determine larval and juvenile growth rates, hatching size, pelgic larval duration (PLD) and size-at-settlement (SAS). These results are discussed in terms of their implications for dispersal and connectivity.

Chapter 5

Chapter 6: General discussion

Chapter 6

The main findings of the previous chapters are integrated in this section. The connectivity patterns detected from population genetics structure are discussed in light of the larval distribution patterns and early life history traits determined for this species.

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CHAPTER 2: EVIDENCE OF ISOLATION BY DISTANCE AT MICRO-GEOGRAPHICAL SCALES IN A TEMPERATE ROCKY REEF FISH, *TRIPTERYGION DELAISI*

Evidence of Isolation by Distance at Micro-geographical Scales in a Temperate Rocky Reef Fish, *Tripterygion delaisi*

F.N. Solomon[1,2], R. Borges[1,3], E.A. Serrão[1], S. Planes[2]

2.1 Abstract

Understanding population connectivity and the scale of larval dispersal, is fundamental for designing management and conservation plans for marine meta-population dynamics, fisheries, and biodiversity reserves. In this study, we investigated local-scale population genetic structure of the black-faced blenny (*Tripterygion delaisi*), along the coastlines adjacent to the Arrábida Marine Park, Portugal. Given the low dispersal capabilities of this species, and the physical characteristics of the study area, it was hypothesized that samples collected over a spatial scale of approximately 100 km would have exhibited detectable genetic structure. In 2012, adults were collected from several sites around the Arrábida Marine Park, and the two nearest rocky reefs to the north and south, Cascais and Sines respectively. In 2013, larvae and juveniles were collected from these same sites in order to test matches with the adults collected in 2012. Using 8 highly polymorphic microsatellites, we found that all pairwise F_{ST} values between adult samples were low and non-significant, suggesting genetic homogeneity throughout the study area. Within this scenario of high gene flow however, we demonstrated that (1) significant isolation by distance; (2) several genetic clusters identified by Structure; and (3) the clustering and separation of sites in multivariate analyses, all alluded to the possible occurrence of restricted dispersal and local scale genetic heterogeneity. When larval and juvenile samples were included in the analyses, the occurrence of some significant pairwise F_{ST} values, the detection of several genetic clusters, and the segregation of sites by the various methods, all indicated the occurrence of temporal genetic variability. Overall, our study detected restricted larval dispersal amidst high gene flow, illustrates the phenomenon of "chaotic genetic patchiness", and highlights the importance of sampling at multiple spatial and temporal scales when attempting to decipher connectivity patterns.

Keywords: Fish, *Tripterygion delaisi*, Portugal, Microsatellite, Isolation by distance, Chaotic genetic patchiness

2.2 Introduction

Connectivity can be defined as the extent to which discrete populations are linked by dispersal (Palumbi, 2003). In demersal reef fish, which usually exhibit limited adult home ranges, scales of connectivity become largely dependent upon the extent of dispersal during the pelagic larval phase (Cowen *et al.*, 2007; Cowen & Sponaugle, 2009). Due to the difficulties of tracking very minute organisms for extended periods of time (i.e. duration of the pelagic larval phase can reach up to several months) in open oceanic water, indirect methods including analyses of population genetic structure have been used to infer the extent of larval dispersal and connectivity (Thorrold *et al.*, 2002; Levin, 2006). The underlying premise is, high connectivity limits the build-up of genetic differences between populations resulting in genetic homogeneity whilst isolated populations build genetic differentiation and substantial structure resulting from limited connectivity (Bohonak, 1999; Hedgecock *et al.*, 2007). Although genetic studies have traditionally focused on evolutionary connectivity through the use of markers such as allozymes and mtDNA, the advent of microsatellites has stimulated an increase in the number of studies addressing ecological questions at contemporary time-scales, using direct parentage analysis or genetic assignment processes (Planes *et al.*, 2009; Saenz-Agudelo *et al.*, 2011; Berumen *et al.*, 2012; Madduppa *et al.*, 2014).

Studies conducted on medium (>300 km) to broad spatial scales (1000's km) are more prevalent in the literature as marine systems have traditionally been regarded as demographically open and connected (Caley *et al.*, 1996). Within recent times however, several studies using a variety of genetic approaches have shown that retention close to natal reefs is more recurrent than previously thought, and that the contribution of self-recruitment can be high in some reef fish species, resulting in population dynamics dependent more on local processes (Jones *et al.*, 1999; Swearer *et al.*, 1999; Jones *et al.*, 2005; Almany *et al.*, 2007; Carreras-Carbonell *et al.*, 2007; Planes *et al.*, 2009; Christie *et al.*, 2010; Berumen *et al.*, 2012; Hogan *et al.*, 2012; Madduppa *et al.*, 2014). Overall, larval dispersal capabilities are highly unpredictable and difficult to model since they depend on a large number of factors including life-history characteristics, larval behaviour, sensory abilities and oceanographic features such as currents, fonts and eddies (Shanks *et al.*, 2003; Gawarkiewicz *et al.*, 2007; Gerlach *et al.*, 2007; Leis, 2007; Leis *et al.*, 2007; Shanks, 2009; Leis *et al.*, 2011). In support of the changing paradigm of predominantly open populations to one of a range or continuum of connectivity facilitated by non-passive larval dispersal, a number of studies have documented genetic structure on relatively small spatial scales (<200 km) (Hoffman *et al.*, 2005; Miller-Sims *et al.*, 2008). This occurrence has been attributed to a number of factors,

including life history traits such as direct development or short PLD, egg spawning mode and egg characteristics, reproductive strategy, and limited adult mobility (Hoffman *et al.*, 2005; Miller-Sims *et al.*, 2008; Ciannelli *et al.*, 2010; Horne *et al.*, 2011; Hirase *et al.*, 2012; González-Wangüemert & Vergara-Chen, 2014); egg and larval retention in currents or eddies (Sponaugle *et al.*, 2002; Ciannelli *et al.*, 2010); environmental heterogeneity (González-Wangüemert & Vergara-Chen, 2014); habitat discontinuity (Johansson *et al.*, 2008) and larval behaviour (Gerlach *et al.*, 2007). Additionally, an increasing number of studies have been documenting random genetic structure within a range of a couple of hundred kilometres down to tens of kilometres (Li & Hedgecock, 1998; Planes & Lenfant, 2002; Selkoe *et al.*, 2006; Arnaud-Haond *et al.*, 2008; Iacchei *et al.*, 2013; Bentley *et al.*, 2014), and the concept of "chaotic genetic patchiness" (Johnson & Black, 1982) has been introduced to account for this unpatterned genetic heterogeneity with no clear driving process, at a fine spatial scale.

In this study, we used microsatellite markers to investigate local-scale population genetic structure of the black-faced blenny, *Tripterygion delaisi* along a small portion of the Portuguese west coast around the Arrábida Marine Park. Due to several life history characteristics (the production of benthic eggs, an inshore larval distribution, limited adult mobility and short PLD) which suggest that it may have low dispersal capabilities, this species has been the focus of several studies aimed at investigating population connectivity and the occurrence of self-recruitment and local retention (Carreras-Carbonell *et al.*, 2006, 2007; Galarza *et al.*, 2009; Schunter *et al.*, 2014). Given the life-history characteristics of our model species and the physical characteristics of the study area, we hypothesize that samples of *T. delaisi* collected over a spatial scale of approximately 100 km along the west coast of Portugal will exhibit limited dispersal and connectivity leading to detectable (significant) genetic structure. We sampled adults in several locations along 100 km of coastline, and juveniles and larvae around the same area to test matches between adults, larvae and juveniles, and to interpret these matches in terms of connectivity. Our hierarchical (nested) sampling protocol and the testing of the different stages also allowed us to investigate the occurrence of "chaotic genetic patchiness".

2.3 Methods

2.3.1 Model species

The black-faced blenny (*Tripterygion delaisi*) is a small demersal fish that inhabits nearshore rocky habitats at depths of approximately 3 to 12 m both in the Mediterranean and Atlantic (Zander, 1986; La Mesa *et al.*, 2004; Bertoncini *et al.*, 2010). Adults exhibit homing

behavior (Heymer, 1977), and during the reproductive season, territorial males, exhibiting a characteristic black head and yellow body, undertake parental care of benthic eggs in a nest surrounded by algae and sponges (De Jonge & Videler, 1989; Gonçalves & Almada, 1998). Two subspecies of *T. delaisi* have been validated, *T. d. xanthosoma* and *T. d. delaisi* , with the phylogeographical break between subspecies most likely occurring between the Atlantic islands and coast (of Europe), and the Mediterranean (Carreras-Carbonell *et al.*, 2005; Domingues *et al.*, 2007). Studies of adult and larval fish assemblages at the Arrábida Marine Park, Portugal, indicate that not only is *T. delaisi* abundant in this area but also that its larvae are restricted to the nearshore waters where it maybe completing its life-cycle locally. This is evidenced by the co-occurrence of multiple larval developmental stages during a prolonged spawning season from March to September each year (Beldade *et al.*, 2006; Borges *et al.*, 2007; Borges *et al.*, 2009).

2.3.2 Sampling

Our sample sites were at the Arrábida Marine Park and the two nearest rocky reef habitats to the north and south, Cascais and Sines respectively. These three locations, Cascais, Arrábida and Sines, span a total distance of approximately 100 km, and are separated by long sandy beaches that are barriers to adult dispersal. The Arrábida Marine Park itself exhibits several physical characteristics that may interact with local oceanographic processes to potentially favor local retention. All sites within locations were 500 m to 2 km apart.

Adults: During the period May-August 2012, 355 *T. delaisi* adults were sampled over the 3 locations of Cascais, Arrábida and Sines. Samples were collected at 2 sites in Cascais (only 2 sites with fish could be found), and 3 sites each in Arrábida and Sines.

Juveniles: In 2013, 53 juveniles were collected from one site in Arrábida during the period August – October that marked the end of the spawning season for this species.

Larvae: In 2012, 44 larvae were collected from one site in Arrábida, and in 2013 additional larvae were collected from one site each in Arrábida (96 individuals), Sines (51 individuals) and Cascais (24 individuals). Adults and juveniles were collected via SCUBA whilst larvae were collected using light traps. Pectoral and caudal fin clips (approximately 0.5-1 cm in length) from adults and juveniles were preserved in 96% ethanol for genetic analyses. Plankton samples were fixed onboard in 70% ethanol; after sorting in the laboratory, larvae were stored in 96% ethanol for further genetic analyses. Table 2.1. provides a breakdown of the number of individuals per year, location, site (geographic coordinates), life stage (adults, juveniles or larvae) and site code. In total, 355 adults, 53 juveniles, and 215 larvae of *T. delaisi* collected in 2012 and 2013, were used for

genotyping. Samples sizes varied between 14 – 96 individuals per site. Two collections, S3_12 (adult, Site #3 of Sines) and C_L_13 (larvae from Cascais collected in 2013) had sample sizes below 30 individuals, thus potentially yielding poorer estimates of the allele frequencies of the population (Hale *et al.*, 2012).

Table 2.1. Summary statistics for each sample site averaged over all eight loci.

Year	Stage	Location	Geographic Coordinates	Sample	N	Mean N_A	Mean A_R	P_A	F_{IS}	H_O	H_E
2012	Adults	Cascais	38°41'56.4"N 9°25'03.7"W	C1_12	50	19.5	11.1	3	0.051	0.795	0.829
	Adults	Cascais	38°41'40.0"N 9°26'30.8"W	C2_12	47	20.1	11.3	3	0.004	0.843	0.838
	Adults	Arrábida	38°26'41.6"N 9°02'11.2"W	A1_12	50	21.4	11.7	1	0.03	0.82	0.836
	Adults	Arrábida	38°26'53.3"N 9°01'26.2"W	A2_12	50	20.9	11.9	3	0.008	0.847	0.845
	Adults	Arrábida	38°26'53.3"N 9°01'26.2"W	A3_12	49	20.8	11.3	3	-0.02	0.857	0.83
	Adults	Sines	37°50'08.5"N 8°47'51.5"W	S1_12	47	20.5	11.7	0	0.004	0.84	0.836
	Adults	Sines	37°51'19.4"N 8°47'44.3"W	S2_12	48	19.9	11.4	2	0.058	0.797	0.836
	Adults	Sines	37°54'00.6"N 8°48'09.9"W	S3_12	14	12	11.6	0	0.067	0.8	0.825
	Larvae	Arrábida	38°26'46.9"N 9°01'49.4"W	A_L_12	44	18.1	11.0	0	0.016	0.83	0.833
2013	Juvenile	Arrábida	38°26'48.9"N 9°01'52.2"W	A_J_13	53	21.6	11.9	2	0.019	0.847	0.855
	Larvae	Cascais	38°41'58.7"N 9°24'48.9"W	C_L_13	24	14.5	10.9	0	0.052	0.798	0.823
	Larvae	Arrábida	38°26'42.6"N 9°02'23.8"W	A_L_13	96	25.9	11.7	6	0.039	0.813	0.841
	Larvae	Sines	37°50'33.7"N 8°47'39.5"W	S_L_13	51	20.8	11.7	2	0.014	0.847	0.85

Number of individuals sampled (N), mean number of alleles (N_A), mean allelic richness (A_R), inbreeding coefficient (F_{IS} – all values non-significant), observed heterozygosity (H_O) and expected heterozygosity (H_E).

2.3.3 Laboratory analyses

DNA Extraction: DNA was extracted from all samples except the preflexion larvae using the automated QIAxtractor by Qiagen and following the Purification of DNA from Soft Tissue Protocol. DNA from preflexion larvae was extracted manually using the Gentra Puregene ™ Tissue Kit following the DNA Purification from Tissue Protocol.

Multiplex microsatellite amplification and genotyping: Samples were genotyped at 11 polymorphic microsatellite loci previously isolated from *T. delaisi* (Carreras-Carbonell *et al.*, 2004, 2006) and deposited in GenBank (Accession Nos. AY490907–AY490916 and AJ971942). These 11 microsatellites were amplified in 2 multiplex PCRs per individual using fluorescently labelled (VIC, 6FAM. NED and PET) forward primers. PCR reactions were carried out in a total volume of 10.0 µl using the Qiagen Type-It Microsatellite PCR Kit and following the protocol "Multiplex PCR for Amplification of Microsatellite Loci". Thermocycling profiles were also performed in accordance with this kit protocol and consisted of an initial activation step of 5 minutes at 95 °C (HotStarTaq Plus DNA Polymerase is activated by this step), followed by 28 cycles of 30 s at 95 °C

(denaturation), 90 s at 60 °C (annealing) and 30 s at 72 °C (extension). The cycling achieved with a final extension time of 30 minutes at 60 °C. Amplified PCR products were screened using an ABI 3700 Automated Sequencer at GenoScreen – (Lille, France). Alleles were sized and scored with the GeneMapper ™ software by comparison to the internal size standard Genescan-500LIZ (Applied Biosystems Inc.). Locus Td9 was removed because it showed an unexpected allele of approximately 650 bp in length that was outside the range of the size marker used in allele scoring and outside the input size range of the software used to detect genotyping errors.

2.3.4 Data analyses

The software MICROCHECKER V 2.2.3. (van Oosterhout *et al.*, 2004) was used to check the complete data set for the occurrence of genotyping errors due to null alleles, stuttering and large allele drop out. Exact tests for departures from Hardy-Weinberg equilibrium (HWE) and for linkage disequilibrium (LD) were performed using GENEPOP v 4.2.2. (Rousset, 2008) applying the Markov chain method (Guo & Thompson, 1992). A dememorization of 10 000 iterations, 100 batches and 500 iterations per batch was used to reduce standard errors below 0.01. Allele frequencies, the mean number of alleles (N_A), private alleles, and observed and expected heterozygosities (H_O and H_E) were determined per locus, per site and per developmental stage using GenAlEx 6.5 (Peakall & Smouse, 2006, 2012). The inbreeding co-efficient F_{IS} and its estimated probability were calculated via 10 000 random permutations using FSTAT 2.9.3.2 (Goudet, 2002) according to the methodology of Weir and Cockerham (1984). FSTAT was also used to calculate rarefied allelic richness (A_R) based on a minimum sample size of 14 individuals (minimum sample size in Sines). The tests performed above were used to identify any inconsistency in the data set and to ensure that all the loci could be used for subsequent analysis of genetic structure.

Genetic differentiation between all samples was evaluated using pairwise F_{ST} values generated in GenAlEx 6.5 using the AMOVA option. P-values generated were adjusted for multiple testing using the false discovery rate (FDR) calculated using the "qvalue" package implemented in R. In all subsequent analyses two data sets were analyzed (1) "Adults" and (2) "All". The "Adults" data set contained all the adult samples collected in 2012 with the exception of S_PG_12, and the "All " data set contained all the samples collected in 2012 and 2013 with the exception of S_PG_12 and C_LAV_13. These two samples were removed *de facto* from the analyses because of insufficient sample size (14 and 24 individuals respectively).

To test the significance of correlations between pairwise genetic and geographic distances (isolation by distance), a Mantel test was performed between the linearized F_{ST} transformation Lin

$F_{ST} = F_{ST}/(1-F_{ST})$ among adult samples only (Slatkin, 1995) and geographic distance (log km) using the software IBDWS v 3.23 (Jensen *et al.*, 2005). Data input (genetic and geographic distances) for the required matrices were generated using GenAlEx.

To visualize the genetic relationships among sites for both the "Adults" and "All" data sets, principal coordinates analyses (PCoA) were performed using pairwise linearized F_{ST} and Nei's genetic distance implemented in GenAlEx, which uses a co-variance standardized method.

Attempts were made to identify genetic clusters using two approaches: (1) minimizing Hardy-Weinberg and Linkage disequilibrium, via the software STRUCTURE 2.3.4 (Pritchard *et al.*, 2000), and (2) performing a discriminant analysis of principal components, DAPC (Jombart *et al.*, 2010), implemented in the R software package Adegenet (Jombart, 2008). In contrast to STRUCTURE, DAPC does not rely on explicit population genetic models, therefore these methods are complementary (Jombart *et al.*, 2010).

STRUCTURE analyses were performed using the "LOCPRIOR model" which is best suited for detecting weak genetic structure (Hubisz *et al.*, 2009). No admixture and correlated alleles (Falush *et al.*, 2003) were assumed, two assumptions that are also best suited for detecting subtle structure (Pritchard *et al.*, 2000). For each data set 15 runs were performed on each K value where K= 1-8 for the "Adults" data set and k=11 for the "All" data set. Burn-in and run length were set to 1000 000 MCMC (Markov Chain Monte Carlo) and 1000 000 repetitions. The best K was inferred using the ΔK method (Evanno *et al.*, 2005) implemented via the online website "Structure Harvester" (Earl & vonHoldt, 2012).

Two DAPC analyses were performed on each data set. First, each individual was assigned *a priori* to its sample using the function DAPC, and the probability of assignment to this sample was determined. Second, the "find.cluster" function in Adegenet was used to run successive k-means clustering for k=1-15 without prior group information. The optimal K was identified as the one showing the lowest Bayesian Information Criteria (BIC). DAPC was then performed using the function DAPC. Membership probabilities were then calculated for each individual, and each individual was assigned to a cluster using its maximum membership probability (*a posteriori* assignment). Since DAPC (function DAPC) uses principal component analysis (PCA) as a prior step, the retention of too many PCs (Principal Components) can lead to overfitting discriminant functions and hence the modeling of any structure and the virtual discrimination of any set of clusters. To avoid this case, the PC optimization procedure proposed by Adegenet was used in both scenarios. In this procedure, an α-score which measures the difference between the proportion of successful reassignment of the analysis (observed discrimination) and values obtained using random

groups (random discrimination) is generated. The number of retained PCs is then chosen to optimize this α-score.

2.4 Results

MICROCHECKER detected the presence of null alleles in Td3 and the occurrence of both null alleles and stuttering in Td11. After trying some new unsuccessful screening and testing new primers, these two loci were removed from all subsequent analyses. All samples were in Hardy-Weinberg equilibrium at loci Td1, Td2, Td4, Td6, Td7 and Td10. Deviations from HWE were only detected in Td5 for C_L_13 and in Td8 for S2_12, A3_12 and A_L_12 samples. No genotypic linkage disequilibrium was detected between any loci pairs ($p<0.05$).

The remaining 8 loci used for genetic analyses were highly polymorphic. A total of 268 alleles were detected among the 8 loci. Alleles per locus ranged between 8 (Td7) and 66 (Td8), with a mean of 33.5 ± 6.8. Expected heterozygosity (H_E) per locus was generally high (>0.748) with the exception of Td7 (0.513). Mean expected (H_E) heterozygosity was 0.837 [Table 2.2.]. Allelic richness over all loci, calculated from a minimum common sample size of 14 individuals, was fairly uniform across all sites, ranging between 10.9 and 11.9. Mean expected heterozygosity (H_E) was high and fairly uniform across all sites (0.823–0.855). The inbreeding coefficient, F_{IS} was generally low ranging between – 0.02 and 0.067 with all values insignificant. Private alleles were in low numbers and only made of rare alleles (1–6) in 9 out of the 13 sites sampled [Table 2.1.].

Pairwise F_{ST} values were low ranging between -0.004 and 0.018. Twenty-four (24) out of the 91 pairwise F_{ST} values were significant after correction for multiple testing [Table 2.3.]. No significant F_{ST} values were observed between any pairwise comparisons of adult sites. All significant F_{ST} values (14) occurred between different life-stages i.e. between adults and juveniles (5), between adults and larvae (6) and between juveniles and larvae (2) even from the same location.

PCoA performed on linearized F_{ST} values demonstrated that for the adult samples only, the first two axes accounted for 60.5% and 36.4% of the total genetic variance [Figure 2.1. (A)]. Sites clustered into two groups, two Sines sites versus the Arrábida and Cascais sites (with the exception A2_12). Some Arrábida and Cascais sites clustered together but never with any of the Sines sites. When all the samples (adults, recruits and juveniles) were analyzed together several other groups could be identified [Figure 2.1. (B)]. Adult samples clustered together, separated from the larval and juvenile stages. Among the adult samples, Arrábida and Cascais clustered together, opposite to the two Sines sites – as seen when only the adults were analyzed.

Table 2.2. Characterization of the 8 microsatellite loci of *Tripterygion delaisi* utilized.

Locus	Size Range	N	N_A	H_E	Forward and reverse primer sequences (5´– 3´)
Td1	156-198	620	18	0.867	**NED**-CACTTTATGACTAAATGACCACTGC ATCAGCGCTGCATTAGTGTC
Td2	401-443	612	18	0.748	**PET**-GCGCTTATTGAGCAACTGTG AGCCTCATGCAGGGTCTACT
Td4	219-279	618	27	0.880	**6FAM**-GCACGGGAACAGACTGATG GTGCTCCTGCGAGGAATAGA
Td5	182-264	615	40	0.935	**VIC**-GTCCAGGAGATAGACGCAGC GCACATCCCAACCCATAAAG
Td6	108-198	617	45	0.937	**6FAM**-GGTCCTCCTGGTTTTTACCTG GACCAGTTGGTTGTGACTGG
Td7	108-122	623	8	0.513	**NED**-TCTTGGAAACACGCTTGTAA GCACGTCTATTTGTCGTCCTC
Td8	300-444	617	66	0.884	**VIC**-AGCGGATTTGACTGAGGAAA GGCTGTTTCTGAGCCAGTTT
Td10	121-221	620	46	0.931	**NED**-GACAAGACCGGCACATTTTC GGGACAAGAGGCAGAAGTTG
Mean			33.5	0.837	
S.E.			6.8	0.014	

Number of individuals genotyped (N), no. alleles (N_A), and expected heterozygosity (H_E). S.E. = Standard Error. GenBank Accession Nos. AY490907-8; AY490910-13; AY490915 and AJ971942

Table 2.3. Pairwise multilocus F_{ST} values between *Tripterygion delaisi* sample sites. Bold values are significant after FDR correction for multiple testing.

	C1_12	**C2_12**	**A1_12**	**A2_12**	**A3_12**	**S1_12**	**S2_12**	**S3_12**	**A_L_12**	**A_J_13**	**C_L_13**	**A_L_13**	**S_L_13**
C1_12	0.000												
C2_12	-0.004	0.000											
A1_12	-0.003	-0.001	0.000										
A2_12	-0.002	0.003	-0.002	0.000									
A3_12	-0.001	0.001	-0.002	0.001	0.000								
S1_12	0.000	0.005	-0.002	0.000	0.002	0.000							
S2_12	0.002	0.004	0.001	0.004	0.003	0.001	0.000						
S3_12	0.008	0.011	0.008	0.010	0.005	0.001	0.001	0.000					
A_L_12	0.003	0.005	0.002	0.001	0.003	0.002	0.002	0.001	0.000				
A_J_13	**0.006**	**0.005**	**0.005**	0.003	**0.006**	**0.007**	0.005	0.004	**0.005**	0.000			
C_L_13	**0.013**	**0.012**	**0.013**	**0.010**	**0.016**	**0.014**	**0.014**	**0.018**	**0.013**	**0.008**	0.000		
A_L_13	**0.004**	**0.006**	0.002	0.002	**0.005**	**0.004**	**0.006**	0.009	**0.008**	0.002	**0.006**	0.000	
S_L_13	0.005	0.004	0.003	0.003	0.005	**0.005**	0.003	0.003	0.004	0.001	0.006	0.001	0.000

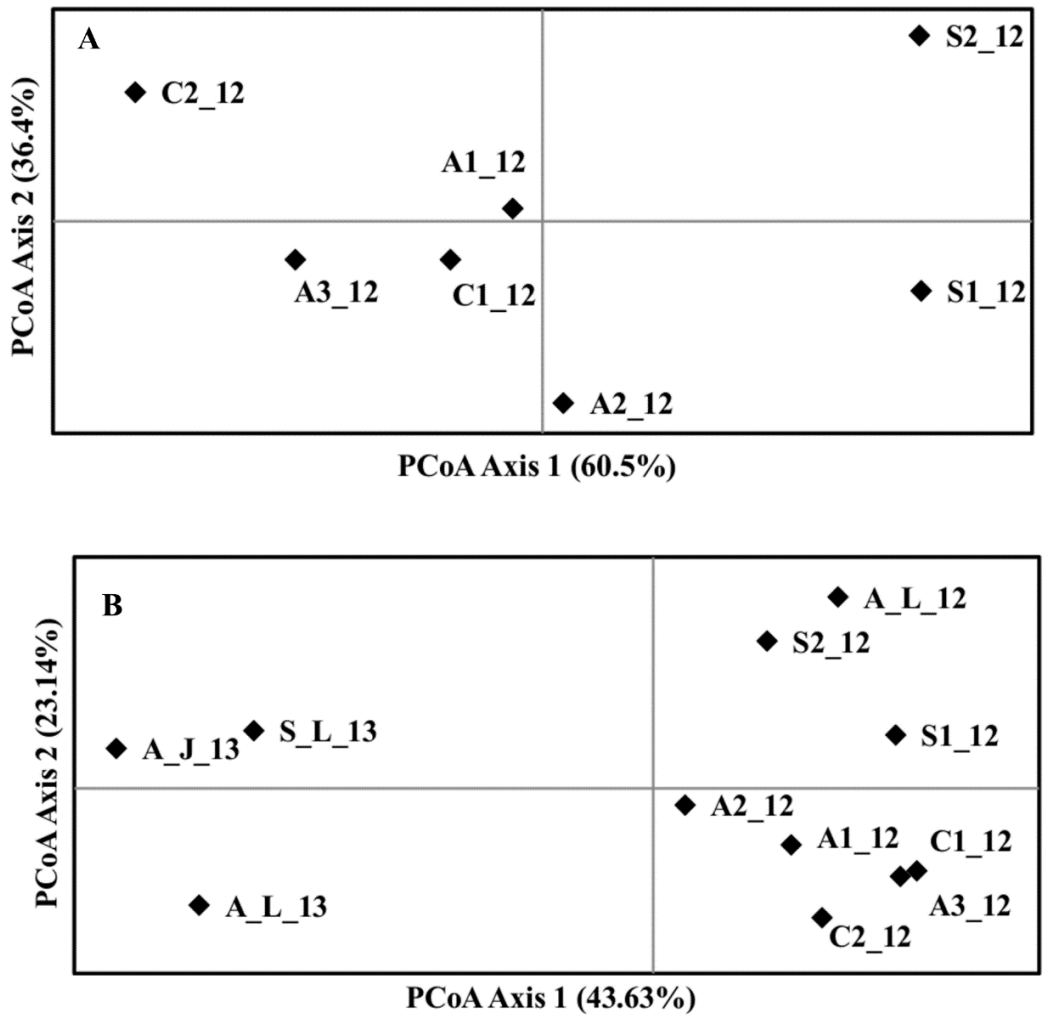

Figure 2.1. Plot of the first two axes of the PCoA performed on pairwise linearized F_{ST} values for (A) the "adult" samples and (B) "all" samples of *Tripterygion delaisi*. The proportion of the variance explained by each axis is given in parentheses.

The mantel test for adult samples indicated that there was a positive significant correlation between genetic distance and geographic distance ($r = 0.4089$; $P = 0.0070$; $R^2 = 0.167$) [Figure 2.2].

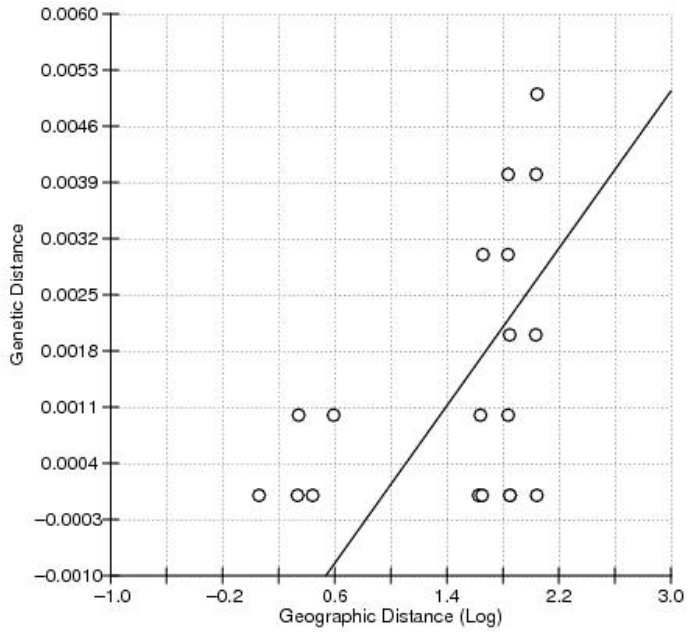

Figure 2.2. The relationship between Genetic Distance (Linearized FST) and Log Geographic Distance for adult *Tripterygion delaisi* samples.

Structure analysis involving the "adults" samples indicated that k=5 was the optimal number of mating genetic groups [Figure 2.3. (A)]. When considering k=5, we can segregate samples into the following units: each Cascais site is a segregated group [(C1_12)] and [(C2_12)], and each Sines site also [(S1_12)] and [(S2_12)], whereas all Arrábida sites are making single genetic entities (A1_12 vs A2_12 vs A3_12) [Figure 2.4.]. The second dataset analyzed included "all" samples (adults, juveniles and larvae) and two peaks in ΔK were detected, a larger peak at k =3 and a smaller peak at k=10 [Figure 2.3. (B)]. When considering K=3, we can separate samples into the following units (C1_12, C2_12), (A1_12, A2_12, A3_12, S1_12, S2_12 and A_L_12) and (A_J_13, A_L_13, S_L_13) [Figure 2.5. (A)] i.e. the three groups are, Cascais adults, Arrábida and Sines adults and the larval samples collected in 2012, and the larval and juvenile stages collected in 2013. When considering K = 10, virtually all the samples (except the larval samples collected in 2013) could be classified into their own genetic group [Figure 2.5. (B)].

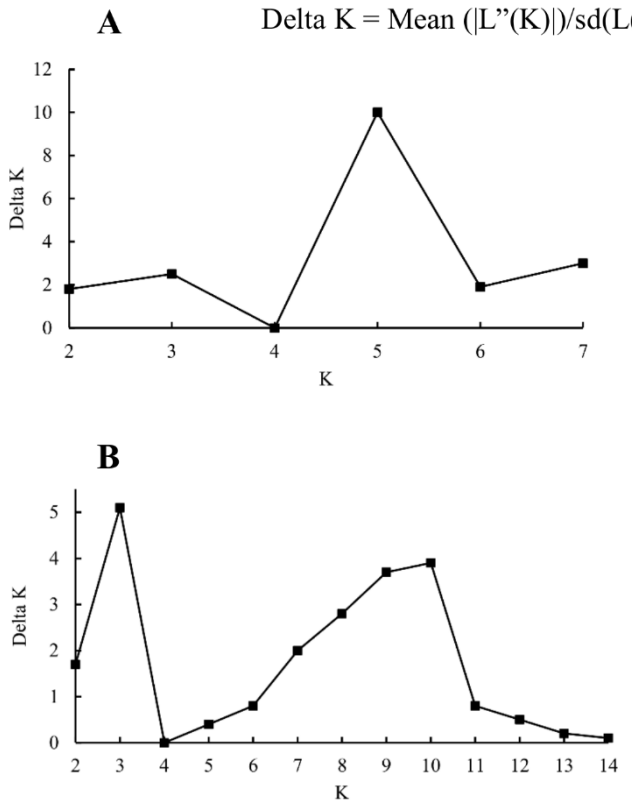

Figure 2.3. Inference of genetic clusters using STRUCTURE for the (A) "adults" and (B) "all" data sets. Plots generated in Structure Harvester.

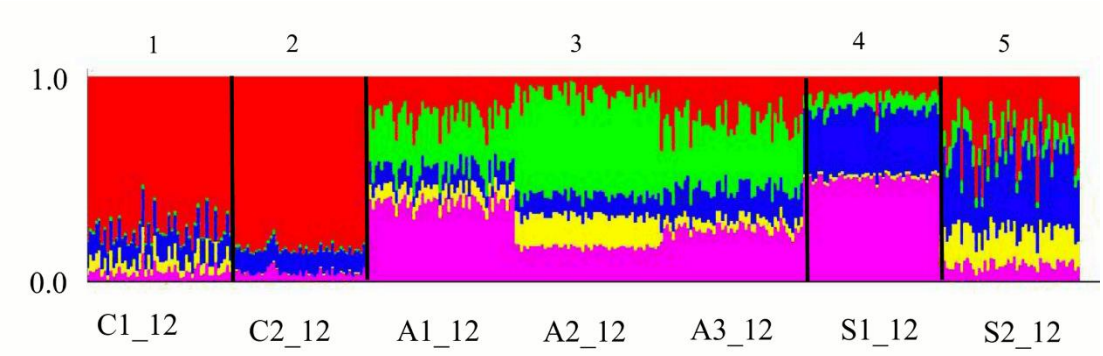

Figure 2.4. STRUCTURE Bar Plot for the "adult" data set, k = 5

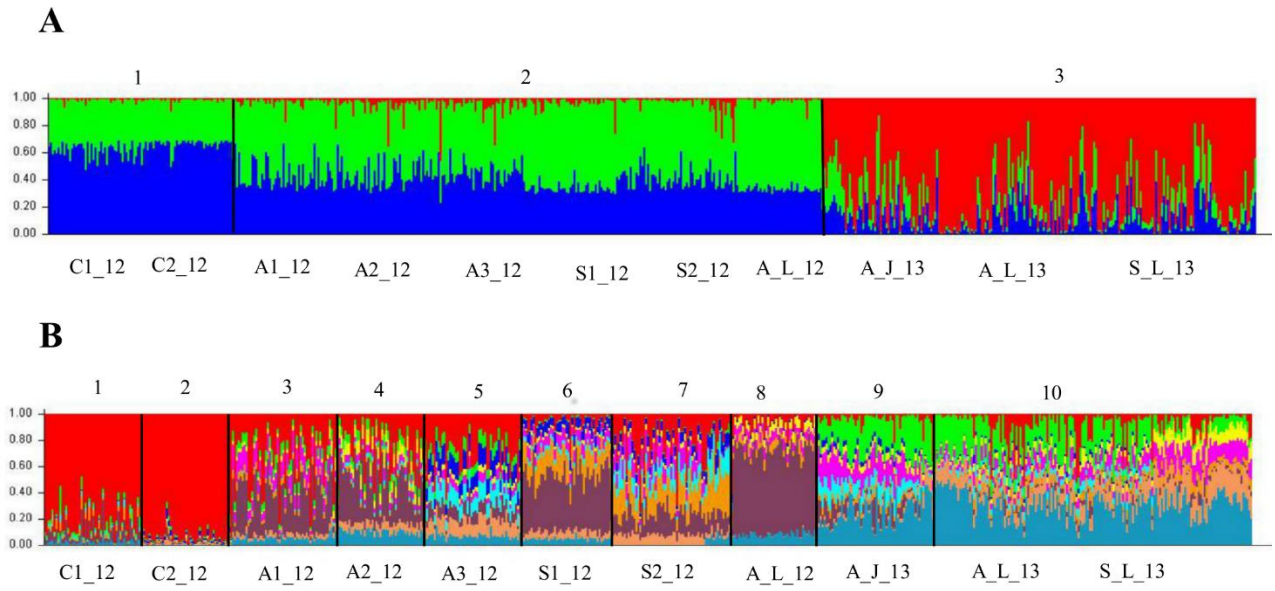

Figure 2.5. STRUCTURE Bar Plots for the "all" data set (A) k = 3 and (B) k = 10

DAPC: A **priori** assignments were performed according to site for both data sets. For the adult data set [Figure 2.6. (A)], although there was a high degree of overlap between clusters, the two Sines clusters (6 and 7), were separated from the others. The probability of assignment to the original sites ranged between 38% - 58%, with the two highest re-assignment to the two Sines sites. When the larvae and juvenile stages collected in 2013 were included in the data analyses (i.e the "all" data set), these clusters (9, 10 and 11) were separated from the adult clusters [Figure 2.6. (B)], occurring more to the right. For the adult analyses, the DAPC plots were in support of the main patterns seen in the PCoA plots. **A posteriori** assignments were also performed for both data sets. When only the "adults" were analysed k = 4 (lowest BIC) was identified as the optimal number of clusters, however the decline in BIC was not sharp and clear but rather gradual from k = 3 to k = 5, indicating that 3, 4, or 5 clusters could adequately describe the data set. When "all" the samples were analysed, k = 6 (lowest BIC) was identified as the optimal number of clusters. Once again the decline in BIC was not sharp indicating that 5–8 clusters may adequately describe the data set. For the DAPC analysis 13 PCs and 4 discriminant functions accounting for 36.4% of the variation was retained. Compoplots generated did not show any discernible geographic pattern.

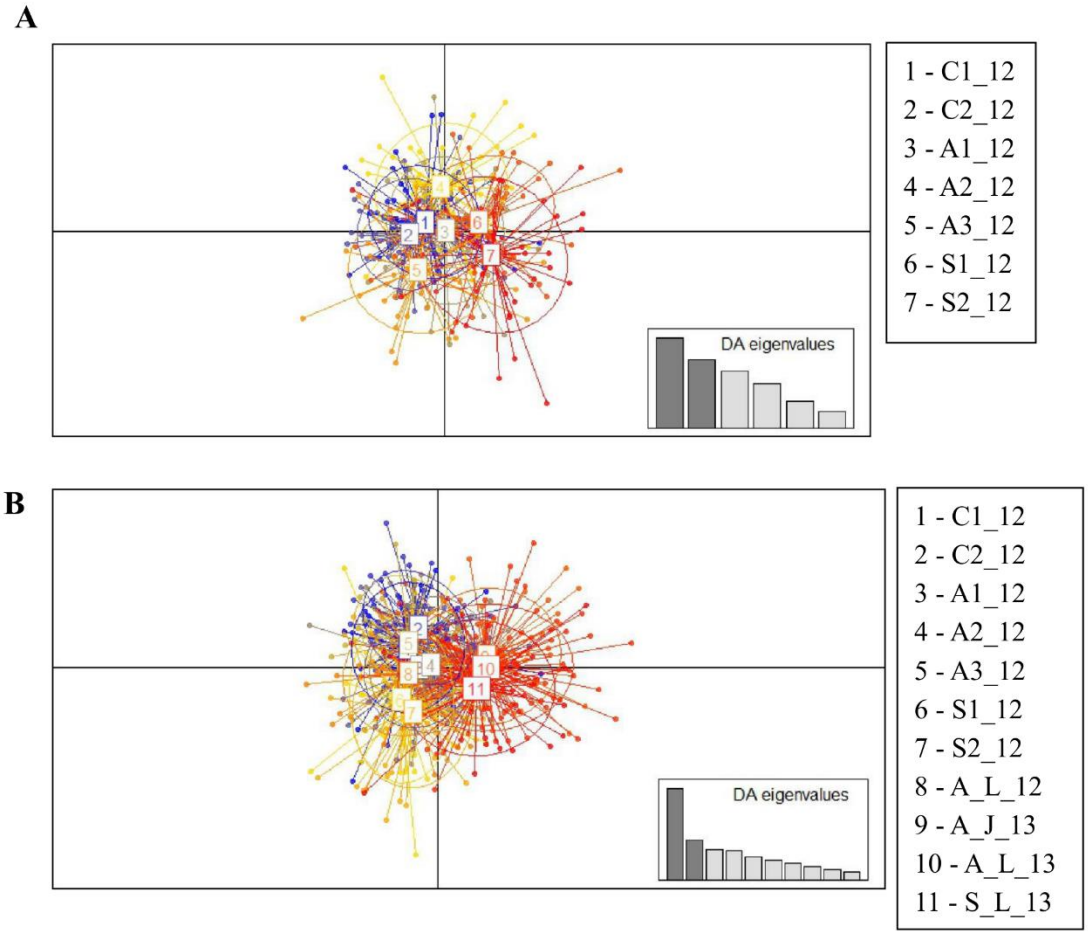

Figure 2.6. Inference of genetic clusters using DAPC for *Tripterygion delaisi* samples. Scatter plot of the first two axes of *a priori* defined populations/clusters based on sample location for (A) "adults" and (B) "all" samples. Each point represents an individual and colors correspond to the different sample locations. The inset graph represents the amount of variation attributed to each principal component.

2.5 Discussion

Overall our analyses revealed significant divergence mainly between samples of different life stages. However, a significant isolation by distance demonstrated some structure over a geographic scale of 100 km of coastline, and clustering based on minimizing HW-disequilibrium segregated the Arrábida and Cascais samples versus the Sines ones. When the larvae and juvenile samples were included, they clustered away from the adult samples suggesting temporal genetic heterogeneity.

2.5.1 Spatial genetic structure

Low and insignificant pairwise F_{ST} values between adult sites suggested a lack of genetic differentiation throughout the study area (high gene flow) over a scale of approximately 100 km. These results are in contrast to what we expected given the low mobility of this species, and characteristics of the study area that may have favored restricted larval dispersal and local retention. *T. delaisi* is regarded as a model species for investigating dispersal and population connectivity due its low dispersal capabilities, and several studies conducted on this species in the Mediterranean have yielded evidence of restricted dispersal with high self-recruitment levels, genetic differentiation between populations, and isolation by distance (Carreras-Carbonell *et al.*, 2006, 2007; Schunter *et al.*, 2014). Compared to these previous studies, our study differs in terms of spatial scale. Overall, Carreras – Carbonell *et al.*, (Carreras-Carbonell *et al.*, 2006, 2007) was conducted on a much larger scale (1500 km) whilst Schunter *et al.*, (Schunter *et al.*, 2014) was conducted on a smaller scale (50 km). Our study area of 100 km is comparable to the distances between some of the localities sampled in Carreras – Carbonell *et al.* (Carreras-Carbonell *et al.*, 2006), and the distance between Cascais and Arrábida, and Cascais and Sines, is similar (45 km and 70 km) to the stretch of coast examined by Schunter *et al.* (Schunter *et al.*, 2014). These similarities allow for some comparisons between studies.

The lack of genetic differentiation between adult samples in our study is due to the small spatial scale and local oceanographic connection. In general marine organisms with a pelagic larval stage have a high potential for gene flow, and hence the detection of local scale genetic structure in the marine environment is far and few between. In cases where it has been documented, local oceanographic processes is often cited as a major factor (Ciannelli *et al.*, 2010; Hirase *et al.*, 2012; Hoffman *et al.*, 2012). The modelling of local oceanographic processes however is very difficult. Whilst this information is not available for our study area, our initial predictions of genetic differentiation were partly based on some of the nearshore processes possibly occurring in the

Arrábida area due to its physical characteristics, and how they may have promoted larval retention at that particular locality based on the literature. For example, the interaction of alongshore tidal currents with irregular shallow bottom topography may create microcirculation patterns serving to keep the larvae within the area, the slowing of water movement by the coastal boundary layer may provide a slow bottom layer for larval retention, and upwelling relaxation events created by the northerly winds on the southward facing Arrábida coastline, may also favor retention. Our results show that if these local processes were indeed occurring, none was sufficient to lead to genetic differentiation between localities at the spatial scale of our study.

We also predicted the occurrence of genetic structure based on habitat discontinuity, in that each rocky shore locality was separated from the other by a stretch of sand. In a previous study, Carreras-Carbonell *et al.* (Carreras-Carbonell *et al.*, 2006) found significant genetic differentiation on a small spatial scale between two pairs of populations, presumably due to habitat discontinuity. One pair, 144 km apart, was separated by deep water and the other pair, 163 km apart, was separated by a large stretch of sand. These two features, which created habitat discontinuity were regarded as barriers to dispersal, leading to restricted gene flow and significant population structure. In contrast, other pairs of populations separated by similar distances, but with either continuous rocky shore or only small sand gaps (<15 km) between them, did not exhibit significant genetic differentiation. Other studies have also shown that discontinuities of sand or deep water may restrict gene flow leading to genetic structure between locations, however most of these studies have been carried out on larger spatial scales (Riginos & Nachman, 2001; Johansson *et al.*, 2008; González-Wangüemert *et al.*, 2010), where the extent of habitat discontinuity was greater. Thus, in our study, the lack of genetic differentiation between the 3 rocky shore localities separated by sand (creating habitat discontinuity) indicates that either the expanse of sand may not have been sufficient (large enough) to act as a physical barrier to dispersal or, other factors which may be promoting larval dispersal, such as physical oceanographic processes are active. Similar results (a lack of genetic differentiation and high gene flow) at a similar spatial scale (70-80 km) were found for the damselfish, *Parma microlepis*, another temperate rocky reef fish, in New South Wales, Australia. Oceanographic processes and habitat continuity were cited as two possible drivers of significant gene flow in this low dispersal species (Curley & Gillings, 2009).

Despite evidence for gene flow between locations and sites, however, significant isolation by distance, the detection of several genetically distinct clusters by STRUCTURE, and the spatial clustering and separation of sites in PCoA and DAPC plots all point to some local scale genetic heterogeneity. The PCoA and the DAPC plots which showed that the Sines sites which were

furthest away from Arrábida, and a greater distance away Cascais than Arrábida, appear to cluster together and away from the other sites, lends support to the significant pattern of isolation by distance detected. The lack of genetic differentiation between sites and localities, as inferred from insignificant F_{ST} values, does not exclude the occurrence of limited or restricted ecological dispersal, as only a few migrants per generation is needed to genetically homogenize populations. In order to better understand ecological patterns of dispersal and connectivity, an increasing number of studies have been applying "isolation by distance" to scenarios exhibiting low or weak genetic differentiation (Purcell *et al.*, 2006; Purcell *et al.*, 2009). Isolation by distance (IBD), based on a stepping stone model of dispersal (Wright, 1943; Kimura & Weiss, 1964), is often applied to populations distributed along a coastline or chain of islands. Its theoretical basis is that, if individuals disperse locally (i.e. dispersal is restricted), individuals that are close to each other will be more genetically similar than individuals that are further apart, i.e., a pattern of decreasing genetic relatedness with geographic distance will emerge. The significant pattern of isolation detected indicates that dispersal is restricted within the study area. This limited dispersal maybe responsible for the local scale genetic heterogeneity seen in the PCoA and DAPC plots where Cascais and Arrábida appear to be more related to each other than to Sines.

2.5.2 Temporal genetic structure

Significant pairwise F_{ST} values, Bayesian clustering, and both PCoA and DAPC plots, all indicate the occurrence of temporal genetic variation (i.e. the larvae and juveniles sampled in 2013 were genetically differentiated from the adults sampled in 2012, and larvae and juveniles sampled in 2013 were genetically differentiated from each other). Commonly referred to as "chaotic genetic patchiness" (Johnson & Black, 1982; Hedgecock, 1994), this phenomenon of unpatterned genetic heterogeneity has been recorded for a number of marine invertebrate (Li & Hedgecock, 1998; Moberg & Burton, 2000) and fish species (Planes & Lenfant, 2002; Pujolar *et al.*, 2006; Selkoe *et al.*, 2006; Liu & Ely, 2009). The definitive causes of this temporal variation in genetic structure are not known, however several plausible reasons have been proposed to account for its occurrence. These include: (1) sweepstake reproduction, whereby variability in adult reproductive spawning success due to stochastic processes, result in cohorts of larvae or new recruits that come from only a fraction of the adult population and are thus less genetically diverse than the global adult population (Hedgecock & Pudovkin, 2011; Pusack *et al.*, 2014); (2) localized post-settlement selection due to micro-geographic variability in environmental conditions (Johannesson *et al.*, 1995); (3) localized pre-settlement selection on individuals resulting in genetically variable larval cohorts through space

and time (Johnson & Black, 1984; Watts *et al.*, 1990; Hedgecock, 1994; Selkoe *et al.*, 2006); and (4) spatial and temporal variability in the genetic composition of recruits caused by fluctuations in the larval source (Purcell *et al.*, 1996; Selkoe *et al.*, 2006).

The exact reason for chaotic genetic patchiness in our study cannot be determined due to limitations of our sampling strategy, however some reasons maybe more feasible than others. Pre and post settlement selection is possible but our neutral microsatellite markers are expected to be insensitive to natural selection, especially involving 8 independent loci, and most loci contributed to significant global F_{ST} values. There is no obvious support for sweepstake reproduction in our results. One would expect that if this were the main mechanism driving the "chaotic genetic patchiness" then juvenile samples would show lower levels of diversity than the adult samples, this was not seen. At Arrábida, larval sampling was conducted using light traps anchored to a fixed position in the nearshore environment where local oceanographic processes maybe promoting larval retention. Some of our samples were derived mostly from a single light trap (i.e. for our A_L_13 sample, 60% of the individuals came from the same light trap sample) comprising individuals spawned in a particular area at a particular time. It is possible that these individual may have originated from a single adult or a small group of adults. Additionally local inshore processes may promote the creation of larval patches, resulting in limited admixing rather than a homogenous larval pool. This maybe driving the chaotic genetic patchiness observed.

Overall, we were able to illustrate that despite high gene flow, larval dispersal in a low dispersing temperate reef species *T. delaisi* may be restricted over a spatial scale of 100 km resulting in pattern of isolation by distance and local scale genetic heterogeneity. The phenomenon of "chaotic genetic patchiness" is also illustrated likely due to high variance in reproductive success among small spawning groups of adults.

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2.8 Supplementary Information

Table 2.8.1S Summary statistics for adult samples of *Tripterygion delaisi* collected along the Portuguese west coast. Shown are sample sizes (N), the number of alleles (N_A), and the observed and expected heterozygosities (H_O and H_E).

SAMPLES		LOCUS								
		Td1	Td2	Td4	Td5	Td6	Td7	Td8	Td10	MEAN
C1_12	N	50	50	50	50	50	50	50	50	
	$N_A(A_R)$	12(9.5)	8(6)	16(9.9)	30(15.9)	27(15.9)	4(2.7)	33(14.8)	26(14.2)	
	H_O	0.900	0.600	0.880	0.940	0.900	0.460	0.740	0.940	0.795
	H_E	0.872	0.702	0.860	0.936	0.943	0.481	0.907	0.928	0.829
C2_12	N	47	47	47	47	47	47	47	47	
	$N_A(A_R)$	13(9.9)	9(6.5)	20(10.9)	26(14.9)	31(16.5)	4(2.8)	35(15.2)	23(14.0)	
	H_O	0.894	0.851	0.872	0.872	0.979	0.511	0.872	0.894	0.843
	H_E	0.878	0.755	0.867	0.921	0.946	0.518	0.892	0.926	0.838
A1_12	N	50	50	50	50	50	50	50	50	
	$N_A(A_R)$	13(10.1)	9(6.3)	19(11.1)	30(15.3)	29(16.4)	4(3.1)	37(15.2)	30(16.1)	
	H_O	0.840	0.720	0.900	0.920	0.920	0.460	0.860	0.940	0.820
	H_E	0.885	0.726	0.886	0.928	0.945	0.479	0.905	0.938	0.836
A2_12	N	50	50	49	49	49	50	50	49	
	$N_A(A_R)$	14(10.1)	10(6.5)	23(13.3)	30(16.5)	28(15.2)	4(3.2)	32(14.7)	26(15.6)	
	H_O	0.900	0.700	0.939	0.939	0.959	0.500	0.900	0.939	0.847
	H_E	0.887	0.730	0.908	0.946	0.931	0.498	0.915	0.944	0.845
A3_12	N	49	49	49	49	49	49	49	49	
	$N_A(A_R)$	15(10.2)	8(5.8)	16(10.1)	26(14.9)	34(16.7)	4(2.5)	34(15.1)	29(14.9)	
	H_O	0.918	0.714	0.939	0.980	0.939	0.633	0.796	0.939	0.857
	H_E	0.880	0.716	0.869	0.932	0.943	0.473	0.902	0.928	0.830
S1_12	N	47	43	47	47	47	47	47	47	
	$N_A(A_R)$	12(9.1)	10(6.4)	20(10.6)	26(15.8)	29(16.6)	4(3.1)	32(15.0)	31(16.7)	
	H_O	0.915	0.674	0.915	0.915	0.957	0.660	0.723	0.957	0.840
	H_E	0.856	0.701	0.880	0.943	0.948	0.513	0.900	0.948	0.836
S2_12	N	48	48	48	48	48	48	48	48	
	$N_A(A_R)$	14(9.3)	11(7.2)	19(10.8)	32(17.4)	26(15.0)	5(3.7)	24(11.6)	28(15.9)	
	H_O	0.854	0.771	0.896	0.958	0.833	0.521	0.583	0.958	0.797
	H_E	0.862	0.765	0.887	0.952	0.927	0.540	0.820	0.939	0.836
S3_12	N	14	13	14	14	14	14	13	14	
	$N_A(A_R)$	11(10.6)	6(6.0)	14(13.3)	17(16.2)	18(17.1)	4(4)	13(13)	13(12.6)	
	H_O	0.786	0.923	0.929	0.857	0.857	0.357	0.692	1.000	0.800
	H_E	0.847	0.790	0.857	0.921	0.926	0.559	0.808	0.890	0.825

Table 2.8.2S Summary statistics for juvenile and larval samples of *Tripterygion delaisi* collected along the Portuguese west coast. Shown are sample sizes (N), the number of alleles (N_A), and the observed and expected heterozygosities (H_O and H_E).

SAMPLES		LOCUS								
		Td1	Td2	Td4	Td5	Td6	Td7	Td8	Td10	MEAN
A_L_12	N	43	39	42	42	42	44	41	42	
	$N_A(A_R)$	11(9.3)	9(6.7)	17(11.6)	27(15.8)	28(15.9)	4(2.6)	26(12.5)	23(13.7)	
	H_O	0.930	0.744	0.952	0.929	0.929	0.523	0.780	0.857	0.830
	H_E	0.869	0.759	0.899	0.939	0.933	0.491	0.867	0.911	0.833
A_J_13	N	53	53	53	53	53	53	53	24	
	$N_A(A_R)$	13(9.5)	10(7.3)	17(11.0)	30(16.6)	28(15.5)	5(3.4)	42(17.1)	22(14.6)	
	H_O	0.755	0.887	0.887	0.962	0.943	0.509	0.887	0.958	
	H_E	0.857	0.802	0.896	0.949	0.938	0.534	0.932	0.933	
C_L_13	N	23	24	23	23	22	24	24	24	
	$N_A(A_R)$	10(8.9)	8(6.8)	12(9.7)	19(14.1)	20(14.4)	4(3.1)	21(14.1)	22(15.8)	
	H_O	0.739	0.875	0.826	0.739	0.955	0.417	0.875	0.958	0.798
	H_E	0.819	0.765	0.836	0.909	0.908	0.536	0.877	0.933	0.823
A_L_13	N	96	95	95	94	96	96	95	96	
	$N_A(A_R)$	13(9.2)	14(7.1)	19(11.1)	34(15.3)	37(16.4)	8(3.5)	48(14.7)	34(15.9)	
	H_O	0.885	0.705	0.905	0.883	0.938	0.406	0.884	0.896	0.813
	H_E	0.870	0.738	0.894	0.936	0.950	0.500	0.897	0.946	0.841
S_L_13	N	50	51	51	49	50	51	50	51	
	$N_A(A_R)$	14(10.6)	11(7.4)	16(10.9)	26(15.7)	32(16.7)	6(3.7)	32(13.5)	29(15.2)	
	H_O	0.980	0.824	0.863	0.918	0.880	0.549	0.860	0.902	0.847
	H_E	0.895	0.779	0.894	0.942	0.947	0.541	0.868	0.934	0.850

CHAPTER 3: DISTRIBUTION OF *TRIPTERYGION DELAISI* LARVAE IN THE ARRÁBIDA MARINE PARK, PORTUGAL

3.1 Introduction

Patterns of larval spatial and temporal distribution can provide important insights into the extent of larval dispersal and ecological connectivity (Leis *et al.*, 2011). In both temperate and tropical environments, the larvae of some species exhibit restricted dispersal by remaining within a few hundred meters of their spawning site (Barnett *et al.*, 1984; Brogan, 1994). Vertical and horizontal sampling of the nearshore environment have also found restricted inshore larval distributions for several species belonging to the families Gobiesocidae and Tripterygiidae (Kingsford & Choat, 1989; Sabatés *et al.*, 2003). Additionally, the occurrence of all the different larval developmental stages of a single species in the nearshore environment, which suggests that some degree of local retention and development maybe occurring, has also been documented (Leis *et al.*, 1998; Leis *et al.*, 2003; Borges *et al.*, 2007b; Borges *et al.*, 2009). While the occurrence of longshore dispersal cannot be definitively excluded based solely on patterns of larval spatial distribution, studies of spatial distribution can be complimentary to other more definitive methods (Leis, et al., 2011).

Tripterygion delaisi is a small demersal reef fish that inhabits nearshore rocky habitats in the Mediterranean and Atlantic. This species is often regarded as a model species for investigating connectivity due to several life-history characteristics (the production of benthic eggs, an inshore larval distribution, limited adult mobility and a short PLD) which suggest that it has low dispersal capabilities. Throughout its range, the larvae of *T. delaisi* are found almost exclusively in coastal waters usually between the coastline and 100 m offshore (Sabatés *et al.*, 2003; Beldade *et al.*, 2006; Borges *et al.*, 2007b). Several studies focusing on the composition and distribution (vertical and horizontal) of larval fish assemblages at the Arrábida Marine Park (AMP), indicate that *T. delaisi* larvae are abundant and occur in the nearshore environment, where all the developmental stages (preflexion, flexion and postflexion) occur (Beldade *et al.*, 2006; Borges *et al.*, 2007a; Borges *et al.*, 2007b; Borges *et al.*, 2009). These previous studies however have all relied on the use of plankton nets for sampling and have all been restricted to the nearshore environment. While this method is the most commonly used method for the collection of plankton samples, it has two main limitations: (1) larger larvae are able to exhibit net avoidance and hence maybe under represented (Brander & Thompson, 1989; Heath & Dunn, 1990) and (2) the force of the flow of water through the nets usually results in physical damage to the larvae or larval mortality, making the collection of live specimens for *in situ* experiments difficult.

An alternative method of collecting plankton samples is the use of light traps. Light traps which are a passive form of sampling that rely on the phototactic abilities of the larvae have been

increasing in popularity given the importance of collecting late stage larvae in order to understand the transition from larvae to juveniles. Though more widely used in the tropics, it have been applied successfully in the temperate region as well (Hickford and Schiel, 1999; Chícharo et al., 2009).

We used both light traps and planktons nets to examine the spatial and temporal distribution of *T. delaisi* in the Arrábida Marine Park in order to gain some insights into the connectivity patterns of this species. More specifically, our aims were (1) to compare larval densities in the inshore and offshore environments, and at the surface and bottom depth strata and (2) to search for the occurrence of ontogenetic vertical distribution and to (3) to compare both methods used.

The use of light traps in this study will compliment previous studies that utilized just plankton nets to describe *T. delaisi* distribution within the area.

3.2 Methods

3.2.1 Study Area

Our study area is the Arrábida Marine Park (AMP) which is located on the west coast of Portugal between Sesimbra and Portinho da Arrábida - roughly between 9°00'15"–9°03'48"W and 38°26'–38°27'N [Figure 3.1.]. The area is positioned in a southerly direction and is thus protected from the north and north-west winds by the adjacent mountain chain of Arrábida. Relatively calm sea conditions persist for much of the year allowing sampling of the very nearshore environment. Local oceanographic processes are dominated by tidal currents that run parallel to the shore, and the underwater rocky substratum created by boulders originating from the Arrábida mountain chain extend offshore for only about some tens of metres (and approximately 13 metres in depth). The AMP is separated from the two nearest rocky reefs areas to the north and south, Cascais and Sines respectively, by sand. As such it can be considered as a continental island with rocky habitat.

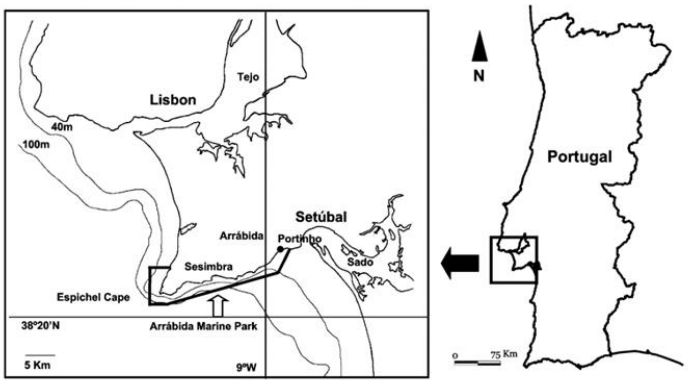

Figure 3.1. Map showing the location of the study area (Adopted from Borges *et al.*, 2007)

3.2.2 Sampling

Scooters: Scooter sampling involved the use of a plankton net attached to an underwater scooter (Figure 3.2). To facilitate ease of manoeuvrability, the mouth diameter: net length ratio was restricted to 1:3. Surface trawls were conducted approximately 1m below the surface, while bottom samples were collected approximately 0.5 m above the substrate at depths of about 4 -6 m. The technique used was: on reaching the bottom, the diver opened the net and commenced the trawl in a direction parallel to the coastline, contouring around obstacles when necessary. After 5 minutes of trawling, the diver closed the net and ascended to the surface. Sampling speed was approximately 1.5 knots and flowmeters were attached to the nets in order to calculate the volume of water passing through the net. Scooter sampling occurred in both 2011 and 2013 during the spring-summer period which is regarded as the spawning season for most species in the AMP (Henriques *et al.*, 2002; Borges *et al.*, 2007), including *T. delaisi*. In both years, samples were collected inshore (max 50 m away from the reefs), in each of two similar sites (Risco and Derrocada) within the fully protected area of the Marine Park; in 2013, two extra sites (Alpertuche and Pedra do Leão) were also sampled. **Table 3.1.** gives a breakdown of the number of scooter samples collected by year, site and position in the water column.

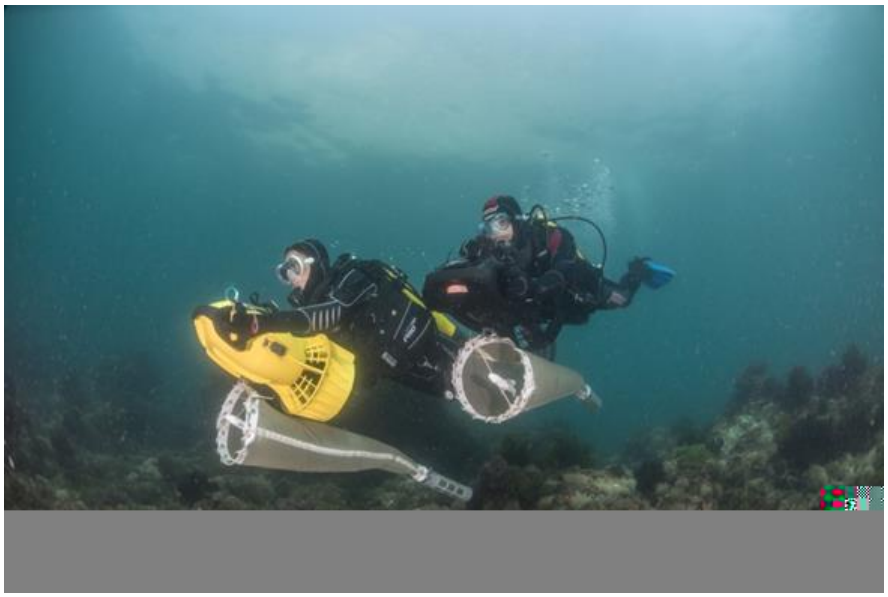

Figure 3.2. Scooter sampling at the Arrábida Marine Park, near the bottom.

Table 3.1. Scooter samples collected in the Arrábida Marine Park according to year, month, site and depth strata. Samples selected for year*depth analysis of larval density are highlighted in grey.

Year	**Month**	**Site**	**Depth**		**Total**
			Surface	Bottom	
2011	6	Risco	2	2	4
		Derrocada	2	2	4
	7	Risco	13	13	26
		Derrocada	13	15	28
		Total	30	32	62
2013	7	Risco	6	5	11
		Derrocada	6	6	12
		Pedra do Leão	6	6	12
		Alpertuche		4	4
	8	Risco	8	8	16
		Derrocada	8	8	16
		Pedra do Leão	8	8	16
		Alpertuche		8	8
	9	Risco	6	6	12
		Derrocada	6	6	12
		Pedra do Leão	4	6	10
		Alpertuche		5	5
		Total	58	76	134

Scooter samples were collected in 2 years, 2011 and 2013. In 2011 two sites were sampled (Risco and Derrocada) in the months of June and July, while in 2013, 4 sites were sampled (Risco, Derrocada, Pedra do Leão and Alpertuche) during the period July – September. Sampling effort was unbalanced between the two years with more than twice the amount of samples being collected in 2013 (N = 134) compared to 2011 (N = 62). The number of samples collected also varied between months and sites. In terms of strata the number of samples collected at the suface and the bottom was even at most sites except for Alpertuche where only bottom samples were collected.

Light Traps: Lights traps modelled according to the AIMS design (Meekan *et al.*, 2001) [Figure 3.3.], were deployed at two sites in the fully protected area (Risco and Derrocada) within the AMP during the periods May – August 2011, April – September 2012 and June – September 2013. During these periods, sampling was scheduled for 2 days every other week. Traps were deployed in both the offshore and nearshore environment. In the nearshore they were set at the surface and at the bottom (maximum depth = 12m), over the reefs where the adults live, while in the offshore

environment they were set at the surface, in midwater, and at the bottom (depths of ca 20 m, sandy bottoms). A night of sampling typically involved setting 4 traps simultaneously for 1 hour (60 minutes) for 3-4 consecutive hours, starting about 30 minutes to 1 hour after sunset. **Table 3.2.** gives an account of the number of light trap samples collected according to year, month, site, distance from shore (inshore vs offshore) and depth strata (surface, midwater, or bottom).

Figure 3.3. Light trap sampling at the Arrábida Marine Park

Light trap samples were collected from Risco and Derrocada in all three years (2011 – 2013). Over the three years, sampling occurred between April and September, and the months of June and July were the only months sampled in all three years. Sampling effort was much greater inshore compared to offshore [Table 3.2.].

Table 3.2. Light trap samples collected at the Arrábida Marine Park, according to year, month, number of year, month, site, distance from shore (inshore vs offshore) and depth strata (surface, midwater, or bottom).

Year	**Month**	**Location**	**Inshore**		**Offshore**			**TOTAL**
		Depth	**Surface**	**Bottom**	**Surface**	**Medium**	**Bottom**	
2011	May	Risco	1	2				3
		Derrocada	3	2				5
	June	Risco	6	6	5	5	4	26
		Derrocada	28	26				54
	July	Derrocada	19	22				41
	August	Derrocada	2	6				8
	TOTAL		59	64	5	5	4	**137**
2012	April	Risco	7	6	6	7	6	32
		Derrocada	3	3	4	3	4	17
	May	Risco	3	3	3	2	1	12
		Derrocada	5	5	6	4	6	26
	June	Risco	9	9				18
		Derrocada	7	7				14
	July	Risco	4	4				8
		Derrocada	4	4	1	1	1	11
	September	Derrocada	1	2				3
	TOTAL		43	43	20	17	18	**141**
2013	June	Risco	10	10	2	1	2	25
		Derrocada	8	8	4	3	6	29
	July	Risco	16	15				31
		Derrocada	13	14				27
	August	Risco	13	14				27
		Derrocada	13	14				27
	September	Risco	2	8				10
		Derrocada	16	14				30
	TOTAL		91	97	6	4	8	**206**

In order to compare the density of *T. delaisi* larvae in inshore samples with those collected offshore, only periods when both inshore and offshore sampling was conducted, was considered. This segregated the sampling into 8 distinct periods: (1) 1-4 Jun 2011; (2) 9-10 April 2012; (3) 23-25 April 2012; (4) 16-18 May 2012; (5) 30-31 May-1 Jun 2012; (6) 10-11 July 2012; (7) 11-13 Jun 2013 and (8) 25-27 Jun 2013 (Table 3.3).

Table 3.3: The total number of Light trap samples collected during each sample period, according to distance from shore (inshore vs offshore), and depth strata (surface, middle and bottom).

	Inshore		**Offshore**			**Total**
Period	**Surface**	**Bottom**	**Surface**	**Midddle**	**Bottom**	
1	8	9	5	5	4	31
2	4	3	4	4	3	18
3	6	6	6	6	7	31
4	3	3	6	5	4	21
5	6	6	3	1	3	19
6	7	7	1	1	1	17
7	10	10	2	2	4	28
8	8	8	4	2	4	26
Total	**52**	**52**	**31**	**26**	**30**	**191**

For analysing vertical patterns in larval density across years, due to the extremely low numbers of larvae offshore, only inshore samples were considered; for that comparison, samples from Risco and Derrocada collected in June and July for the three years sampled were included. The sampling design is shown in Table 3.4.

Table 3.4. Light trap samples used to examine vertical patterns in larval density.

Year	**Bottom**	**Surface**	**Total**
2011	54	53	107
2012	24	24	48
2013	47	47	94
Grand Total	**125**	**124**	**249**

3.2.3 Laboratorial analysis

For both the light traps and the scooters, the plankton samples collected were stored in either 80% alcohol, or seawater with sodium borate buffered 4 % formalin, for at least one month before larvae were sorted and identified to the lowest possible taxonomic level. *T. delaisi* larvae were identified based on known morphological characteristics (see Chapter 4), enumerated, measured to the nearest 0.1 mm and assigned into one of four developmental stages (preflexion, early flexion, late flexion and post-flexion) according to the degree or extent of notochord flexion. Preflexion and postflexion stages were assigned following Leis and Carson Ewart, 2000. We used a finer classification for the

flexion stage, dividing these larvae into either early flexion (angle between urostyle and ventral body line < 45º) or late flexion (angle > 45º). Given the potential biases in measuring larval length when using two distinct fixatives (as different shrinkage can be expected), we restricted the ontogenetic analyses to the just the developmental stages observed as opposed to analyses by body length.

3.2.4 Data analysis

Spatial and Temporal Variation in Larval Density

Scooter

In the preliminary stages of data analysis, samples with volumes that could be considered as outliers were excluded, in order to prevent biases that maybe due to undetected sampling or reading problems. The detection of outliers was based on the method proposed by Leys *et al.*, 2013. This method utilises a "median ± median absolute deviation" as opposed to a "mean ± S.D." where

$$MAD = b\ M_i\ (|x_i - M_j\ (x_j)|)$$

x_j is the number of original observations and M_i is the median of the series. Usually, b = 1.4826. A rejection criteria of 2 (i.e. volumes were considered outliers when outside of the range Median ± 2MAD) which is considered poorly conservative was utilized. 9 samples were considered as outliers and were not used in analyses.

In each sample, the density of *T. delaisi* larvae was expressed as the number of *T. delaisi* larvae per m^3 of water. The density of each developmental stage was calculated in a similar manner.

Mean density of larvae per month was plotted in order to possibly visualize changes in patterns.

For spatial and temporal (interannual) comparisons, larval density of two similar periods for the two years sampled, were considered: 24th June – 27th July 2011 (62 samples) and 3rd – 31st July 2013 (23 samples). These periods were selected because they were sampled in both years and these were the months with the highest larval densities allowing for comparisons. Samples from Alpertuche were exclude from the analyses due to (1) the lower number of samples collected in that area and (2) the shallow nature of that site which would have made depth stratified sampling superficial. In order to keep an orthogonal design, samples from Pedra do Leão were also excluded from the analysis. A two-way ANOVA was used to investigate the relationship larval density and year and depth, after testing for homoscedascity and normality assumptions with the Levene's test, and residual analysis, respectively, after square root data transformation.

Light Traps

For the light trap samples, the density of individuals was expressed as the number of larvae collected per hour of sampling.

The number of larvae collected offshore was very low, hence only the periods with higher larval densities were analysed. Assumptions for parametric analysis were not met even after data transformation hence a Mann-Whitney U test was performed.

Temporal and inshore vs offshore differences in larval densities among samples could not be investigated with ANOVA because assumptions were not met, even after Log(x+1) and square root transformation of the data.

For analysing the effect of depth on the density of larvae collected with light traps in the different years, as parametric assumptions was not met after data transformation, a univariate PERMANOVA test was used for comparisons, after Log(x+1) data transformation, and using Euclidean distance as resemblance measure. Two factors were considered: depth as fixed factor, and year as a random factor. A Type III (partial) Sums of squares, given its most conservative nature for unbalanced designs. The Permutation method was the Permutation of residuals under a reduced model, considering 9999 permutations. Dispersion of significant factors was investigated with PERMDISP, also using 9999 permutations.

Vertical Ontogenetic Structure

Scooter and Light Traps

In order to compare the existence of possible vertical ontogenetic structure in the samples collected with both the scooter and the light traps, the relative abundance of each of the four developmental stages of *T.delaisi* was considered in each sample, considering all the samples collected with both scooter and light traps inshore in the month July.

The ontogenetic structure and its vertical pattern was investigated in samples collected by both the scooter and light traps method inshore near the reefs in July, by using a multivariate PERMANOVA test, after Log(x+1) data transformation, and Bray Curtis similarities. The two- way analysis considered Depth and Method as two fixed factors. A Type III (partial) Sums of squares was selected, given its most conservative nature for unbalanced designs. The Permutation of residuals were made under a reduced model, considering 9999 permutations. Given a significant interaction between factors, PERMANOVA pair-wise tests were also conducted, following the same procedures. Dispersion of significant factors was investigated with PERMDISP, using 9999 permutations.

3.3 Results

3.3.1. Temporal and spatial patterns of larval density with the Scooter method

The total number of *T. delaisi* larvae collected over the three years in the surface and bottom sample were 1236 and 1297 respectively. After excluding samples considered as outliers, the mean volume filtered per scooter sample was 9.41± 2.72 m^{-3} (N=196; range = 3.67 – 15.71).

For all months, mean larval densities were generally low. The highest value was obtained in July 2013 (1.46 larvae $m^{-3)}$ and the lowest value (0.47 larvae $m^{-3)}$ in August 2013 [Figure 3.4.A]. In terms of the developmental stages, the density of preflexion larvae dominated the scooter samples and it was much higher than that of the other three developmental stages, during all the months examined and across years. The density of preflexion larvae or that of more developed stages did not exhibit any clear temporal pattern of growth or development [Figure 3.4.B].

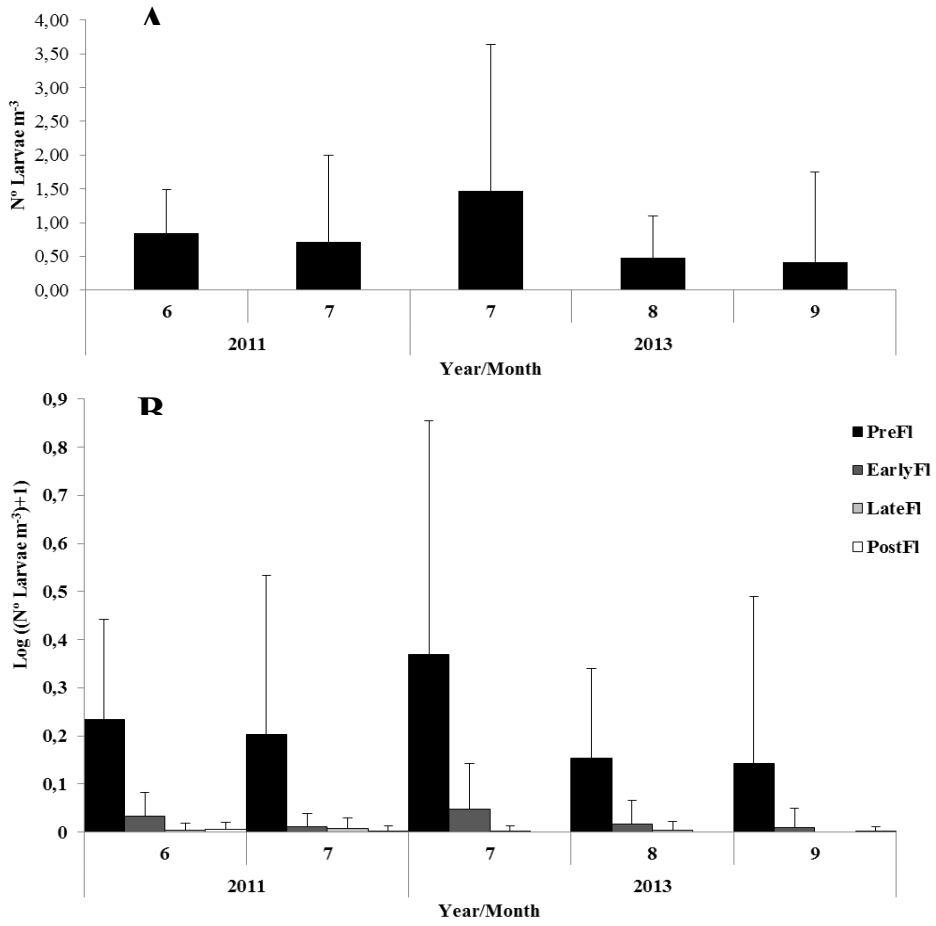

Figure 3.4. Bar plots of the mean density of *T.delaisi* larvae. (A) total larval density; (B) Density per developmental stage. Error Bars = S.D.

The results of the 2-way ANOVA (with square-root transformed data) for the period late June-July, revealed no significant differences between the density of *T. delaisi* larvae among depths (F (1, *N*=85) = 0.46, *N.S.*). Samples from 2013 however, had significantly higher mean larval densities than those of 2011, for the period considered (*F* (1, *N*=85) 6.55, *P*<0.05). There was no significant interaction between factors (*F* (1, *N*=85) = 1.02, *N.S*). In fact, there was no clear vertical pattern in any of the years investigated, with a great dispersion of the data being obvious, with some extreme values detected in both depths. [Figure 3.5.]

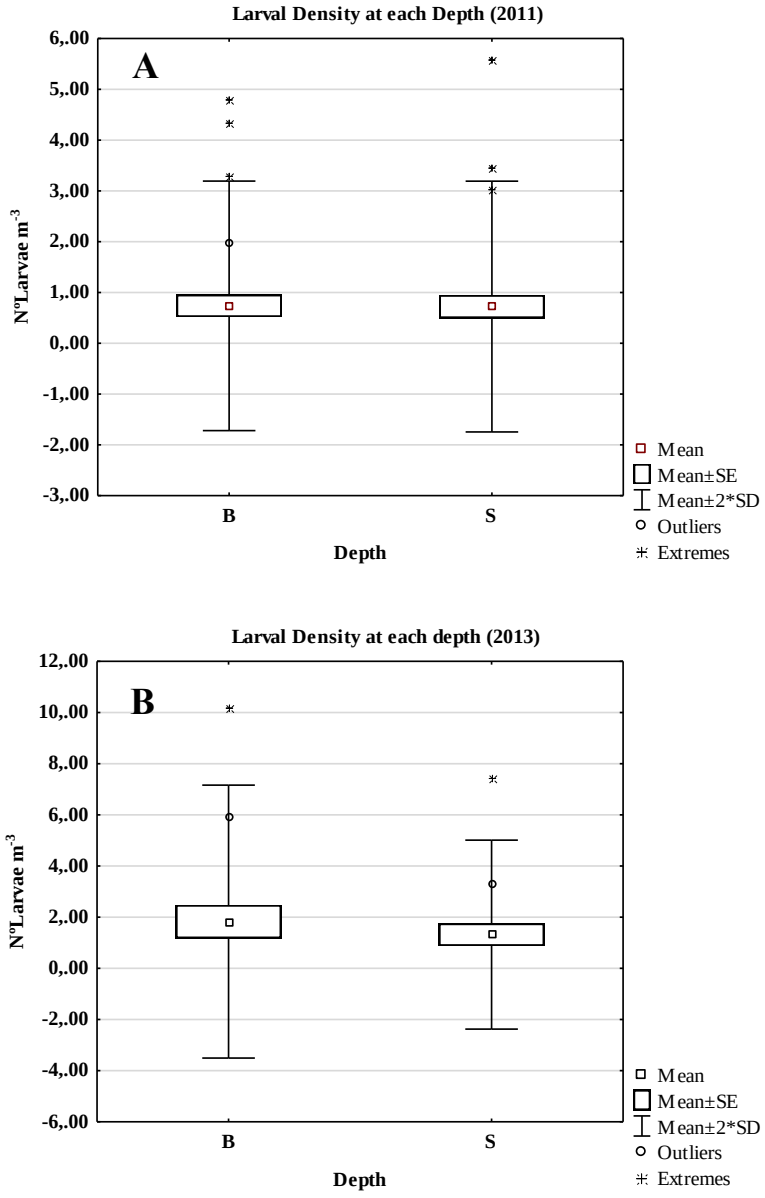

Figure 3.5. Box plots of larval density at the surface and bottom for scooter samples in (A) 2011 and (B) 2013.

3.3.2. Temporal and spatial patterns of larval density with the light traps method

A total of 1656 *T. delaisi* larvae were identified from all the light trap samples collected. 1647 larvae were from inshore samples and 9 from offshore samples. The total number and average number of larvae per hour in offshore and inshore samples in each of the selected periods is shown in Table 3.5.

Spatial Comparison (Inshore vs Offshore): Inshore samples had significantly more larvae/h than offshore samples. Considering only the two periods (1 and 2) in which more larvae were collected, in period 1 inshore samples had significantly higher larval density than offshore samples ((N= 17 Inshore, 14 Offshore), Z=3.57, p<0.001) and in period 2 no larvae were collected offshore [Figure 3.6].

Table 3.5: The mean larval density (no. of larvae/hr) in inshore and offshore samples in each of the selected periods

	INSHORE				**OFFSHORE**				**TOTAL**			
Periods	N	Total No. of Larvae	Mean	SD	N	Total No. of Larvae	Mean	SD	N	Total No. of Larvae	Mean	SD
1(1 - 4 Jun 2011)	17	239.38	14.08	34.11	14	3.87	0.28	0.43	31	243.25	7.85	25.87
2(9 - 10 April 2012)	7	332.84	47.55	99.28	11	0	0	0	18	332.84	18.49	63.62
3(23 - 25 April 2012)	12	33.93	2.83	5.78	19	1	0.05	0.23	31	34.93	1.13	3.76
4(16 - 18 May 2012)	6	5.78	0.96	1.9	15	0	0	0	21	5.78	0.28	1.05
5(30-31 May - 1 Jun 2012)	12	35.89	2.99	6.6	7	0	0	0	19	35.89	1.89	5.37
6(10 - 11 July 2012)	14	16.23	1.16	3.1	3	0.923	0.31	0.53	17	17.153	1.01	2.82
7 (11 - 13 Jun 2013)	20	44.09	2.2	5.14	8	0	0	0	28	44.09	1.57	4.43
8(25 - 27 Jun 2013)	16	9.9	0.62	1.29	10	1.935	0.19	0.61	26	11.835	0.46	1.09
Grand Total	104	718.04	6.9	30.18	87	7.728	0.09	0.31	191	725.768	3.8	22.48

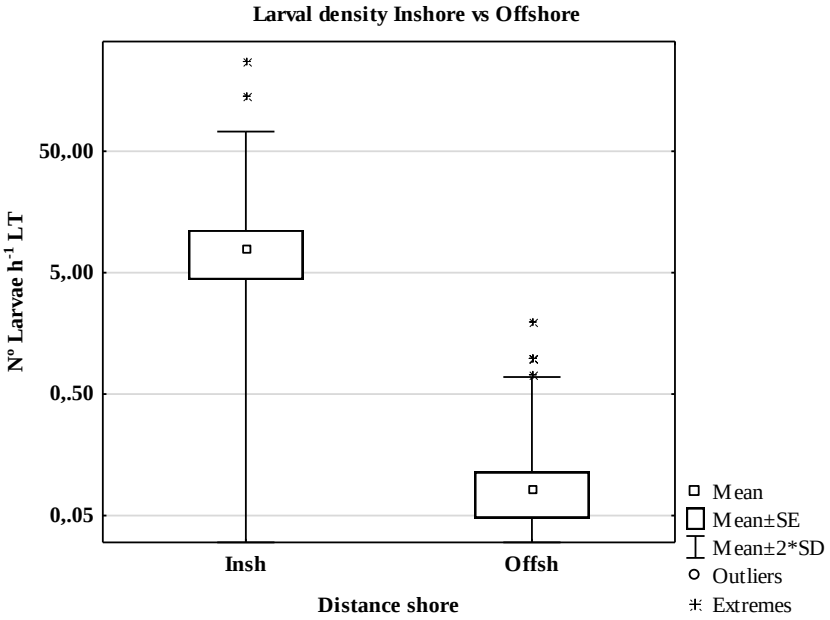

Figure 3.6: Box plots of larval density for the light trap samples collected in the inshore and offshore waters.

When only inshore samples of June and July from the three years sampled [Table 3.6] were analysed, no vertical differences on larval density were was found, but a significant intrennual variation was detected. The number of permutations possible was, nevertheless, low for the factor depth. The Permdisp result revealed that dispersion between years was not significantly different (Deviations from Centroid F = 5.3573, df 1 = 2, df 2 = 246, *N.S.*). Variation was very high at both depths in all the years sampled, with some extreme values (Figure 3.7).

Table 3.6: PERMANOVA Table of results

Source	df	SS	MS	Pseudo-F	P(perm)	Unique Perm
Year	2	5.5376	2.7688	3.018	0.0495	9956
Depth	1	0.21528	0.21528	0.50747	0.5093	335
Year x Depth	2	0.77274	0.38637	0.42113	0.6626	9950
Residual	243	222.94	0.91745			
Total	248	229.3				

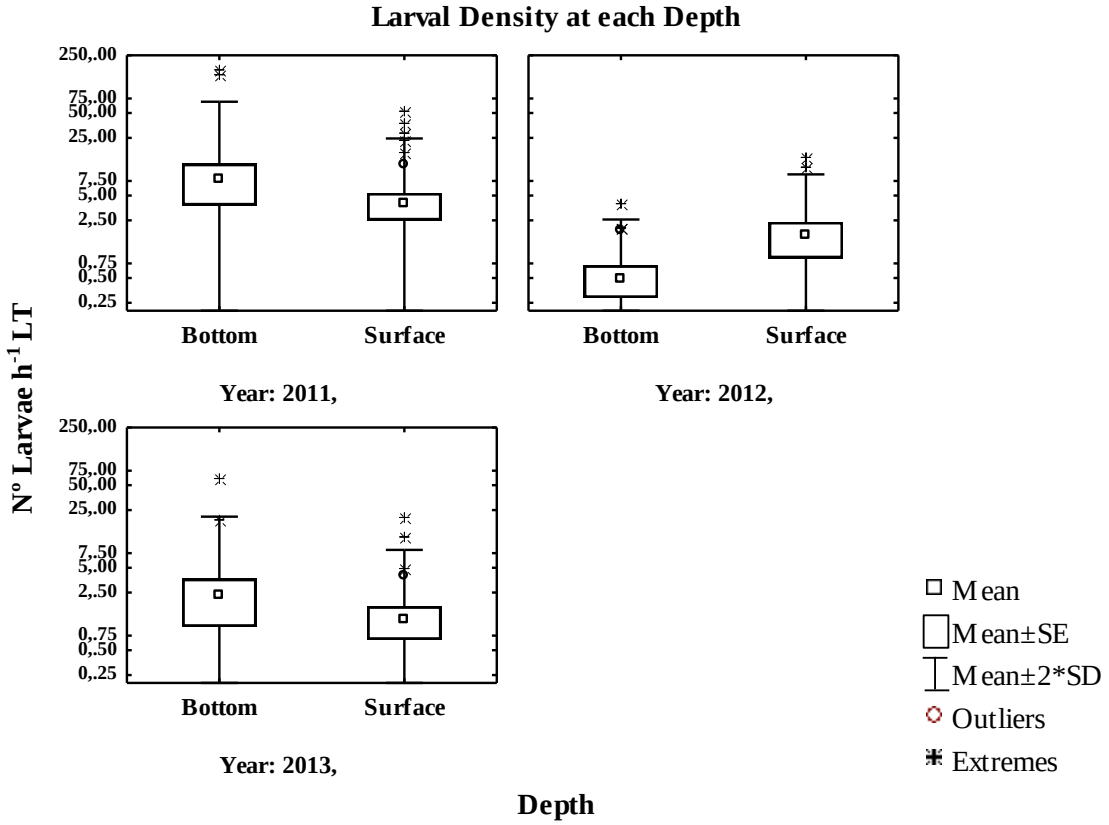

Figure 3.7. Box plots of larval density at each depth strata for light trap samples collected in (A) 2011 (B) 2012 and (C) 2013 plotted on a log scale.

Method efficiency

When considering only samples that collected larvae (positive samples), more larvae were collected with scooter despite the lower number of samples. Furthermore, more than 90% of samples had T.delaisi; opposite to this, only 7.7% (offshore) to 33.8 % (inshore) of Light trap samples had larvae of this species(Table 3.7).

Table 3.7: Percentage of positive samples collected with each method, containing T. delaisi larvae.

		SCOOTER				LIGHT TRAPS			
		NºTdel	Nº Samples with Tdel	Total Nº samples	% samples w/ Tdel	NºTdel	Nº Samples with Tdel	Total Nº samples	% samples w/ Tdel
Inshore	Surface	1080	72	73	98.6	915	70	207	33.8
	Bottom	1030	66	73	90.4	732	63	211	29.8
Offshore	Surface					3	3	32	9.4
	Middle					2	2	26	7.7
	Bottom					4	3	31	9.7

3.3.3. *Ontogenetic structure*

When plotting the mean density of each developmental stage of larvae collected with the scooter method across all the samples, it can be seen that the samples contained mostly pre-flexion stage larvae, at both depths. Variability was very high (Figure 3.8). On the other hand, in ligh trap samples, the mean density of preflexion stage larvae was lower than the more developed larvae; mean abundance of the more developed larval stages was higher in bottom samples, but variability was extremely high, at both depths and to all the developmental stages (Figure 3.9).

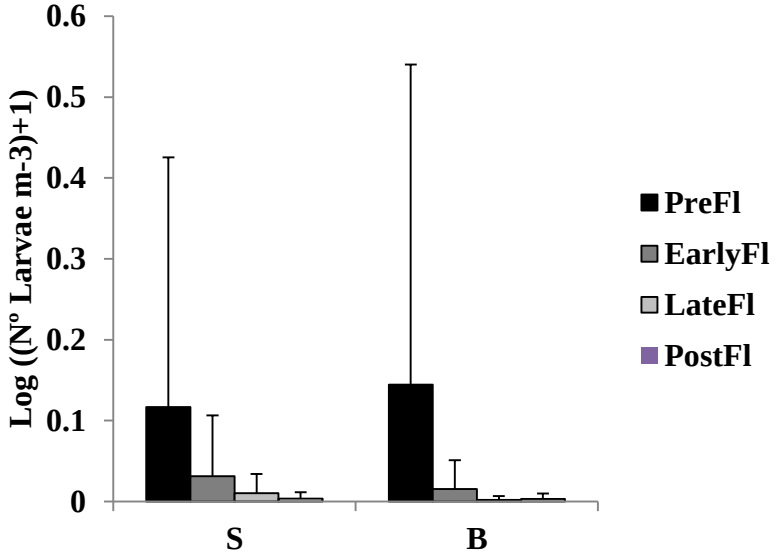

Figure 3.8 Bar plot of mean larval density (log (x+1) transformed) of each developmental stage at the surface and bottom for the scooter samples (all data pooled). S=Surface; B=Bottom. Error Bars =SD.

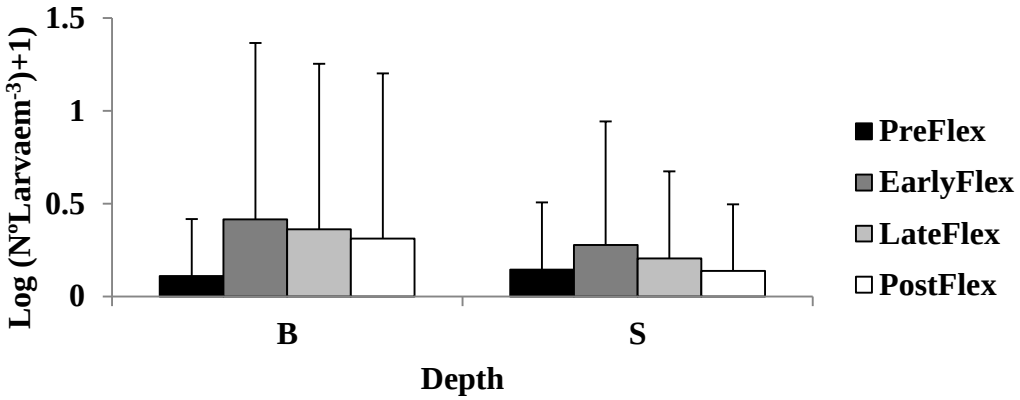

Figure 3.9 Bar Plot of mean larval density (log (x+1) transformed) of each developmental stage at the surface and bottom for the Light trap samples. S=Surface; B=Bottom. Error Bars =SD.

Vertical structure

When pooling all the larval data together (rather than mean numbers of each developmental stage as above), from all samples collected with the scooter and the light trap method, the resulting pattern is similar: larvae in all the four developmental stages are represented in both methods, but with more developed larvae proportionally more represented in LT samples than in the scooter samples. The number of larvae collected in the offshore samples were too few for such ontogenetic comparisons. This low number of larvae collected offshore also prevented inshore vs offshore comparisons on the ontogenetic structure [Figure 3.10].

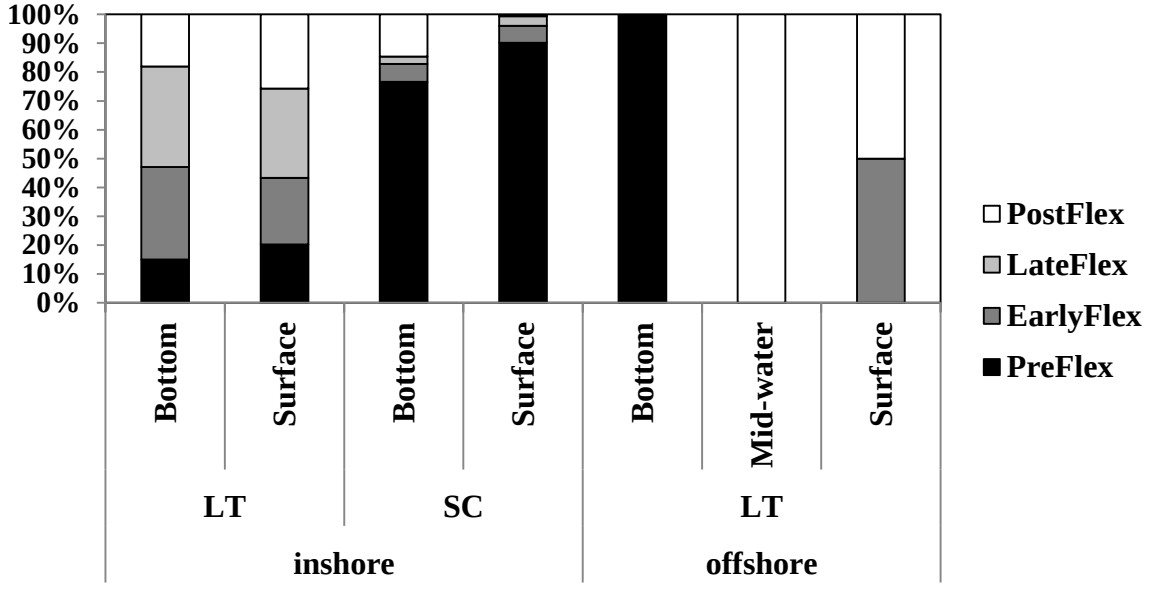

Figure 3.10 Bar plot showing ontogenetic differences between light trap and scooter samples.

When analysing the multivariate structure of all the samples with larvae, a significant interaction between depth and method was found (Table 3.7). Pairwise-PERMANOVA revealed differences in the vertical structure only in samples collected with the scooter method (Table 3.8; Figure 3.11).

Table 3.7: PERMANOVA Table of results for factors Method and Depth.

Source	**df**	**SS**	**MS**	**Pseudo-F**	**P(perm)**	**Unique Perm**
Method	1	2.2507E5	2.2507E5	147.78	0.0001	9966
Depth	1	3544.5	3544.5	2.3273	0.0863	9967
MethodxDepth	1	9501.9	9501.9	6.2391	0.0005	9968
Residual	295	4.4927E5	1523			
Total	298	6.8802E5				

Table 3.8: Results of PERMANOVA pair-wise comparisons for Bottom vs Surface samples, for each method.

METHOD	**t**	**PPerm**	**Unique P(perm)**
Light trap	1.185	0.2469	9961
Scooter	4.029	0.0001	9958

The PERMDISP result (F=310.87, df1 1, df2 297, PPerm < 0.001) showed significantly different dispersion between both methods. Nevertheless, the MDS graphic reveals a clear spatial separation of scooter and light trap samples (Figure 3.11).

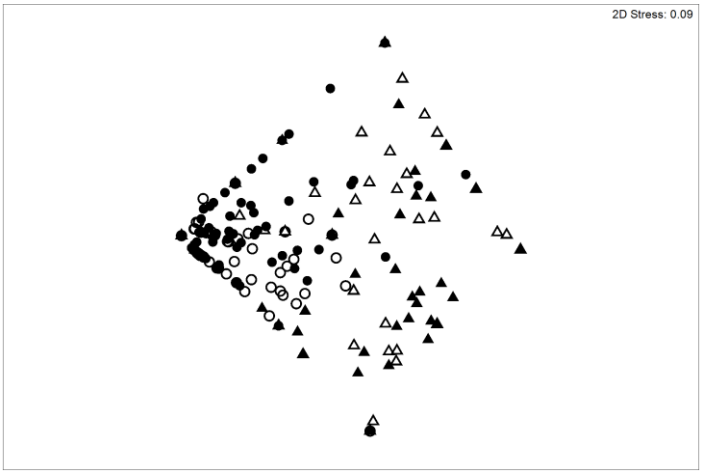

Figure 3.11 Structure of inshore samples. Triangles = Light trap; circles = scooter, open symbols = surface, closed symbols = bottom.

Pair-wise comparisons within each depth, also revealed a significant effect of the method on the ontogenetic structure of the samples (P(perm)<0.01).

3.4 Discussion

An examination of the spatial and temporal distribution of *T. delaisi* larvae in the Arrábida Marine Park showed that (1) all the different larval ontogenetic stages of *T. delaisi* (preflexion, flexion and postflexion) were present in the nearshore environment; (2) the abundance of *T. delaisi* larvae was greater inshore compared to offshore; (3) there were differences in the ontogenetic composition of *T. delaisi* caught in the scooter and light traps - the ratio of preflexion larvae to more developed larvae was greater in the scooter samples compared to the light trap samples (4) vertical differences in the ontogenetic structure of samples collected at distinct depth strata could be detected.

The mainly inshore larval distribution coupled with the occurrence of all the larval ontogenetic stages inshore, indicates that *T. delaisi* larvae can be locally retained within the nearshore environment where it may be completing its larval life. These results support previous studies which describes species belonging to the Family Tripterygiidae as being species retained "close to the reef" (Marliave et al., 1986; Kingsford and Choat, 1989; Sabatés et al., 2003). In the Mediterranean, a very restricted larval distribution near to the inshore rocky areas was detected for *Tripterygion tripteronotus*, also suggesting that local retention maybe occurring (López-Sanz et al., 2011) for this species belonging to the same Genus. Nevertheless, since larval origin is unknown,

there is no direct evidence that the larvae present in the area were spawned from adults in that area, however, it is highly likely given the physical characteristics of the study area. The AMP is separated from the two nearest rocky reef habitats to the north and south, Cascais and Sines respectively by a large expanse of sand. As such it can be considered as a continental island with rocky habitat.

For demersal fishes such as *T. delaisi* which inhabit mainly nearshore rocky habitats, small-scale local processes can be influential. Nearshore environments tend to be characterized by shallow depths, the influence of continental shelf features, freshwater inputs, and a multitude of diverse local hydrodynamic processes (tides, fronts, eddies, internal waves, bores, and coastal boundary effects) which make water flow in these environments very complex and different/distinct from the open ocean (Pineda *et al.*, 2007). Small-scale local processes occurring within the AMP are unknown. There are however several features which can (based on the literature) function to facilitate or promote local retention. The interaction between shallow depths, bottom topography and prevailing alongshore currents can create multi-directional layers of flow. Such conditions can result in the reduction of water flow near the epibenthic boundary layer increasing the chances that larvae that stay near the bottom can be retained (Pineda *et al.* 2007). In the nearby coastal area of the AMP, upwelling events occur. In such situations it is expected that strong winds combined with the Coriolis effect would drive surface waters together with larvae offshore (Ekman transport), potentially reducing local retention and recruitment, leading to increased population connectivity. In this scenario, it is only during periods of relaxation due to weaker winds or a reversal of wind direction that onshore transport of larvae may occur (Farell *et al.*, 1999; Roughgarden et al., 1988). Results in contrast to these expectations however are increasingly being found i.e. high levels of local retention in areas of intense upwelling have been documented. Departure from the traditional expectation has been attributed to physical factors such as jet cores (Harrison and Siegel, 2014), the influence of coastal topographic forms such as headlands (Roughan, 2005), the coastal boundary layer (CBL) (Morgan et al., 2009), and larval behaviour such as vertical migrations in the water column (Marta-Almeida et al., 2006; Weidberg *et al.*, 2015; Morgan et al., 2009; Morgan and Fisher, 2010).

Additional support for local retention is also provided by the detection of vertical ontogenetic structure which indicates that larvae are able to exert some level of control over its vertical position in the water column. This vertical positioning behaviour can be used to mediate horizontal movement

There were differences in the ontogenetic composition of *T. delaisi* caught in the scooter and light traps in that scooter samples comprised mainly of preflexion larvae whilst the light traps collected all the stages including the post-flexion stages. Our findings support previous studies.

In a comparison of both methods, Hickford and Schiel, 1999 found that fish larvae collected in the light trap samples were larger (had a significantly higher mean standard length) than those collected in the plankton samples for all taxon collected. Additionally, size frequency analyses of the samples showed that smaller individuals were more common in the plankton nets.

Several studies comparing the use of light traps and plankton nets have drawn one or more of the following general conclusions: (1) light traps collect larger larvae on average (2) the size structure of the larvae collected by both methods usually vary and (3) a combination of both methods is beneficial as it results in the sampling of a more complete range of size classes, ages and developmental stages (Brogan, 1994; Hernandez and Lindquist, 1999; Hickford and Schiel, 1999; Hernandez and Shaw, 2003; Thorrold, 1992). Scooter samples were more consistent in collecting larvae that were essencially little developed; On the other hand, despite light traps were more efficient in collecting more developed larvae, captures were not so consistent as compared to scooter samples. The passive nature of light trap sampling as well as the clustered distribution of larvae in the water, in particular of those more developed, can help explaining these differences.

3.5 References

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CHAPTER 4: LARVAL DEVELOPMENT AND ALLOMETRIC GROWTH OF THE BLACK-FACED BLENNY, *TRIPTERYGION DELAISI*

Larval development and allometric growth of the black-faced blenny, *Tripterygion delaisi*

F.N. Solomon[1], D.D. Rodrigues[2], E.J. Gonçalves[2], E.A. Serrão[1] and R. Borges[1,2*]

4.1 Abstract

This study describes larval development and allometric growth patterns of the black-faced blenny *Tripterygion delaisi*, from a larval series caught by light traps. This rocky subtidal species which occurs in both the Atlantic and the Mediterranean, is a good model for investigating population connectivity and the occurrence of self-recruitment and local retention, due to its high abundance of early stages near rocky reefs, and some evidence of local growth and limited dispersal. Morphologically, *T. delaisi* larvae exhibit some shared characteristics with the larvae of other species of the Suborder Blennioidei and the Family Tripterygiidae. Here we describe the distinctive morphometric and meristic characteristics which are central to identifying this species from related taxa. Growth coefficients generated from bivariate morphological relationships indicated that most of the body proportions of *T. delaisi* exhibited allometric growth during larval development. When inflexion points of growth were detected, growth was biphasic with the inflexion points occurring within a very narrow range of body length (L_B) = 8.7 to 8.9 mm. Considering allometric growth patterns and ontogenetic descriptions together, the 1st developmental phase includes the preflexion and flexion stage larvae, while the 2nd phase characterises the postflexion larvae prior to the transition from larvae to juvenile. This change in growth (inflexion point) was accompanied by a marked change in body form, from elongated to deeper bodied. Previous descriptions of *T. delaisi* early life stages are scarce and incomplete, and there is still a large gap in detailed morphometric and meristic data for many temperate reef fish species. This detailed information that allows unequivocal identification of fish early life stages is key for taxonomic and ecological studies, especially in temperate waters where efforts have historically been more centred on commercial species rather than on other components of marine biodiversity.

Keywords: *Tripterygion delaisi*; ontogenetic larval development; allometric growth

4.2 Introduction

The pelagic larval phase of most demersal marine fish is a vital stage of their life-cycle with important consequences for recruitment, population dynamics, and dispersal and connectivity (Bergenius *et al.*, 2002; Cowen & Sponaugle, 2009). Despite their importance, the accurate identification of fish larvae, which are morphologically very different from the adults, is a major obstacle in many studies aimed at understanding their early life history and ecology. Difficulties in larval sampling, coupled with small larval size and significant changes in body proportions and pigmentation patterns during development, make larval taxonomy an extremely challenging field. Larval descriptions, which continue to play an important role in identification, despite the advent of genetic approaches (Leis, 2014), are frequently absent or incomplete making identification to the species level tentative at best and many times impossible. These limitations are magnified in temperate nearshore reef environments which are difficult to sample, have received much less attention from marine taxonomists, and are thus often less studied than offshore temperate systems and tropical coral reef environments.

One group of fish that is abundant in the temperate coastal waters of the northeast Atlantic and Mediterranean is the triplefin blennies belonging to the genus *Tripterygion* (Family: Tripterygiidae) (Sabatés *et al.*, 2003; Bertoncini *et al.*, 2010). This genus comprises the species *Tripterygion melanurus (T. melanurus)* (Guichenot, 1850), *Tripterygion tripteronotus* (*T. tripteronotus)* (Risso, 1810) and *Tripterygion tartessicum* (*T. tartessicum)* (Carreras-Carbonell *et al.*, 2007b) which are endemic to the Mediterranean, and *Tripterygion delaisi (T. delaisi)* (Cadenat & Blache, 1970) which occurs in both the Atlantic and the Mediterranean (Wirtz, 1980; Zander, 1986). Two subspecies of *T. delaisi* have been recognized, *T. delaisi xanthosoma* and *T. delaisi delaisi* (Zander & Heymer, 1970), with the recent validation of two genetically distinct clades occurring in the Atlantic islands, and the in the Atlantic coast of Europe and the Mediterranean (Carreras-Carbonell *et al.*, 2005; Domingues *et al.*, 2007).

Previous studies on the composition of larval fish assemblages at our study site (the Arrábida Marine Park, Portugal), indicate that *T. delaisi* larvae are abundant in this area and that their distribution appear to be restricted to the nearshore environment where they maybe completing their life-cycle locally, as evident by the co-occurrence of multiple developmental stages (Beldade *et al.*, 2006; Borges *et al.*, 2007; Borges *et al.*, 2009). Due to its early life history characteristics (the production of benthic eggs, an inshore larval distribution, limited adult mobility and short PLD) which may favour low larval dispersal, this species has been

the focus of several studies aimed at investigating population connectivity and the occurrence of self-recruitment and local retention (Carreras-Carbonell *et al.*, 2006; Carreras-Carbonell *et al.*, 2007a; Galarza *et al.*, 2009; Schunter *et al.*, 2014). Its relatively high abundance in a subtidal environment also makes it fairly accessible for both capture and *in situ* observations and hence a good species to investigate various aspects of territoriality (Gonçalves & Almada, 1998), breeding behaviour (De Jonge & Videler, 1989) and habitat selection in rocky reef fish communities (La Mesa *et al.*, 2004; La Mesa & Vacchi, 2005; La Mesa *et al.*, 2006).

Morphological descriptions of *T. delaisi* adults and juveniles exist (Wirtz, 1980; Zander, 1986; Orlando-Bonaca & Lovrenc, 2010) however, there are no detailed descriptions of the larval stages for this species. In our study site, *T. delaisi* is the only triplefin present. Relatively calm conditions at this site allow sampling of the very nearshore marine environment, where larvae in different developmental stages can be collected. This provides ideal conditions to collect and describe the larval development of this species based on wild-caught specimens.

Also of relevance during early larval development, is the phenomenon of allometric growth whereby different body parts develop at different rates, leading to complex shape changes during the transition from juvenile to adult (Fuiman, 1983; Osse & Van Den Boogaart, 1995; Van Snik *et al.*, 1997). The objective of this study is therefore to describe the ontogenetic development and allometric growth patterns in the *T. delaisi* larvae caught by light traps near the reefs inhabited by adults.

4.3 Methods

4.3.1 Sampling

Larvae (N=235) were sampled at the Arrábida Marine Park (AMP) (38º 28’ N, 8º 59’W), Portugal, using light traps (AIMS design) (Meekan *et al.*, 2001) deployed for 1 hour periods in the extreme nearshore environment, both below the surface and close to the bottom, at maximum depths of 8m on the reef, during the period April to September 2007 and 2008. Samples were stored in 4% saline formalin buffered with sodium borate for at least one month. To complete the collection, two yolk sac larvae caught by light-traps from the AMP in 2013 were also used only for morphological descriptions of this stage. These specimens which were preserved in 80% alcohol were not included in the allometric analyses.

After sorting the plankton samples, *T. delaisi* larvae were identified, measured (to the nearest 0.01mm) and assigned to one of four developmental stages (yolk-sac, preflexion,

flexion and postflexion) as described by Kendall *et al.*, 1984 and by Leis & Carson-Ewart, 2000. Each individual was examined in further detail in order to (1) document relevant aspects of ontogenetic development and (2) measure morphological parameters. Sorting, examination and measurement of all the larvae were conducted using an Olympus SZ-PT binocular microscope equipped with an ocular micrometre and coupled with an Olympus SC35 camera. Early recruits (N=5) ranging between 12.98-16.61 mm L_B, were collected near the reefs using hand nets during SCUBA in August and September 2014 and were included to illustrate the change in pigmentation patterns during the transformation from postlarva to juvenile. They were stored in 80% alcohol.

4.3.2 Ontogenetic development

The ontogenetic description of each individual assigned to a developmental stage (yolk sac, preflexion, flexion and postflexion) was detailed with the main ontogenetic events noted: (1) notochord flexion; (2) fin development; (3) vertebral ossification; and (4) changes in pigmentation patterns.

Fuiman's Ontogenetic Index

Fuiman's Ontogenetic Index (Fuiman, 1994) was calculated in order to express the state of a larva at any point in time in its ontogeny, as a percentage of a logarithmic developmental period, where

$$\mathbf{I_O} = \log L_B / \log L_{JUV} \times 100$$

$L_{JUV} = L_B$ at the beginning of the juvenile period

This index represents the percentage of development that has taken place before a given size. Using L_{JUV} to calculate I_O corrects for interspecific size differences allowing comparisons within and between taxa, while the logarithmic transformation reflects the multiplicative nature of ontogeny (Fuiman & Higgs, 1997).

4.3.3 Allometric growth patterns

Allometric growth patterns were examined using bivariate morphological relationships. Morphometric measurements were recorded to the nearest 0.01 mm. These measurements included: body length (**L_B**) from the tip of the snout to the end of the notochord in the preflexion and flexion larvae, and to the last vertebrae or the caudal

peduncle (usually referred as standard length) in the postflexion stages; total length **(L_T)** from the tip of the snout to the end of the caudal fin in postflexion larvae; pre-anal length (**L_{PA}**) from the snout to the anus; head length (**L_H**) from the tip of the snout to the end of the operculum; head depth (**D_H**) from the bottom of the mouth cavity to the top of the head; eye diameter (**D_E**) in an anterior-posterior plane; and body depth at the anus (**D_{BA}**). Postanal length (**L_{POA}**) was calculated by subtracting pre-anal length from body length (**L_B**). Allometric growth was then modelled by a power function of X (X=**L_B** or X=**L_H**) using non-transformed data as $Y=aX^b$ where Y is the measured character, or dependent variable, and b is the growth coefficient (Fuiman, 1983). Equations were then log transformed and the null hypothesis of isometric growth (H_0: b=1) was tested using the T-test for $\alpha=0.05$. When b=1 isometric growth is occurring, when b>1, positive allometric growth is occurring and when b<1, negative allometric growth is occurring. Additionally, linear regressions were performed on log-transformed data (L_B or L_H as the independent variable) and the inflection points, when existent, represented by the X values where the slope of growth changes, were calculated according to van Snik *et al.*, 1997. The x-y data set for each morphological character measured was sorted in ascending order of x. Regression lines were then generated for x_{min} to $x_{intermediate}$ and for $x_{intermediate}$ to $x_{max,}$ where $x_{intermediate}$ varied iteratively from $x_{min}+2$ to $x_{max}-2$. *T*-tests ($\alpha=0.05$) were subsequently performed to determine whether the growth coefficient of each pair of regression lines generated differed significantly. The $x_{intermediate}$ value that iteratively showed the largest t was defined as the inflexion point.

4.4 Results

4.4.1 Ontogenetic development

A description of ontogenetic development for the four developmental stages (yolk sac, preflexion, flexion and postflexion) was performed. In total two hundred and thirty five *T. delaisi* larvae ranging between 3.3 and 12.1 mm L_B were examined [Table 4.1]. Whilst all attempts were made to provide as much details as possible, damages to fins from sampling and handling during the sorting process made the accurate counting of fins and rays difficult in some instances. Emphasis was placed on characteristics that were easily distinguishable and verifiable (as these would be more useful for identification purposes of wild-caught larvae), rather than ones that were highly variable and exhibited a high degree of overlap.

Table 4.1. *Tripterygion delaisi* larvae examined according to developmental stage, mean body length (**L_B**) and size range. S.D. = Standard deviation; N = no. of individuals

Stage	**N**	**Mean L_B ± S.D. (mm)**	**Size Range (mm, L_B)**
Yolk-sac	2	3.80 ± 0.28	3.6–4.0
Preflexion	98	5.08 ± 0.82	3.30–7.06
Flexion	81	6.98 ± 0.86	5.13–9.19
Postflexion	54	9.44 ± 1.48	6.10–12.10

(1) ***Yolk-sac*** larvae [Figure 4.1. (A)] measured 3.80±0.28 mm L_B (N = 2; L_B = 3.6 and 4.0 mm). These larvae were translucent and had fully pigmented eyes, a clearly discernible circular yolk sac (approximately 0.6 mm and 1.0 mm in diameter, respectively), pectoral fin buds and an open mouth. The body was completely surrounded by a finfold and the anus, which was also open, was anteriorly located, about 33% L_B along the body. Although both preanal and postanal myomere counts were difficult, at least 12 preanal and 33 postanal myomeres were enumerated. Pigmentation at this stage was sparse, occurring in the form of pigmented eyes, pigments in the dorsal region of the gas bladder and at the end of the anus (opening), and a row of regularly spaced postanal ventral punctuate melanophores (visible from the 4^{th} or 5^{th} post ventral myomere and ending on the second to last) [Figures 4.1. (A) and 4.2.]. The otoliths were already clearly visible.

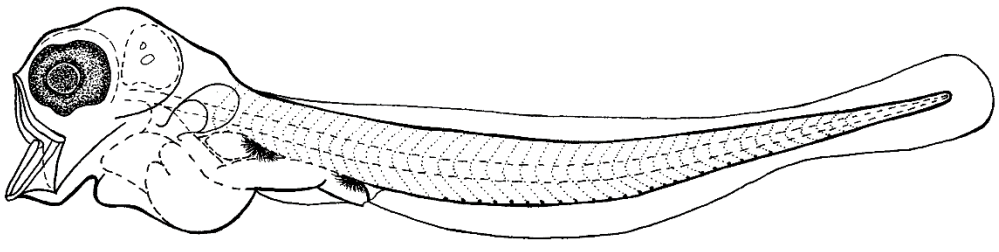


Figure 4.1. (A) *Tripterygion delaisi* yolk sac larva (4mm L_B)

(2) ***Preflexion*** specimens [Figure 4.1. (B)] ranged between 3.3 to 7.06 mm L_B (N=98) with a mean of 5.09±0.08 mm L_B. The yolk sac was not visible (having been completely reabsorbed), the entire body was still completely surrounded by its finfold and, although the caudal fin anlage appeared in 38% of the individuals (N=37; 5.0–6.58 mm L_B; mean ±SD = 5.83±0.39 mm L_B) indicating the beginning of caudal fin development [Figure 4.2.], the notochord had not yet started to flex. The start of vertebral ossification was observed in 56 individuals (57% of preflexion larvae) ranging in size from 5.03 to 7.06 mm L_B (mean±SD = 5.66±0.48 mm L_B) and the presence of teeth was observed in 13 individuals (13% of preflexion larvae) ranging in size from 6.0 to 7.06 mm L_B (mean±SD = 6.34±0.31 mm L_B). Pigmentation at this stage was similar to the yolk sac stage. Post ventral melanophores varied from14-27 in number. Due to this variability, the number of pigments was not considered a good character to be used in identification and hence was not subsequently enumerated.

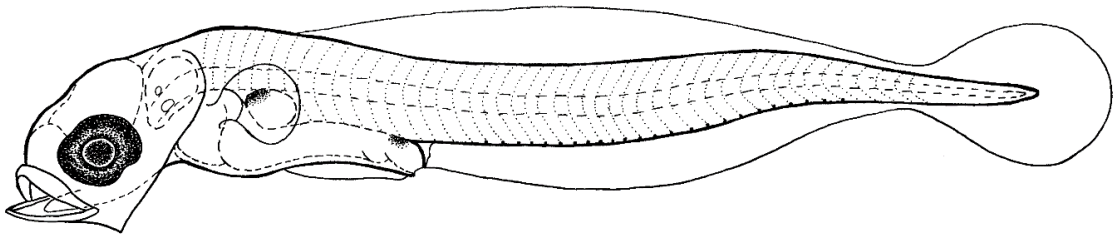
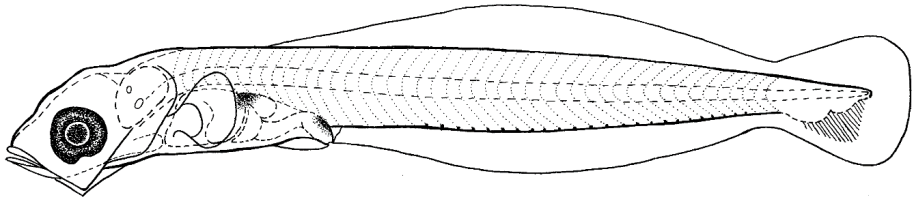


Figure 4.1. (B) *Tripterygion delaisi* preflexion larva (4.25mm L_B)

(3) The ***flexion*** stage specimens (N=78) ranged between 5.13–9.19 mm L_B (mean±SD = 6.97±0.87 mm L_B) [Figures 4.1. (C) and 4.1. (D)] and was characterized by the upward inclination (flexion) of the posterior part of the urostyle, the coiling of the gut, the continuation of caudal fin development and the appearance of dorsal pigmentation in the head region [Figures 4.1. (D) and 4.2.]. Head pigmentation (N =15(20%); range = 6.87-9.19 mm L_B; mean±SD = 7.97±0.67 mm L_B) comprised of either a single internal midline melanophore in the posterior region, or this single melanophore together with one or two additional external melanophores on the hind brain. In some specimens (N = 6 (22%); range = 8.13-9.19 mm L_B; mean±SD = 8.54±0.39 mm L_B) the 3rd dorsal fin and the anal fin had commenced development [Figure 4.1. (D)]. These individuals were in the late stages of flexion with the

notochord tip having already reached its final position at an angle of about 45 degree and the principal caudal fin rays and supporting skeletal elements nearing the adult longitudinal position.

C



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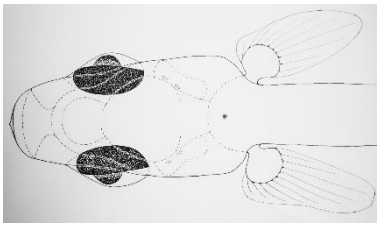
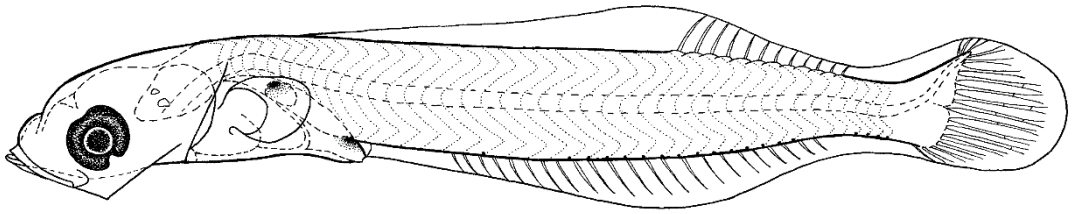




Figures 4.1. *Tripterygion delaisi* (C) flexion larva (6.29 mm L_B) and (D) late flexion larva (7.16 mm L_B) Inset = head pigmentation

(4) The ***postflexion*** stage [Figure 4.1. (E)] (N = 53; range = 6.1–12.1 mm L_B; mean±SD = 9.42±1.4 mm L_B) was mainly characterized by fin development. The 3rd dorsal fin and the anal fin had begun development in all postflexion specimens examined [Figure 4.2.]. Throughout development these two fins developed in close synchrony and were always present together. Based on a comparison of the extent of ray development, it is most likely that the 3rd dorsal started to develop just prior to the anal. The pectoral fins were next (N = 21 (40%); range = 9.19-12.1 mm L_B; mean±SD = 10.64±0.82 mm L_B) with the actual rays only becoming visible then, even though the buds were detected from as early as the yolk sac stage. The pelvic fins, which followed, were difficult to detect and first visible at 9.36 mm L_B (N = 12(15%); mean±SD = 10.84±0.82 mm L_B). The 2nd (N = 3(4%); range = 11.61-12.1 mm L_B; mean±SD = 11.90±0.26 mm L_B) and 1st dorsal fins (N = 2(2%); range = 12.0-12.1 mm L_B; mean±SD = 12.05±0.07 mm L_B) were the two last fins to initiate development, in that order. Complete fin development was recorded for only two individuals (L_B = 12.0 and L_B = 12.1 mm). Diagnostic fin counts were: dorsal (III+XVI+13 and III+XVIII+12) and anal (II+26 and II+27) which were in agreement with that recorded for adult *T. delaisi* (Zander, 1986). Vertebral ossification was complete in these two individuals having commenced during the preflexion stage.

The pigmentation pattern at this stage was the same as the previous stages with just some minor changes. Head pigmentation in some individuals (N=43(81%); range = 7.55-12.1 mm L_B; mean±SD = 9.91±0.95) showed an increase in the number of melanophores on the hind brain, ranging from (1-4) [Figure 4.1. (E)]. The most common pattern (N=27) was two small external melanophores on the hind brain and a larger internal posterior midline melanophore. The midline melanophore could easily be overlooked in some individuals due to an increase in the density of the overlying tissue. Caudal fin pigmentation in the form of several individual (1-6) punctate melanophores along the vertical base of the caudal fin was observed for the first time during this stage (N=26 (37%); range = 9.0-12.1 mm L_B; mean±SD = 10.31±0.91) [Figures 4.1 E and 4.2]. In the late postflexion stages, the postanal ventral pigments, which were present from the yolk sac stage, now resemble a series of short L-shaped slashes of black pigment (**L_B** = 12.0 and **L_B** = 12.1 mm) [Figure 4.1. (E)].

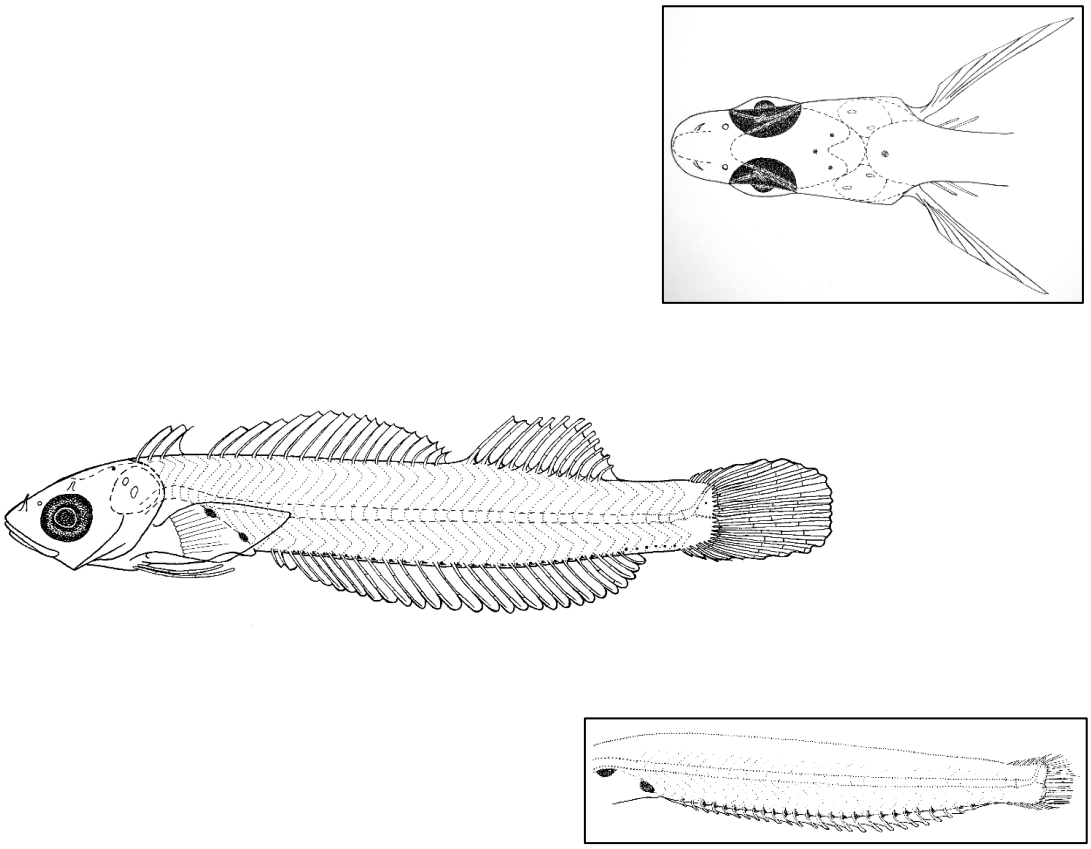


Figure 4.1. (E) *Tripterygion delaisi* postflexion larva (11.61 mm L_B). Inset: above - head pigmentation; below – postanal ventral pigmentation

Early recruits [Figure 4.1. (F)] were much more heavily pigmented than the larvae, with dermal pigmentation in both the head and body area. The five broad vertical bars on the flanks which are characteristic of the adults are already present though somewhat incomplete and not as well defined. The last bar forms a spot on the caudal peduncle which extends out onto the base of the caudal fin. Fin development was complete and in agreement with that recorded in the literature for the adults. The size and age at which *T. delaisi* started to settle was not determined in this study however we estimate size-at-settlement to range between 12-13 mm L_B (unpublished data).

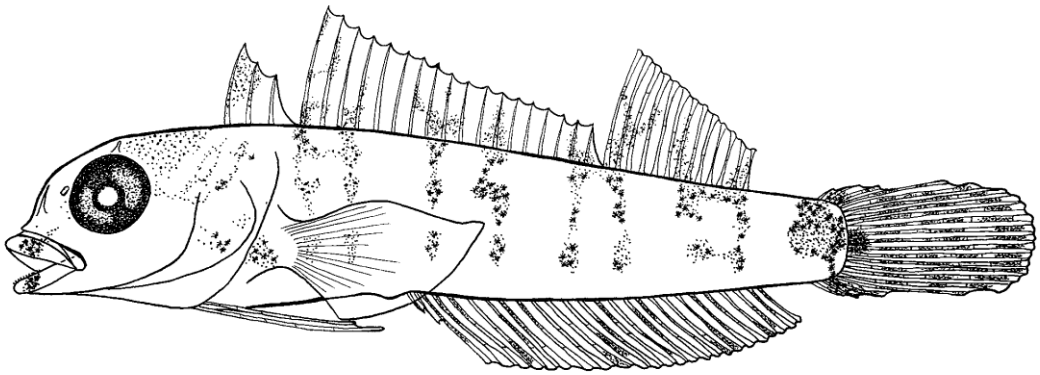
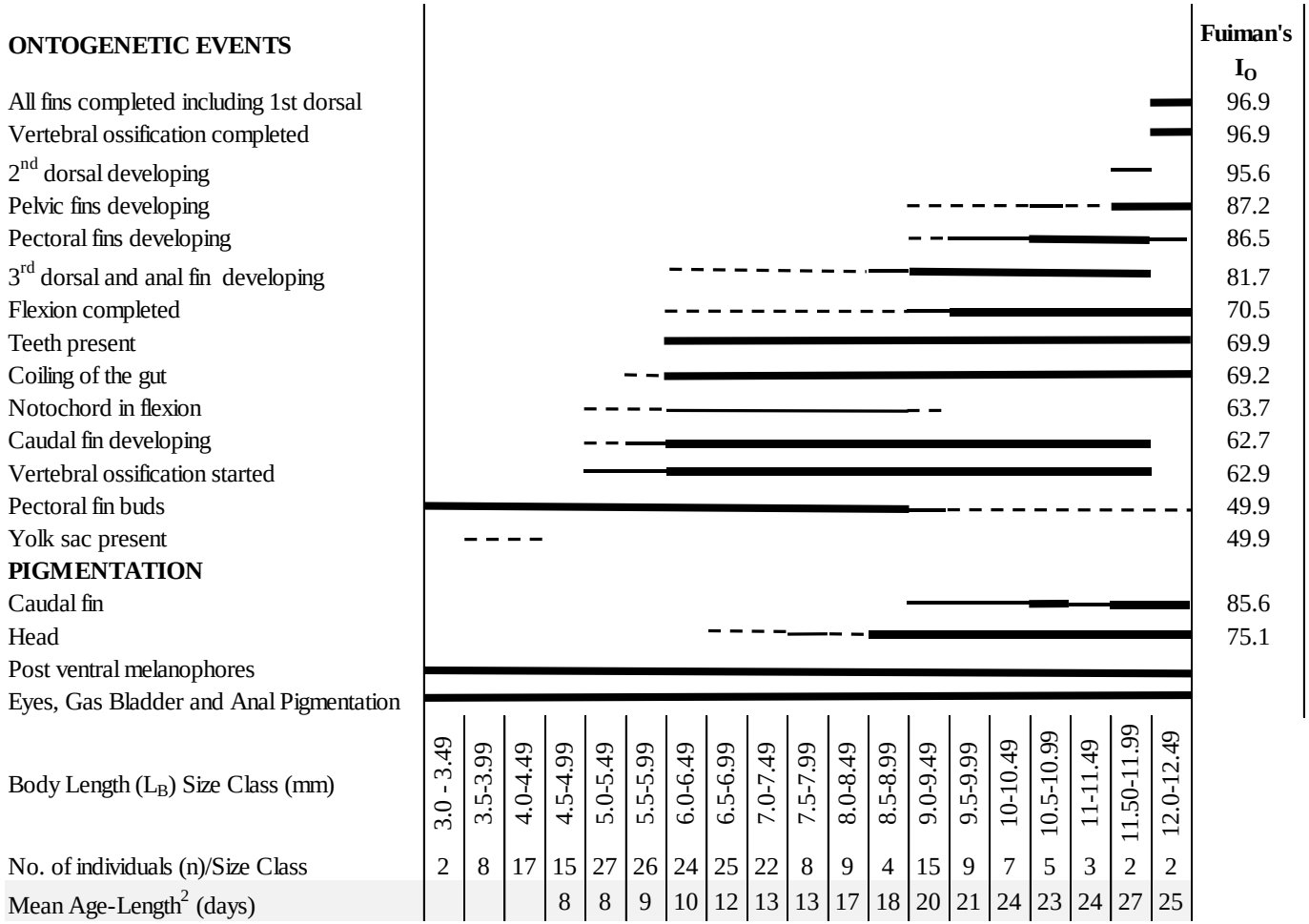


Figure 4.1. (F) *Tripterygion delaisi* early recruit

Myomere counts: myomere counts were performed in 197 individuals (2 yolk sac, 79 preflexion, 38 flexion and 78 postflexion). In total, the minimum number of myomeres recorded, for any individual, was 37 and the maximum 45. Preanal myomeres ranged from 7-13, whilst postanal myomeres ranged from 29-33. Myomeres were generally easier to count during the preflexion and flexion stages, as these larvae were more translucent. During the preflexion stage care must be taken not to over count myomeres at the posterior region of the notochord.

Using L_{JUV} = 13.0 mm LB based on field observations, Fuiman's Ontogenetic Index (I_o) ranged between 49.9 to 96.9 with much of the fin development, (with the exception of the caudal fin) occurring in the later stages of development (I_o = 81.7 -96.9).



Body Length (L_B) Size Class (mm)	3.0 - 3.49	3.5-3.99	4.0-4.49	4.5-4.99	5.0-5.49	5.5-5.99	6.0-6.49	6.5-6.99	7.0-7.49	7.5-7.99	8.0-8.49	8.5-8.99	9.0-9.49	9.5-9.99	10-10.49	10.5-10.99	11-11.49	11.50-11.99	12.0-12.49
No. of individuals (n)/Size Class	2	8	17	15	27	26	24	25	22	8	9	4	15	9	7	5	3	2	2
Mean Age-Length[2] (days)				8	8	9	10	12	13	13	17	18	20	21	24	23	24	27	25

Figure 4.2. The sequence of main ontogenic events including the development of pigmentation patterns during larval development of *Tripterygion delaisi*.

4.4.2 Allometric growth patterns

Two hundred and thirty three *T. delaisi* larvae ranging between 3.3 and 12.1 mm $L_{B,}$ including preflexion, flexion and postflexion stages were measured for allometric analyses. [Table 4.1.]. T.he allometric growth relationships between the six measured body segments (L_T, L_{PA}, D_H, D_E, D_{BA}, L_{POA}), and body length (L_B) or head length (L_H), during larval development (all stages included), are presented in Figures 4.3. (A) – (F)**.**

With respect to $\mathbf{L_B}$, three (D_E, D_H and L_{PA}) out of the six morphometric characters examined exhibited negative allometric growth during the entire period of larval development [Figures 4.3. (A), (B) and (D)]. L_{POA} and D_{BA} exhibited positive allometric growth [Figures 4.3. (E) and (F)], whilst $\mathbf{L_H}$ exhibited isometric growth [Figure 4.3. (C)]. The relationship between $\mathbf{L_H}$ and $\mathbf{D_H}$ was negatively allometric [Figure 4.4.].

Piecewise linear regressions performed on the log transformed data of each morphometric character revealed that D_H and D_{BA} exhibited a biphasic growth pattern with inflexion points at 8.9 and 8.7mm $L_{B,}$ respectively [Figure 4.5. (A) and 4.5. (B)]. D_H [Fig. 4.5. (A)] exhibited negative allometric growth (b=0.84) during the early stages of larval development but then, after an inflexion point at L_B = 8.9 mm, it showed positive allometric growth (b=1.59). For D_{BA} [Figure 4.5 (B)], a slow positive allometric growth (b=1.18), until 8.7 mm $L_{B,}$ was followed by a faster positive allometric growth (b=1.5), although there was no change in allometry from negative to positive. The relationship between L_H and D_H could also be divided into two distinct phases – a period of negative allometric growth early on (b=0.66) followed by positive allometric growth (b=1.29) after an inflection point at 1.46 mm L_H ([Fig. 5(c)], reflecting the same kind of relationship than that exhibited between D_H and L_B.

According to the morphometric ratios commonly used in larval identification (Leis & Carson-Ewart, 2000), *T. delaisi* larvae body are elongated (% D_{BA}: L_B=9.23±1.41) with a moderate head size (% L_H: L_B=20.95±1.98), eyes (% D_E: L_H=32.8±4.93) and gut (% L_{PA}: L_B=38.8±2.93).

A

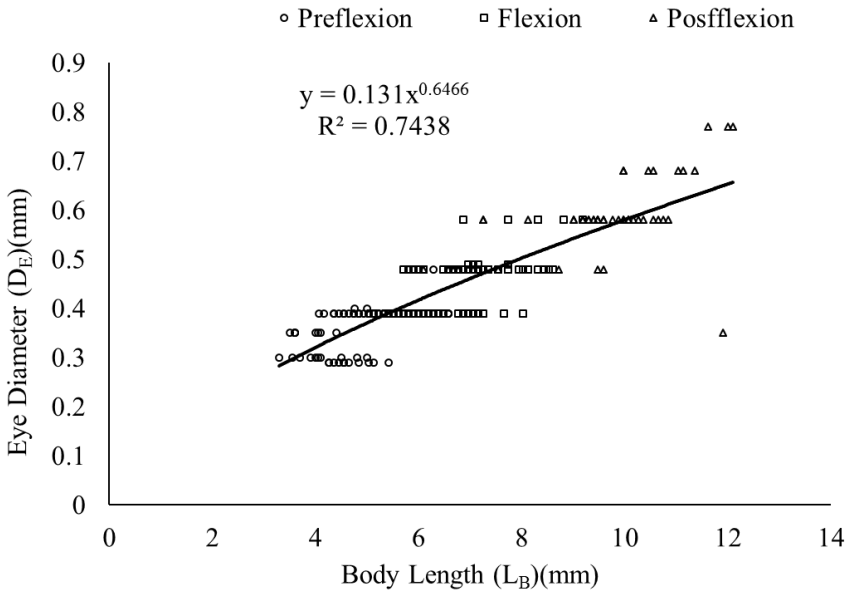


Figure 4.3. (A) Allometric growth equation and relation between Eye Diameter (D_E) and Total Body Length (L_B) for *Tripterygion delaisi* during larval development.

B

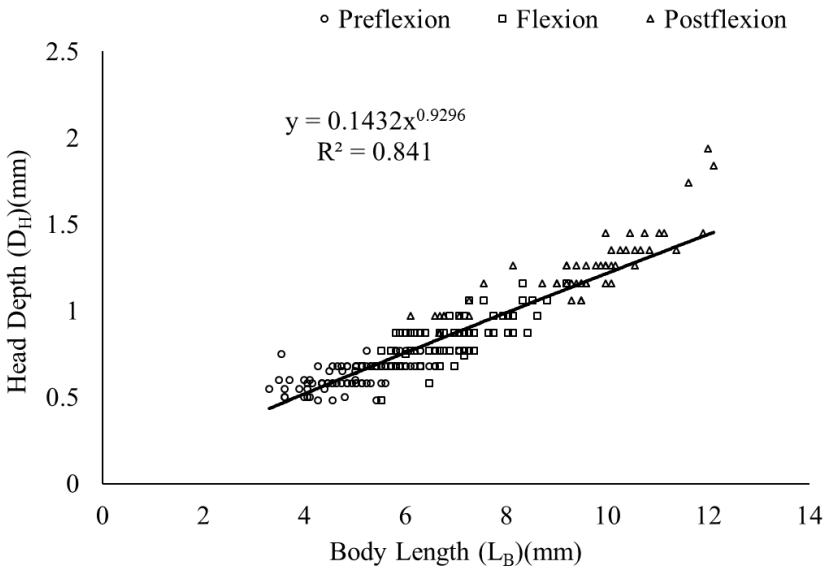


Figure 4.3. (B) Allometric growth equation and relation between Head Depth (D_H) and Total Body Length (L_B) for *Tripterygion delaisi* during larval development.

C

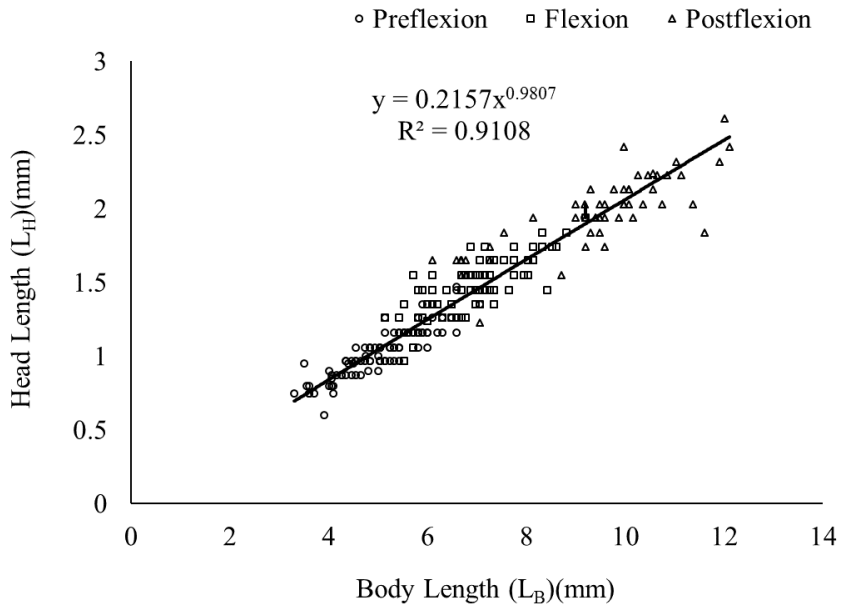


Figure 4.3. (C) Allometric growth equation and relation between Head Length (L_H) and Total Body Length (L_B) for *Tripterygion delaisi* during larval development.

D

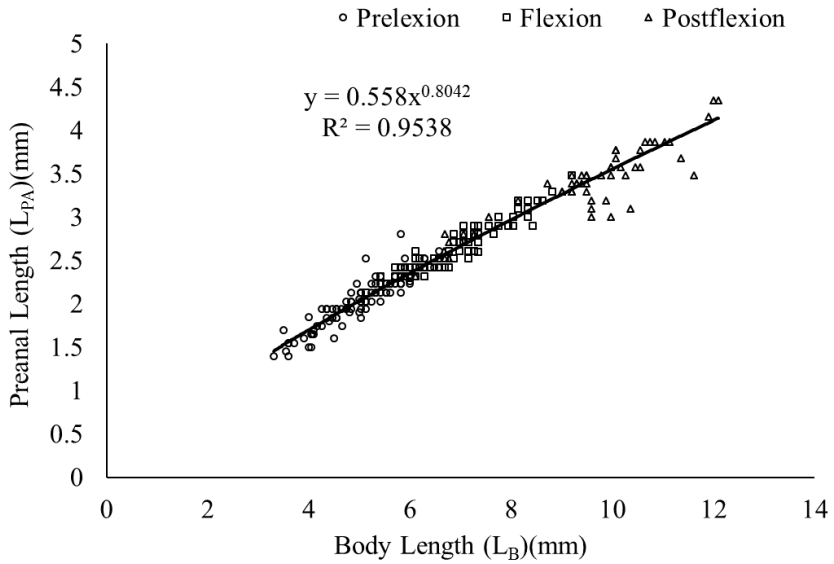


Figure 4.3. (D) Allometric growth equations and relations between Preanal Length (L_{PA}) and Total Body Length (L_B) for *Tripterygion delaisi* during larval development.

E

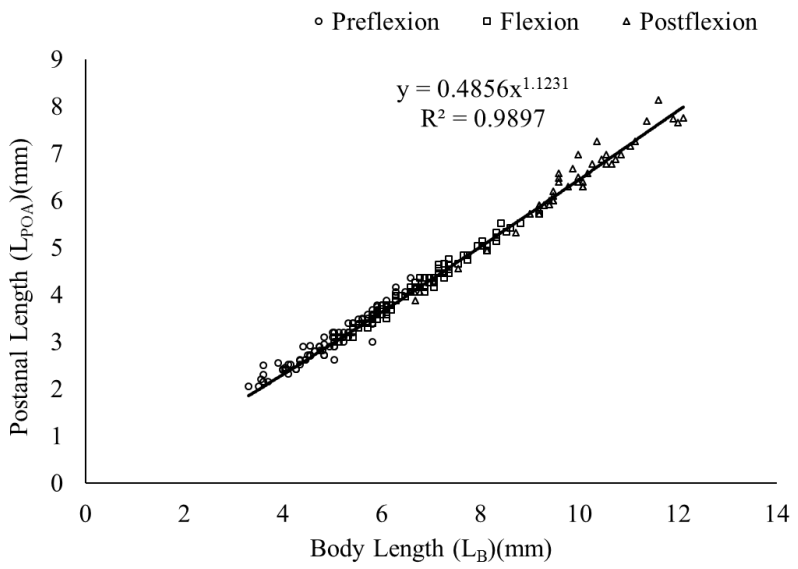


Figure 4.3 (E) Allometric growth equations and relations between Postanal Length (L_{POA}) and Total Body Length (L_B) for *Tripterygion delaisi* during larval development.

F

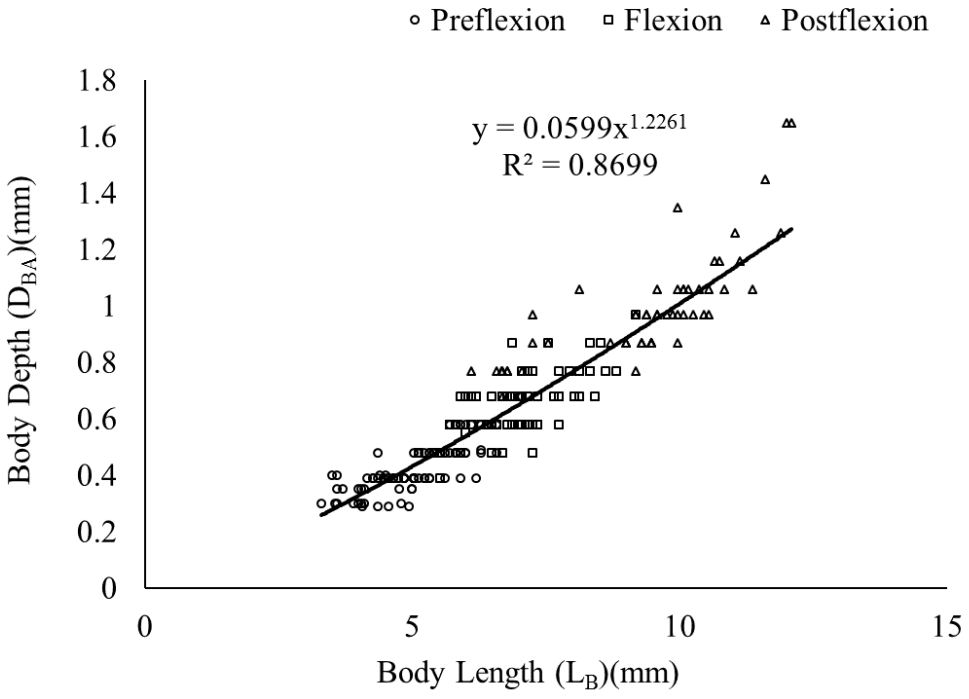


Figure 4.3 (F) Allometric growth equations and relations between Body Depth (D_{BA}) and Total Body Length (L_B) for *Tripterygion delaisi* during larval development.

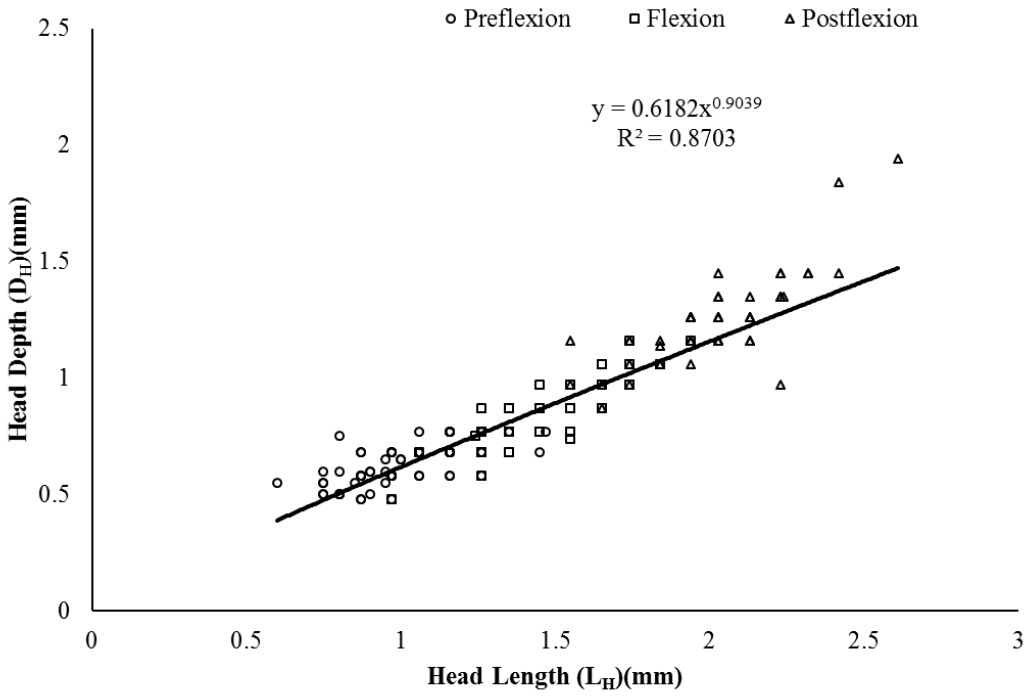


Figure 4.4. Allometric growth relation between Head Depth (D_H) and Head Length (L_H), for *Tripterygion delaisi* during larval development.

A

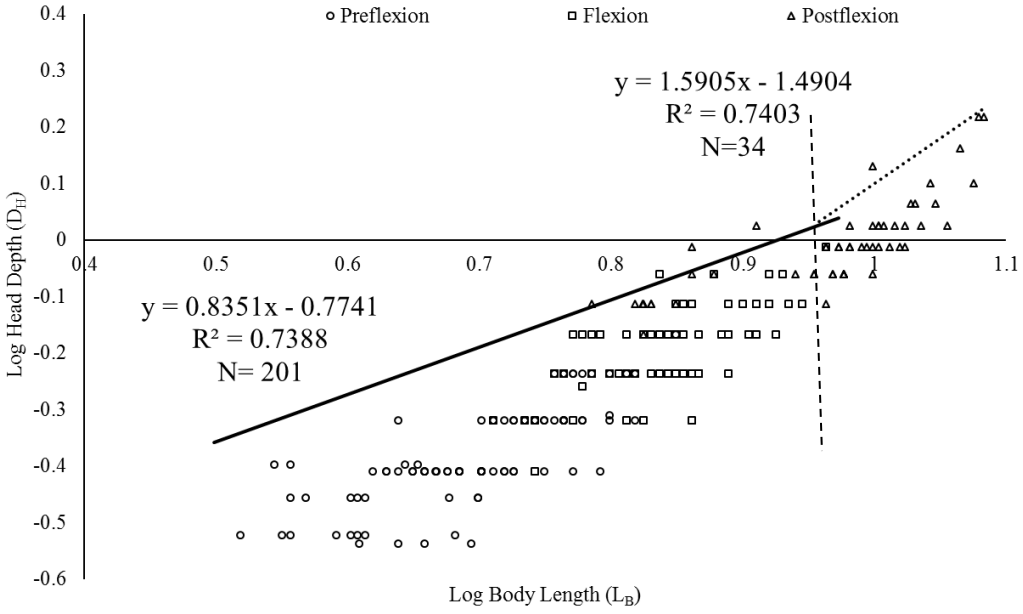


Figure 4.5. (A) Allometric growth relationship and regression equation of Body Length (L_B) vs. Head Depth (D_H). Dotted lines indicate the inflexion point. Note the Log axes.

B

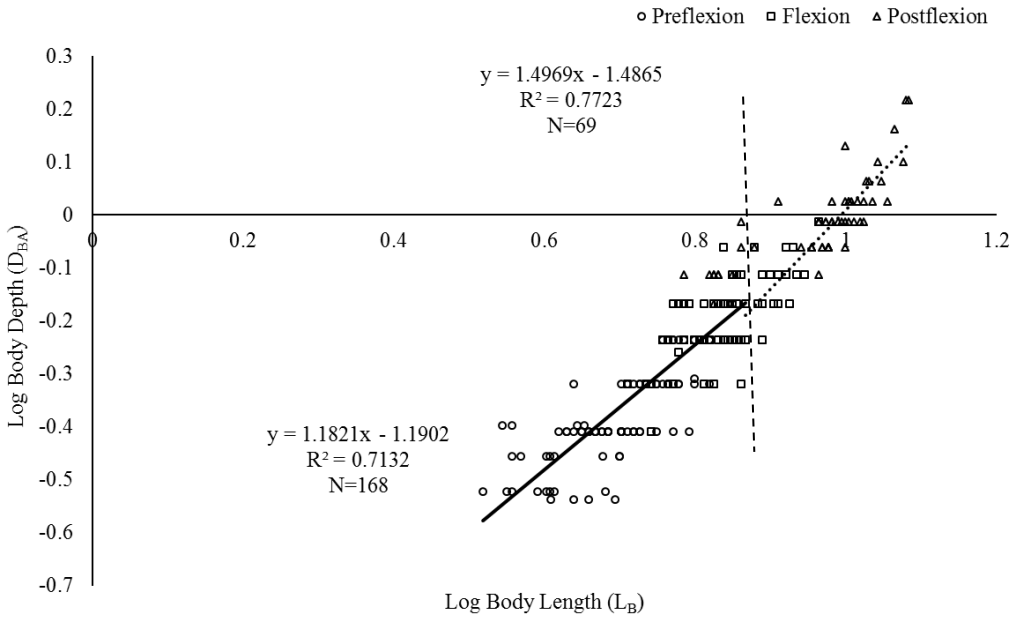


Figure 4.5. (B). Allometric growth relationship and regression equation of Body Length (L_B) vs. Body Depth (D_{BA})

C

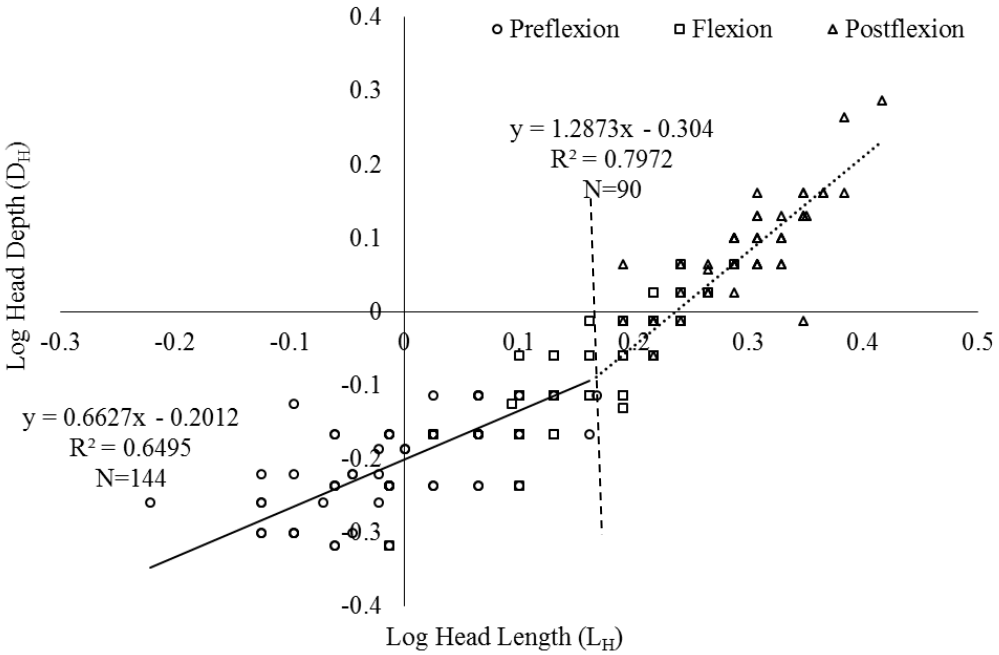


Figure 4.5 (C). Allometric growth relationship and regression equation of Head Length (L_H) vs. Head Depth (D_H). Dotted lines indicate the inflexion points. Note the Log axes.

4.5 Discussion

Ecological studies aimed at understanding fish early life history traits and the processes they influence are often affected by difficulties in accurately identifying the larval stages. The abundant occurrence of all larval developmental stages of *T. delaisi* in the nearshore environment, coupled with ease of sampling via light traps, facilitated the description of morphological and ontogenetic larval development which could be used for identification to the species level.

T. delaisi larvae exhibit morphological characteristics typical of the suborder Blennioidei and in particular the family Tripterygiidae. These include a moderately elongated body, sparse pigmentation, and melanophores on the ventral margin of the trunk which eventually give the appearance of an L shape in the late postflexion stage (Ditty *et al.*, 2005; Herrera & Lavenberg, 2002; Matarase *et al.*, 1984; Watson, 1996). The sequence of initiation of fin development (caudal, 3rd dorsal and anal, pectoral, pelvic, 2nd dorsal and 1st dorsal) was similar to that commonly reported for many teleosts (Moser, 1996) as well as for the family Tripterygiidae where the majority of fin development occurs during the postflexion stage (Watson, 2009). This was supported by the Fuiman's Ontogenetic Index (I_O) which indicated that the majority of fin development occurred at a relatively late stage during larval development. Pigmentation was sparse, consisting of pigmented eyes, gas bladder pigmentation in the dorsal region, anal pigmentation, and a row of regularly spaced postanal ventral melanophores. This pigmentation pattern was present from as early as the yolk-sac stage and persisted throughout all stages with just the addition of head and caudal pigmentation during the flexion and postflexion stages, respectively. Myomere counts (37-45) were similar to that recorded for other larval Tripterygiid and on completion of fin formation, the dorsal and anal fin ray counts were in agreement with that documented for adult *T. delaisi* (Watson, 2009; Zander, 1986): dorsal (III+XVI+13 and III+XVIII+12) and anal (II+26 and II+27).

T. delaisi larvae are most likely to be confused with sympatric species belonging to the Family Blenniidae, one of the most representative family in the rocky reefs of the northeast Atlantic and the Mediterranean (Gonçalves *et al.*, 2002). Larvae of these species have several morphological characteristics in common with *T. delaisi*., which may vary on a species basis, and include body form, myomere counts, post-anal ventral pigmentation and the occurrence of a pectoral bud and open mouth at hatching (Faria *et al.*, 2002; Faria *et al.*, 2006; Faria *et al.*, 2005; Watson, 2009). Unlike *T. delaisi* however, many blennies exhibit peritoneal pigmentation and well developed pigmented pectoral fins from as early as the yolk sac stage (Faria *et al.*, 2002; Faria *et al.*, 2005; Watson, 1996). In the absence of these two features, other differentiating characteristics include the location and shape of the gut and the degree of myomere definition during the preflexion stage. In

T. delaisi the anus is located further along the body (33% L_B), the gut is more elongated as opposed to triangular, and the myomeres tend to be better defined, compared to the blennies. Subsequently, whilst pigmentation increases during the flexion and postflexion stage for most blennies, *T. delaisi* maintains its sparse pigmentation with just the addition of several melanophores on the head and caudal fin. Postflexion *T. delaisi* can also be distinguished from blennies by the occurrence of three dorsal fins, and just prior to juvenile transformation, the dorsal and anal fin ray counts. Early recruits are much more heavily pigmented as the five broad vertical bars on the flank which are characteristic of the adults have already commenced development. The saddle spot of the last bar which extends onto the base of the caudal fin is also evident and can be used to differentiate *T. delaisi* from other *Tripterygion spp*. (Orlando-Bonaca & Lovrenc, 2010).

The growth coefficients indicate that most of the body proportions examined exhibited allometric growth during larval development (i.e. $b \neq 1$) thus supporting the hypothesis of ontogenetic priorities during development (Osse & Van Den Boogaart, 2004). Not all body proportions exhibited significant inflexion points of growth but when they were detected, growth was biphasic with the inflexion points occurring within a very narrow range of L_B (8.7-8.9 mm), which is close to the mean length (9.44±1.48 mm L_B) of postflexion larvae.

Allometric growth, which involves changing body proportions (Fuiman, 1983), has been observed during larval development in many fish species (Gisbert, 1999; Khemis *et al.*, 2013; Osse & Van Den Boogaart, 2004). Due to differential growth, this change in body proportions is often viewed as an adaptive response to environmental conditions as organs and systems (involved in primary functions such as feeding, swimming, and respiration) exhibit functionally optimal growth for survival (Osse & Van Den Boogaart, 2004). This is in contrast to isometric growth where the body proportions remain constant and do not change in size.

The biphasic pattern of growth exhibited by *T. delaisi* has also been documented for the California halibut (*Paralichthys californicus*) and the thick-lipped grey mullet (*Chelon labrosus*), based on allometric growth patterns. In those studies, the 1st phase involved an early larval phase characterized by rapid organogenesis and differentiation whilst the 2nd phase involved a late larval phase where most of the morphological changes were related to the transition from larvae to juvenile (Gisbert *et al.*, 2002; Khemis *et al.*, 2013). In *T. delaisi*, although there are two phases, these seem to differ in character compared to the previous species. The 1st phase includes the preflexion and flexion larvae and is characterized by negative allometric growth or isometry of all the head characters (head depth, eye diameter, head length, preanal length) and positive allometric growth of all body characters (body depth and postanal length). The 2nd phase includes the

postflexion larvae and is characterized by positive allometry of some of the head characters (head depth both in relation to body length and head length) and continued positive allometric growth of body characters (body depth and postanal length). When considered together with the observations of ontogenetic larval development, we hypothesize that the 1st phase primarily relates to the development of the digestive system (coiling of the gut occurs during this stage), and the swimming abilities of the larvae (development of the myomeres in terms of length and width, and caudal fin development occurs during this stage). During the 2nd phase, although locomotory development may continue with fin formation and development, the transition from larvae to juvenile resulted in an increase in head depth and the overall body depth leading to a marked change in body form, from elongated to deeper bodied.

Also, in contrast to the results obtained here, some studies (for the thicked-lipped mullet, *Chelon labrosus*; the California halibut, *Paralichthys californicus*; the Siberian sturgeon, *Acipenser baeri* and the burbot, *Lota lota L.*) (Gisbert, 1999; Gisbert *et al.*, 2002; Khemis *et al.*, 2013; Kupren *et al.*, 2014) report positive allometric growth for head characters that generally occurs during the preflexion stage. This is usually associated with the development of the nervous, sensory, respiratory and feeding systems of the larvae immediately after hatching (Gisbert, 1999; Gisbert *et al.*, 2002; Khemis *et al.*, 2013; Kupren *et al.*, 2014). Such systems are considered as being essential for survival during this high mortality stage. Failure to detect this positive allometry at the preflexion stage in our study maybe due to several reasons: (1) in *T. delaisi* the rapid growth of these systems occur during the yolk sac to preflexion stage, which we were unable to adequately represent due to an insufficient number of yolk sac larvae, (2) these systems were already somewhat developed at hatching, hence growth was not rapid as would be indicated by positive allometry but rather more slow and consistent and, (3) development of these systems was delayed to the later postflexion stage which was when positive allometric growth of the head characters in *T. delaisi* was detected.

The 2nd hypothesis seem to be supported by the fact that, in several species that exhibit male parental care, larvae hatching from demersal eggs are known to be larger and better developed, with more advanced sensory and swimming capabilities compared to those hatching from pelagic eggs (Thresher, 1984). Additionally, the ability of preflexion *T. delaisi* larvae to detect and react to light stimulus from the light traps indicate that this sensory function is already well developed at this stage. Moreover, the occurrence of larvae of all developmental stages near the reefs indicates that behavioural and hence neural and sensorial capabilities allowing larvae to react and interact with the environment must develop soon in ontogeny. A better representation of yolk sac larvae as well as

studies on sensory development and larval swimming abilities are needed to help clarify these issues.

4.6 Acknowledgements

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CHAPTER 5: EARLY LIFE HISTORY CHARACTERISTICS OF THE BLACK-FACED BLENNY, *TRIPTERYGION DELAISI* INFERRED FROM OTOLITH MICROSTRUCTURE ANALYSES

5.1 Abstract

The pelagic larval stage is a critical stage in the life cycle of benthic fish. Early life history traits during this stage may influence important processes such as settlement, post settlement survival, and dispersal and connectivity. In this study, early life history characteristics of the black-faced blenny, *Tripterygion delaisi* was investigated from larval and early recruit samples collected along the Portuguese west coast in 2012 – 2014. Microstructure analyses of the sagittal otoliths was used to determine larval and juvenile growth rates, size-at-hatching, pelagic larval duration (PLD) and size-at-settlement (SAS). In total 267 larvae from preflexion to pre-settlement (3.5 – 12.5 mm L_B) and 36 early recruits (12.8 - 22.5 mm L_B) were examined. Daily growth increments (DGIs) ranged between 3 – 33 and 44 – 71 days, in the larval and recruits samples, respectively. Instantaneous growth rates derived from the age vs size relationships ranged between 0.23 – 0.28 mm^{-1} day for both larvae and juveniles and are consistent with that recorded for other demersal reef fish. PLD estimates based on the back calculations from the settlement mark in the early recruits range between 29 and 34 days, with a mean ± SD of 31.75 ± 1.54. These values are higher than those obtained from previous studies conducted in the Mediterranean. Hatching size (3.1 – 3.7 mm L_B) and size-at-settlement (10.10 – 12.25 mm L_B; 11.15±0.53) match those obtained from field observations. With the exception of PLD, these estimates are the first for this species.

5.2 Introduction

In organisms with multiple life-stages such as benthic fish with a pelagic larval stage, early life history traits (ELHTs), particularly growth related traits, may influence important processes such as settlement (Shima & Findlay, 2002), post settlement survival (Shima & Findlay, 2002; Macpherson & Raventós, 2005; Gagliano *et al.*, 2007; Hamilton *et al.*, 2008), and dispersal and connectivity (Macpherson & Raventós 2006; Shima & Swearer, 2010; Sponaugle, 2010). Despite the importance of these early life history traits in shaping processes at the juvenile and adult stages however, there is usually a paucity of information on these key parameters such as size at hatching and settlement, pre and post-settlement growth rates and pelagic larval durations.

Daily otolith microstructure analyses have become a very important tool for determining early life history traits (ELHTs) such as, size and time at hatching (Contreras *et al.*, 2013; Betti *et al.*, 2014; Palacios-Fuentes *et al.*, 2014), larval and juvenile growth rates and patterns (Sim-Smith *et al.*, 2012; Contreras *et al.*, 2013; Plaza *et al.*, 2013; Landaeta *et al.*, 2015), pelagic larval duration (PLD) (Victor, 1986; Wellington & Victor, 1989; Raventós & Macpherson, 2001; Beldade *et al.*, 2007; Ishihara & Tachihara, 2011; Mansur *et al.*, 2014), and size-at settlement (Ishihara & Tachihara, 2011; Spies *et al.*, 2014). Otoliths which are paired calcium carbonate structures located in the inner ear cavity of all teleost, function in balance and hearing (Campana, 1999). Given the challenges often associated with sampling the larval stages, most studies rely on the otoliths derived from early recruits or juveniles to infer characteristics about the larval period, and studies providing early life history information such as growth patterns, derived from both the larval and juvenile stages of a singles species are scarce. This is particularly so for small cryptic species like those belonging to the Genus *Tripterygion*, which comprise some of the most abundant species in temperate nearshore rocky reef systems (Illich & Kotrschal, 1990; Sabatés *et al.*, 2003; Beldade *et al.*, 2006b).

The black-faced blenny, *Tripterygion delaisi*, is a small demersal fish that inhabits nearshore rocky habitats both in the Mediterranean and Atlantic (Zander, 1986; La Mesa *et al.*, 2004; Bertoncini *et al.*, 2010). Due to several life history characteristics (the production of benthic eggs, an inshore larval distribution, limited adult mobility and a short PLD) which suggests that it may exhibit limited dispersal capabilities, this species is often regarded as a model species for investigating population connectivity and the occurrence of self-recruitment and local retention (Carreras-Carbonell *et al.*, 2006, 2007; Galarza *et al.*, 2009; Schunter *et al.*, 2014). Apart from PLD estimates of 16-21 days based on otolith microstructure analyses of newly settled individuals

(Raventós & Macpherson, 2001; Macpherson & Raventós 2006), there is no information on life history parameters during the early life stages of this species.

T. delaisi is the only *Tripterygion sp.* present in the Atlantic and by extension along the Portuguese coast. Along the west coast of Portugal, juveniles are abundant in nearshore rocky areas including the Arrábida Marine Park (AMP), the target area for the present study. In the AMP previous studies indicate that *T. delaisi* larvae are also abundant and that their distribution appear to be restricted to the nearshore environment where they may be completing their life-cycle locally as evident by the co-occurrence of multiple developmental stages (Beldade *et al.*, 2006a; Borges *et al.*, 2007; Borges *et al.*, 2009). This, together with the relatively calm conditions that occur at the AMP allows for nearshore sampling and the collection of both the juvenile and larval developmental stages. Such circumstances provide an ideal opportunity to investigate several early life history traits of this species and to compare information from the larval period obtained from both larval and recruit otoliths.

In this study, we used otolith microstructure analysis of both larval and juvenile *T. delaisi* individuals in order to provide insights into some of the early life history characteristics of this species. Sampling for two consecutive years also allowed us to examine inter-annual variability in some of these characteristics. Our results are compared to those recorded for other demersal reef fish, and discussed in terms of the possible implications for important processes such as recruitment and connectivity. Knowledge of these early life history characteristics is important for fully understanding life-cycles and the ecology of a species.

5.3 Methods

5.3.1 Sampling

Larvae*:* Larval samples were collected using light traps at two sites along the Portuguese west coast: the Arrábida Marine Park (AMP), and Sines. At the AMP samples were collected during the period April - September 2012 (months corresponding to the spawning season of this species) and June – September 2013. During these extended periods sampling was scheduled for two consecutive nights every other week. Traps were deployed in both the nearshore and offshore environment. In the nearshore environment they were set at the surface and at the bottom at depths of 5 – 10 m and in the offshore environment they were set at the surface, in midwater and at the bottom at depths of roughly 20 m. A night of sampling typically involved setting four traps simultaneously for 1 hour for 3-4 consecutive hours, starting about 30 minutes to 1 hour after sunset. At Sines, 4 samples were collected for just one night in August 2013 at depths of 4 - 10 m,

also in the nearshore environment. Plankton collections from the light traps were stored in 80% alcohol. After sorting the plankton samples, *T. delaisi* larvae were identified based on morphological characteristics (*T. delaisi* is the only *Tripterygion sp*. present in the Atlantic) and assigned to one of the three developmental stages (preflexion, flexion and postflexion) depending on the degree of notochord flexion as described in Kendall *et al.,* (1984) and Leis and Carson-Ewart, (2000). Each individual was measured for body length (**L_B =** length from the tip of the snout to the end of the notochord in the preflexion and flexion larvae, and to the last vertebrae in the postflexion stage - standard length) to the nearest 0.01mm using an ocular eyepiece in a dissecting microscope. **Early Recruits:** Early recruits were collected at two localities in the AMP - Risco and Derrocada, via SCUBA in the months of August and September 2014. This period marks the end of the spawning season for this species in this area. Specimens collected were stored in plastic vials, in 80% alcohol. Just prior to otolith removal the body length (**L_B**) and total length (**L_T**) of each individual was measured to the nearest 0.01mm using a digital Vernier caliper. For recruits **L_B was measured as** standard length.

5.3.2. Otolith extraction and preparation

Larvae: Larval otoliths were extracted under a dissecting microscope using the "teasing method" as described by Secor *et al.,* 1992. Initially, both sagittae and lapilli were extracted, however preliminary work showed that daily growth increments (DGIs) were most clearly visible on the sagittae hence subsequently only these otoliths were extracted for analysis. Following extraction, the left and right sagittal otolith from each specimen was mounted on a single glass slide using heat activated Crystal Bond Resin. Otoliths were sufficiently transparent to be viewed directly under a light microscope hence no further preparation was needed prior to microstructural analyses. **Early Recruits**: Both the sagittal and lapillar otoliths were extracted from all specimens using the "open-the-hatch method" (Secor *et al.,* 1992) with slight modifications. Otoliths were stored mounted on a glass slide (one per with slide) with Crystal Bond Resin. To allow for comparisons between the larval and juvenile stages, the sagittal otoliths were chosen for analysis. The right sagittae of each individual was subjected to further preparation for microstructural viewing using the method described by Wilson & McCormick, 1997. The approximate location of the core of each otolith was identified using a dissecting microscope. The Crystal Bond Resin was then reheated and the otolith was positioned horizontally on the glass slide such that the distal end was protruding over the edge of the slide. Care was taken to ensure that the core was positioned on the slide and not protruding. The protruding section was then ground off using polishing paper (grit 12 um) until the

core was exposed. The otolith was then repositioned with the polished end mounted face down on the slide. Using a decreasing series of polishing paper (12, 9, 5, 3 and 1 um) the rostral end of the otolith was ground down in a similar way to produce a thin transverse section that incorporated the nucleus. A drop of immersion oil was placed on each section and slides were stored in slide boxes until further analysis.

5.3.3. Microstructure analyses

General: Otolith microstructure interpretation was guided by Campana (1992). Both larval otoliths and early recruit otolith sections were viewed under transmitted light at 1000x magnification using an Imaging system comprised of a Zeiss Microscope connected to a computer screen via a monochrome digital camera (Cybernetics©). The number of DGIs was first enumerated in live view with frequent focal adjustments in order to obtain rough estimates. Images were then captured using the digital camera and the Image-Pro Plus image analysis software. Photos were viewed on the computer screen and DGIs counts were enumerated and matched up with the live counts previously recorded. If the live counts and the photo counts differed by > 3, the otolith was not used in analysis. Final increment counts and measurements were taken from the captured digital images using the Image-Pro Plus software. **Larvae**: For each right larval otolith the following measurements were recorded: maximum otolith diameter (µm), core diameter (µm), and maximum radius (µm). The hatch mark (**HM**) was identified as the first dark prominent increment surrounding the "core" or "central area" and age was determined by counting the number of DGIs from the hatch mark to the otolith edge. Three independent readings (along different axes) were performed per otolith. When the increment counts of the three readings were within 10% of each other, modes or averages (if all counts were different) were calculated and used for data analyses. If the increment counts between readings differed by more than 10% of each other, the otolith was discarded and not used in analyses. Increment width measurements were taken from the hatch mark to the edge along the longest radial axis. When the increments were not clear along this axis, the position of the unclear rings were extrapolated from adjacent clearer areas. **Early Recruits**: Measurements recorded for each transverse otolith section included core diameter, otolith diameter and otolith radius (longest axis). Similar to the larvae, the hatch mark (**HM**) was identified as the first dark prominent increment surrounding the "core" or "central area" and age was determined by counting the number of increments from the hatch mark to the otolith edge along the longest axes. Each otolith was read by two independent readers; the primary reader enumerated increments and performed increment measurements whilst the secondary reader only enumerated increments. The

settlement mark **(S)** was identified as an abrupt settlement-mark (Type **Ia**) characterized by a rapid decrease in increment width across the settlement mark and by the completion of the settlement transition over one increment (Wilson & McCormick, 1999). This was further verified by plots of increment no. vs increment width for each individual.

Whilst daily ring formation has not been validated for this species and is hence assumed, it has been validated for a number of other Tripterygiids including *Forsterygion nigripenne, F. capito, F. varium*, *Ruanoho whero* (Kohn & Clements, 2011), *Helcogrammoides chilensis*, and *H. cunninghami* (Mansur *et al.*, 2014). The following analyses were conducted: (1) specimens (larvae and recruits) were aged by enumerating the number of daily growth increments from the hatch mark to the edge (2) a brief description of key otolith morphological characteristics and microincrement patterns was developed by describing changes in parameters such as otolith diameter, core size, and DGI counts and widths, over the three developmental stages (3) the relationship between otolith radius (OR) and body length (L_B) was examined to determine if otolith growth was a good indicator of somatic growth (4) the relationship between age (no. of DGIs) and otolith radius (OR) was examined (5) larval and early recruit **growth rates** were determined from linear models of the relationship between size (body length) and age (**DGIs**), where the slope of the graphs represented mean instantaneous growth rates and the intercepts represented size at hatching (6) the pelagic larval duration (PLD) of each recruit was obtained by counting the number of increments from the hatch mark (HM) to the settlement mark. (7) size-at-settlement (SAS) for each recruit was back-calculated from the linear regression of body length vs otolith radius in the form $L= mx + c$ where L = body length, m = slope, x = otolith radius at settlement and c = y intercept (8) growth trajectories derived from average daily increment widths ± S.E. were plotted to look for variability in growth over time as well as for inter-annual variability.

5.4 Results

In total 142 (4.5 – 12.5 mm L_B) and 125 (3.5 -12.4 mm L_B) larvae collected in 2012 and 2013 respectively, and 36 early recruits (12.8 to 22.5 mm L_B) collected in 2014 were successfully subjected to otolith microstructure analyses. Table 5.1. gives a breakdown of the number, size and age (DGI counts) of individuals analysed by year, location, developmental stage (preflexion, flexion, postflexion and early recruits) and sample date. Also included are water temperature (temperature at the sample collection site) and the position at which the light trap was set in the water column (i.e. surface, midwater or bottom).

All larval samples were collected in the nearshore environment. In 2012, all of the larvae analysed were collected from surface samples whilst in 2013, 54 out of the 125 (43%) larvae examined came from bottom samples. Water temperature at light trap sampling sites ranged between 15 – 17.5°C in 2012 and 15 – 20°C in 2013.

Larval developmental stages (preflexion, flexion and post flexion) showed a high degree of overlap in both L_B and DGI counts. Overall, the L_B of each stage was significantly higher in 2012 compared to 2013 (two sample t-test, preflexion ($p<0.05$); flexion ($p<0.05$) and postflexion ($p<0.0001$)), and the mean age was significantly higher in 2012 for the flexion and postflexion stages (two sample t-test ($p<0.05$).

Table 5.1. Breakdown of the number, size and age (DGI counts) of individuals analysed by year, location, developmental stage (preflexion, flexion, postflexion and early recruits) and sample date.

Year	Location	Stage	Sample Date	No. of Larvae	S,B	Water Temp °C	N	Size Range (L_B mm)	Mean L_B ± S.D.	Age Range (Days)	Mean Age ± S.D. (Days)
2012	Arrábida	Preflexion	9th April	58	58	16	70	4.5 – 7.6	6.2±0.85	3 - 18	9.6±2.95
	Arrábida	Preflexion	17th May	1	1	16.7					
	Arrábida	Preflexion	14th June	3	3	16					
	Arrábida	Preflexion	11th July	8	8	15					
2012	Arrábida	Flexion	9th April	41	41	16	44	5.7- 10	8.09±0.85	8 - 23	15.4±3.5
	Arrábida	Flexion	17th May	1	1	16.7					
	Arrábida	Flexion	11th June	1	1	16					
	Arrábida	Flexion	11th July	1	1	15					
2012	Arrábida	Postflexion	9th April	20	20	16	28	8.5 - 12.5	10.2±1.02	17 -31	22.2±3.59
	Arrábida	Postflexion	17th May	3	3	16.7					
	Arrábida	Postflexion	31st May	1	1	16.5					
	Arrábida	Postflexion	14th June	1	1	16					
	Arrábida	Postflexion	28th June	1	1	17.5					
	Arrábida	Postflexion	10th July	1	1	15					
	Arrábida	Postflexion	11th July	1	1	15					
2013	Arrábida	Preflexion	12th June	5	5	15	12	4.0 - 6.7	5.3±0.85	4 - 13	8.3±2.87
	Arrábida	Preflexion	10th July	6	1,5	20					
	Arrábida	Preflexion	11th July	1	1	19					
2013	Arrábida	Flexion	12th June	3	3	15	13	4.5 - 8.3	6.76±1.16	9 - 18	13.4±3.12
	Arrábida	Flexion	26th June	1	1	15					
	Arrábida	Flexion	10th July	8	1, 7	20, 19					
	Arrábida	Flexion	22nd August	1	0,1	19.5, 18.5					
2013	Arrábida	Postflexion	12th June	4	4	15	56	5.5 - 12.4	8.1±1.66	10 - 33	18.9±4.68
	Arrábida	Postflexion	26th June	7	6,1	15					
	Arrábida	Postflexion	10th July	38	0,37	20, 19					
	Arrábida	Postflexion	11th July	3	3	19					
	Arrábida	Postflexion	5th August	1	1	18					
	Arrábida	Postflexion	22nd August	3	0,3	18.5					
	Arrábida	Postflexion	5th September	1	1	19.5					
2013	Sines	Preflexion	20th August	44	44	ND	44	3.5 - 6.6	4.8±0.83	3 - 11	6.3±1.86
2014	Arrábida	Early Recruit	2nd August	1		ND	36	12.8 – 22.5	17.9±1.94	44-71	59.0±6.12
	Arrábida	Early Recruit	26th August	1		ND					
	Arrábida	Early Recruit	1st September	1		ND					
	Arrábida	Early Recruit	3rd September	15		ND					
	Arrábida	Early Recruit	5th September	8		ND					
	Arrábida	Early Recruit	8th September	2		ND					
	Arrábida	Early Recruit	9th September	7		ND					
	Arrábida	Early Recruit	10th September	1		ND					

5.4.1 Otolith morphology and microstructure

Larvae: Analyses of otolith parameters measured (i.e. otolith diameter, core diameter) showed that there were no significant differences between years (two sample t-test, $p<0.05$) hence larval samples collected in **2012** and **2013** were pooled for the description of otolith morphological characteristics and microstructural patterns. For all developmental stages, growth increments were well defined over most of the sagitta, and the hatch mark (**HM)** was identified as the first prominent ring outside the central area. The core which encompassed the **HM** to the centre of the otolith measured between 15.4 and 25.7 µm in diameter (mean±SD = 20.2±1.51 µm; N=262) in all developmental stages combined.

During the preflexion stage (range = 3.5 – 7.6 mm L_B; mean±SD = 5.61 ± 1.07 mm L_B; N = 124) sagittal otoliths were spherical and disk shaped (otolith length: otolith width = 0.8 - 1.13; mean±SD = 0.99 ± 0.05 µm; N = 124) ranging between 26.91 - 55.48 µm in diameter (N = 125; mean±SD = 42.42 ± 5.6 µm) [Figure 5.1. (A)]. Micro-increment counts ranged between 3 and 18 (mean±SD = 9.27 ± 3.0; N = 124) and increment widths varied between 0.56 and 2.8 µm.

During the flexion stage (mean±SD = 5.2 – 10 mm L_B; range = 7.82 ± 1.0 mm L_B; N = 57) the circular shape was maintained (otolith length: otolith width = 0.91 - 1.106; mean±SD = 0.99 ± 0.05 µm; N = 57) however, there was a general increase in otolith diameter 44.68 – 66.16 µm (N = 57; mean±SD = 54.20 ± 5.32 µm). Micro-increment counts ranged between 8 and 23 and increment widths varied between 0.54 and 2.95 µm [Figure 5.1. (B)].

During the postflexion stage (range = 5.5 – 12.5 mm L_B; mean±SD = 8.71 ± 1.76 mm L_B; N = 80) otolith diameter continued to increase (range = 55.08 – 120. 37 µm; mean±SD = 75.21 ± 14.57 µm; N=80), and some of the otoliths were no longer spherical as they began to elongate along the post-rostral axis. The micro-increment widths became wider at the rostral edge with sub-daily increments becoming visible and clearly discernible between these wider bands [Fig. 5.1(C) and (D)]. No accessory growth centres were visible in any of the otoliths examined. Micro-increments ranged between 10 and 33 and increment widths varied between 0.534 and 7.997 µm.

Early Recruits: In the early recruits, sagittal otoliths were processed along a transverse plane and measurements were taken from the core to the edge along the longest possible axis which ranged between 142.8 (L_B=12.98 mm) and 241.8 (L_B = 21.15 mm) µm. Core diameter which was consistent with the values obtained for the larvae ranged between 17.3 and 26.17 µm (mean±SD = 19.9±1.6 µm). Similar to a previous study (Raventós & Macpherson, 2001), the settlement mark (**S)** was identified as an abrupt settlement-mark (Type **Ia**), characterized by a rapid decrease in increment width across the settlement mark and by the completion of the settlement transition over

one increment (Wilson & McCormick, 1999) [Figure 5.2.]. Pre-settlement increments ranged between 0.72 and 10.42 µm in width whilst post settlement increments ranged between 1.19 and 7.29 µm. Micro-increment counts ranged between 44 and 71. Knowledge of the pattern of daily growth increments close to the core in the larvae assisted in the interpretation and enumeration of daily growth increments close to the core in the early recruits.

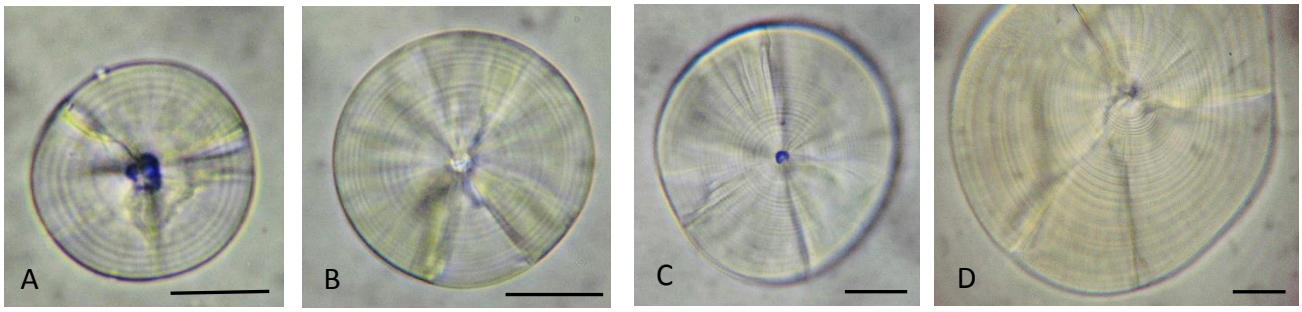


Figure 5.1. Sagittal otolith morphological development in *Tripterygion delaisi* during the larval stages (A) preflexion at 3.5 mm L_B (B) flexion at 6.0 mm L_B (C) early postflexion at 7.0 mm L_B (D) late postflexion at 11.0 mm L_B (Scale Bar = 20 µm).

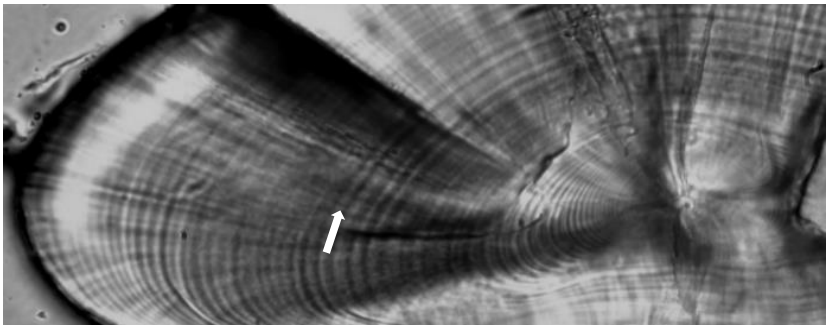


Figure 5.2. Pictograph of polished transversal section of the sagittal otolith of an early recruit *Tripterygion delaisi*. Growth increment counts and measurements were made along the longest axis from the core to the edge. S = settlement mark (Scale Bar = 20 µm)

5.4.2 Morphometric relationships

Otolith size vs body size: The relationship between larval body length (L_B) and otolith radius (OR) was best fitted by a linear function in 2012 (r^2 = 0.8017; p <0.0001; N=141) [Figure 5.3 (A)]. In 2013, a comparison of the linear regressions (best fit) of otolith radius vs body length for preflexion Sines (L_B=0.19OR+2.96; r^2= 0.59; N=42) and preflexion Arrábida (L_B=0.269OR+2.11; r^2= 0.61; N=12) larvae showed that these two regressions were not significantly different from each other (ANCOVA; F =1.48 , df = 1, 51, p>0.05(ns)) and hence the Sines larvae were pooled together with the Arrábida samples for analysis. On pooling the samples, the relationship between larval

body length and otolith radius was fitted to a linear regression (r^2 = 0.7426; p <0.0001; N=125) [Figure 5.3 (B)]. Overall, the slope of the line was less steep (lower) in 2013, showing that the otolith radius of a larvae sampled in 2013 was generally greater at a given body length compared to a larvae collected in 2012. This difference appears to be due mainly to postflexion larvae caught in the bottom samples in 2013. These larvae exhibited great variability in the otolith radius to body length relationship. A comparison of the 2012 and 2013 regressions, showed that both regression lines were significantly different (ANCOVA, F = 64.36, df = 1, 260, p<0.0001). Early recruits were best fitted to a linear relationship (r^2 = 0.63; p <0.0001; N=36) [Figure 5.3 C].

A

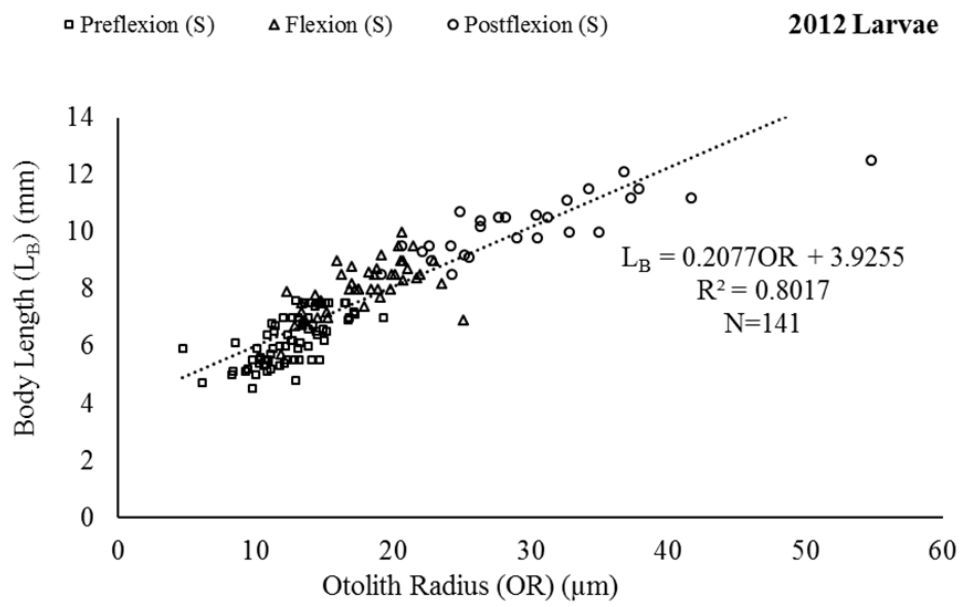
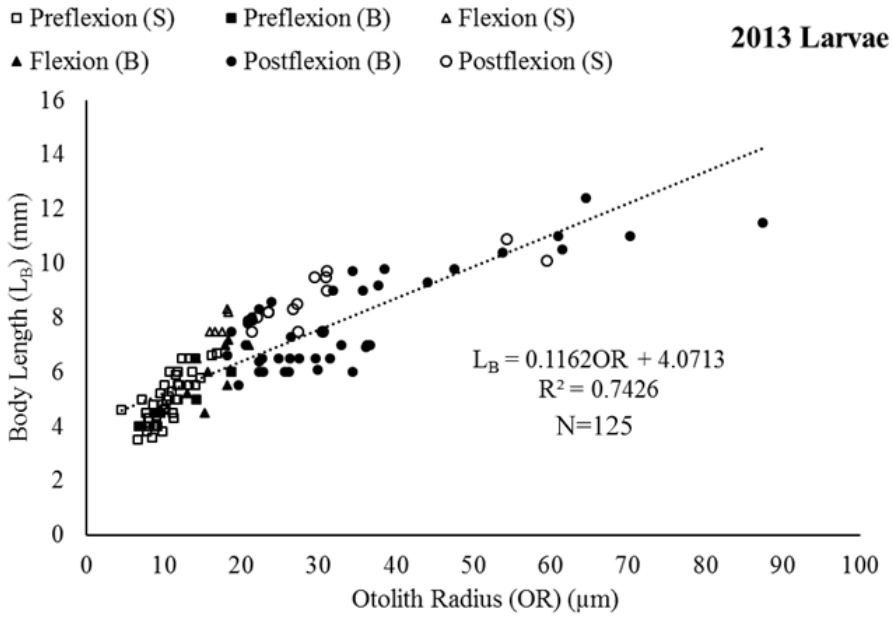


Figure 5.3 (A). Relationship between body length (**L_B**; mm) and otolith radius (OR; µm) for larvae caught in 2012. The different developmental stages (preflexion, flexion and postflexion) and the location (S=surface; B=bottom) at which the light trap was set in the water column is differentiated for the larvae.

B



C

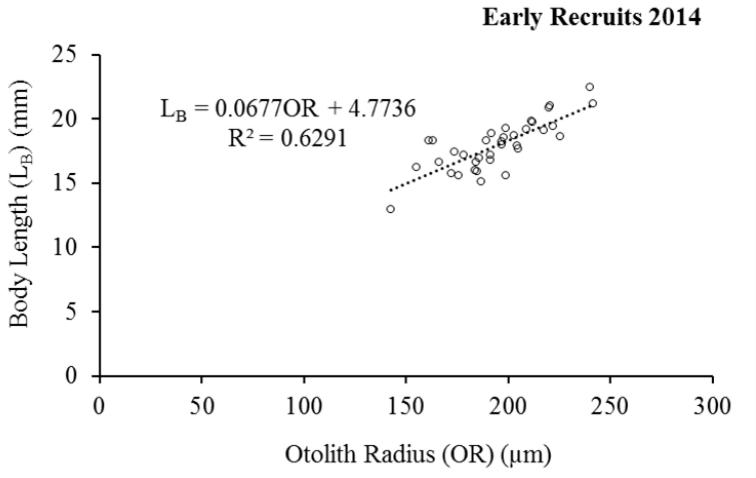
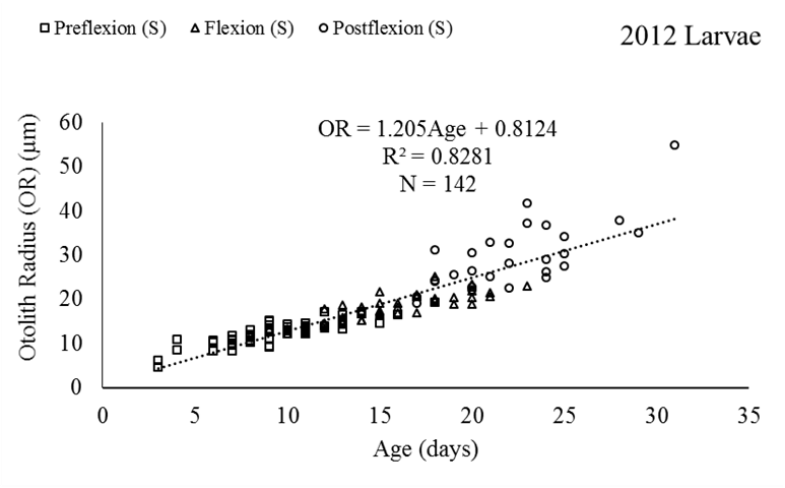


Figure 5.3 Relationship between body length (**L_B**; mm) and otolith radius (OR; µm) for larvae caught in (B) 2013, and (C) early recruit samples caught in 2014. The different developmental stages (preflexion, flexion and postflexion) and the location (S=surface; B=bottom) at which the light trap was set in the water column is differentiated for the larvae.

Age vs otolith size: In 2012 the relationship between age and otolith size was linear (r^2 = 0.83; p <0.05; N=142) [Fig. 5.4 A], whilst for 2013 the relationship was curvilinear (r^2 = 0.88; N=125), best fitted to an exponential function [Fig. 5.4 B]. There were several outlier points at the

top of the curve which appear to be responsible for the curvilinear fit of the graph as opposed to a linear fit. These individuals were mainly postflexion larvae caught at the bottom.

A



B

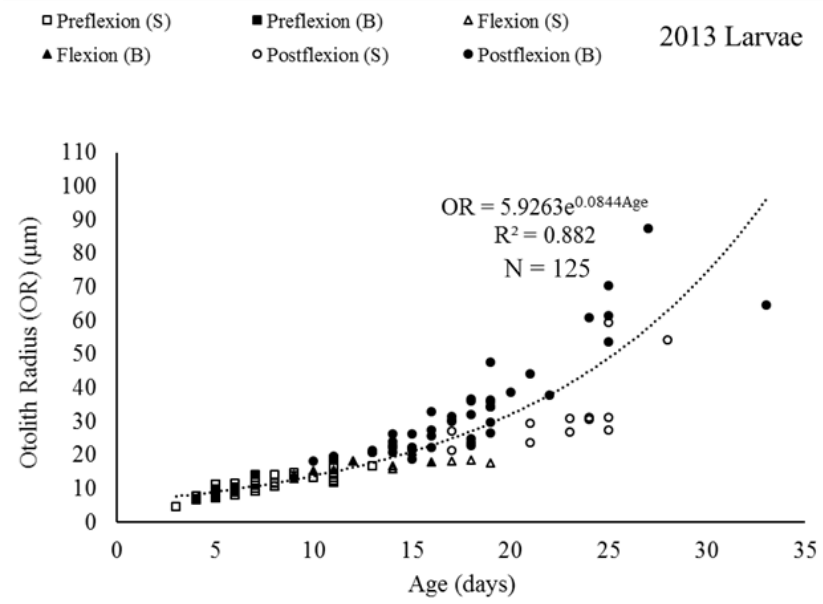


Figure 5.4. Relationship between otolith radius (**OR**; µm) and age (days) for larvae caught in (A) 2012 and (B) 2013. The different developmental stages (preflexion, flexion and postflexion) and the location (S=surface; B=bottom) at which the light trap was set in the water column is differentiated for the larvae.

Size vs Age: Length at age regression models were linear (r^2 = 0.83; p <0.0001; N=142 and r^2 = 0.83; p <0.0001; N=125) for larval samples collected in both years, estimating mean instantaneous growth rates of 0.28 mm day^{-1} in 2012 and 0.27 mm day^{-1} in 2013 [Fig. 5.5(A) and (B)]. Size at hatch (the intercept) were estimated to be 3.7 and 3.1 mm in 2012 and 2013

respectively. A comparison of regressions showed that the intercepts of the regressions were significantly different (ANCOVA; F= 58.23, df = 1, 263, $p<0.0001$) but the slopes were not (ANCOVA; df = 1, 262; F = 0.93; $p>0.05$). This indicates that although the rate of growth was similar, the 2012 individuals were larger in size (L_B).

Figure 5.5 C shows the size age regression models for the juveniles only (instantaneous growth rate = 0.26 mm day^{-1}), as well as for all specimens combined. The combined regression model showed a strong significant linear relationship ($r^2=0.95$; $p<0.0001$) (instantaneous growth rate = 0.24 mm day^{-1})

Pelagic Larval Duration: PLD estimates for the early recruits ranged between 29 – 34 days with an average of 31.75 ± 1.54 (N=36) days.

Size-at-settlement: Based on back-calculations **s**ize at settlement ranged between 10.10 – 12.25 mm L_B (mean±SD =11.15±0.53).

Growth Trajectories [Fig. 5.6 and 5.7]: Individual larval growth trajectories for 2012 and 2013 are presented inset to show the high variability among individuals. Larval growth trajectories constructed from mean increment widths however show that despite this variability, a general pattern across both years could be deciphered. Growth was initially slow but fairly uniform from hatch until age 10 - 13 days after which there was a consistent increase. At the time of examination, both larval growth trajectories were still increasing. Juvenile growth trajectories [Fig. 5.7] were initially similar to the larvae, showing that growth was slow and relatively uniform from hatch to age 13 days. This was followed by a steady increase until age 29 days, a rapid decrease in growth until age 37 days, after which growth became relatively uniform.

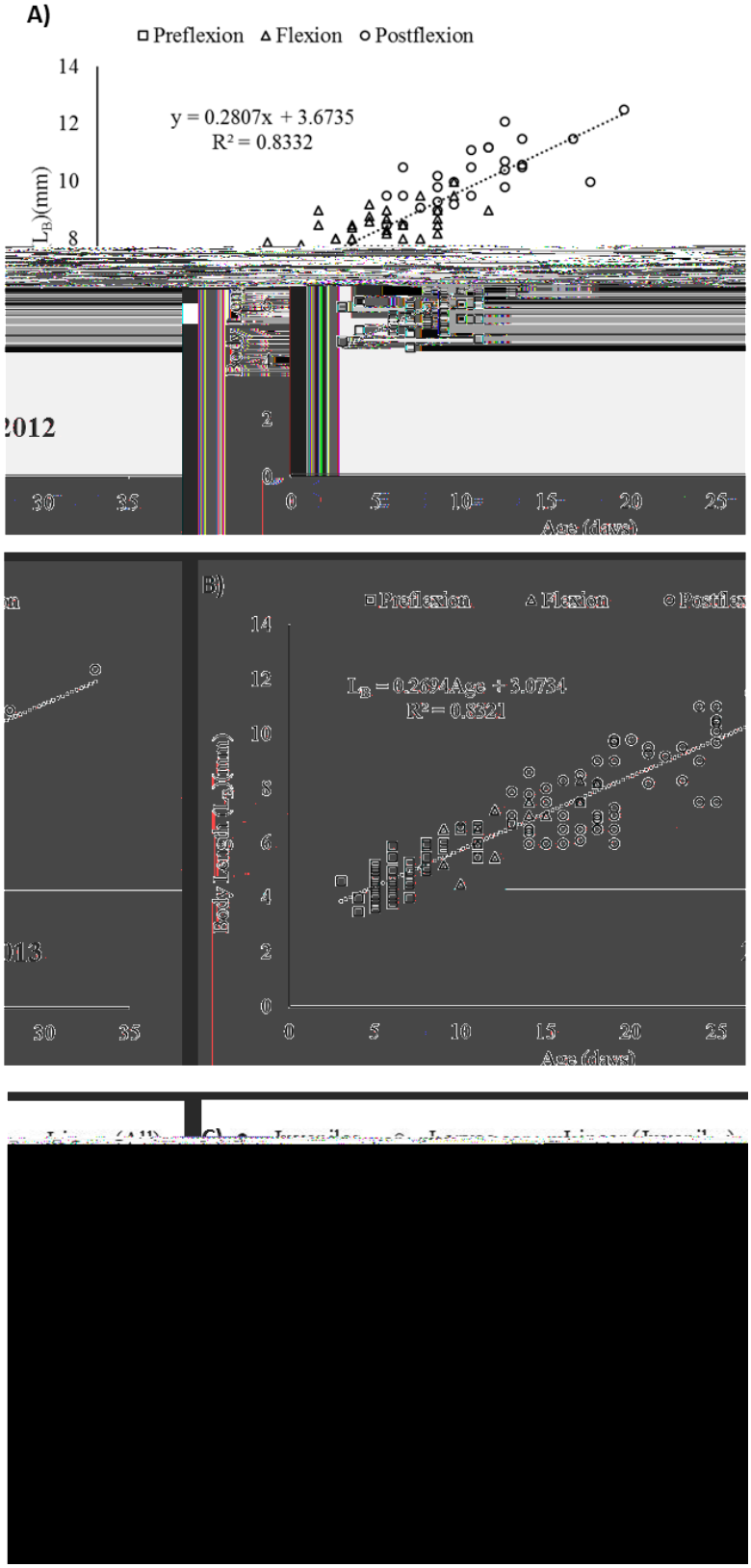


Figure 5.5 Estimated linear growth model for *T. delaisi* larvae collected along the Portuguese west coast in 2012: (A) 2012; (B)2013; (C)juveniles and all individuals combined.

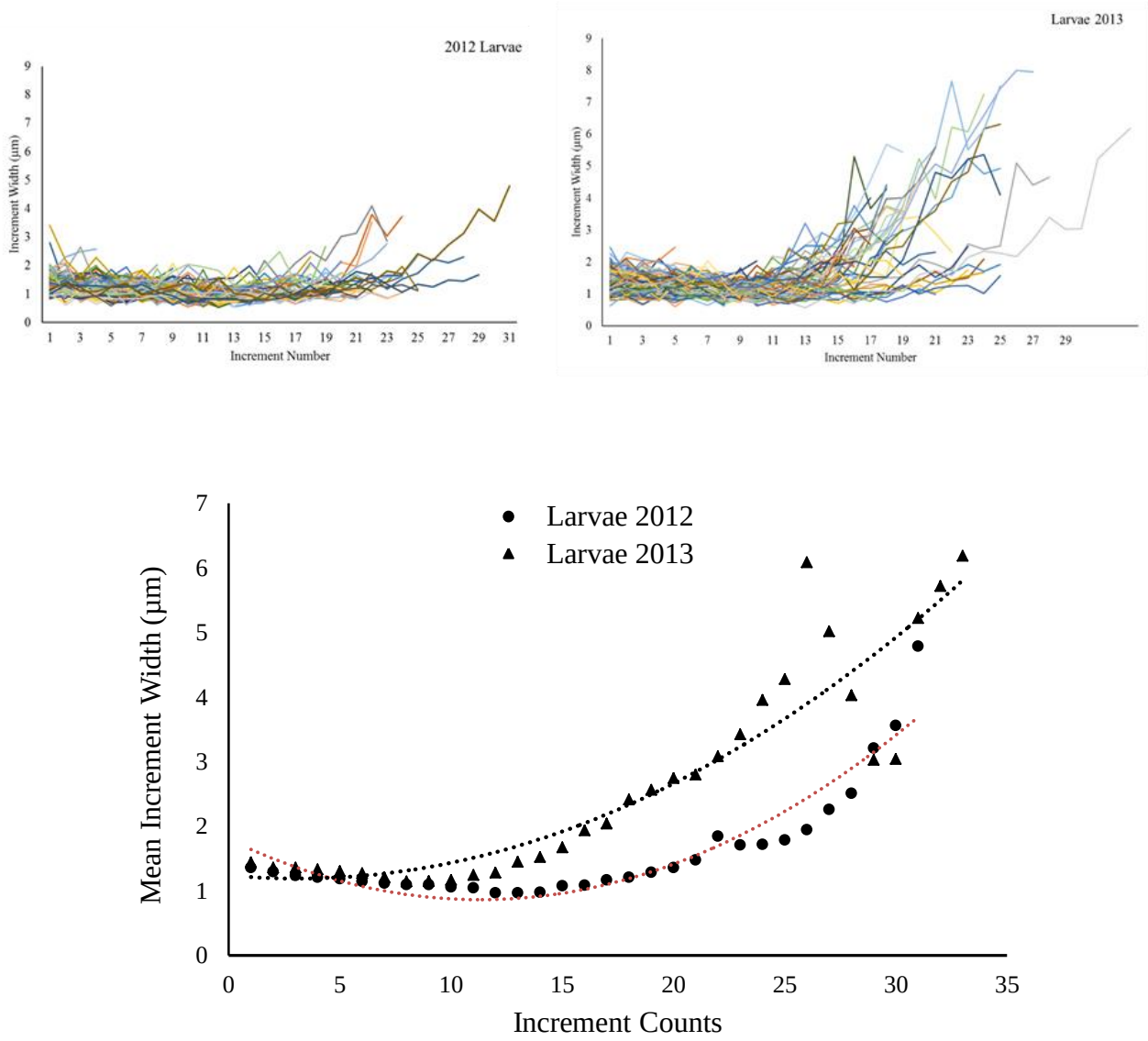


Figure 5.6. Mean increment width profiles for (A) 2012 and 2013 larvae (Inset: individual growth trajectories).

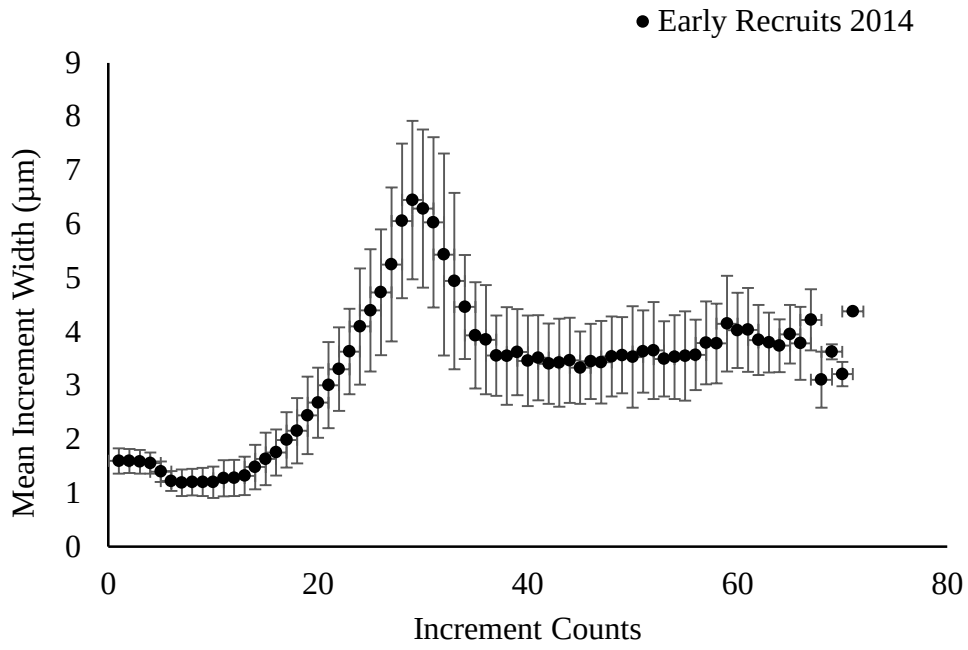


Figure 5.7. Mean increment width profiles for early recruits 2014.

5.5 *Discussion*

In this study, the occurrence of well-defined daily growth increments on the sagittal otoliths of larval and juvenile *T. delaisi* individuals, facilitated the use of otolith microstructure analyses to derive several early life history parameters for this species. The only previous ELH parameters available for *T. delaisi* were PLD estimates based on back-calculations from the settlement marks on the lapillar otoliths of juveniles from the Mediterranean ((Raventós & Macpherson, 2001; Macpherson & Raventós 2006). In those studies, the lapillar otoliths were found to be more reliable than the sagittal otoliths for age estimations due to better increment clarity and definition. In our study, we found that the sagittal otoliths from both the larvae and the juveniles exhibited good increment clarity and definition and were hence suitable for ageing. When sub-daily rings were observed (as microincrement widths began to widen during the postflexion stage) on the sagittal otoliths, they were clearly discernible from the more prominent daily rings. The use of the sagittal otoliths allowed us to make comparisons between larvae and early recruits as daily increment rings were not clearly discernible on the lapillar otoliths of the larvae, especially during the preflexion stages.

Both larval and juvenile otolith morphology (changes in otolith size and shape) and microincrement patterns (changes in width) were similar to that recorded for most teleost fish The increase in otolith increment width late during the postflexion stage has been recorded and is

usually associated with the metamorphosis from the larvae to the juvenile phase (Wilson and McCormick, 1999)

A strong linear relationship between otolith radius and body size indicated that otolith growth was a good indicator of somatic growth in both the larval and juvenile stages and this relationship formed the basis of all subsequent analyses.

Growth Rates: Larval and recruit instantaneous growth rates derived from the relationship between size and age ranged between 0.239– 0.281 mm day^{-1}. While these growth rates are higher than those recorded for another triplefin, *Helcogrammoides chilensis* (0.15 and 0.18 mm day^{-1}) (Plaza *et al.*, 2013; Palacios-Fuentes *et al.*, 2014) in Chilean waters, they are similar to the slower growth rates recorded for demersal some reef fish compared to pelagics. Growth rates documented for the larvae and juveniles of some demersal reef fish include: the clingfishes *Gobiesox marmoratus* (0.24 mm day^{-1}) and *Sicyases sanguineus* (0.14 mm day^{-1}) (Contreras *et al.*, 2013), the rockfish *Sebastes capensis* (0.14 mm day^{-1}) (Landaeta & Castro, 2006), the cardinalfish *Ostorhinchus doederleini* (0.35 mm day^{-1}) (Kingsford *et al.*, 2014) and the sea bream *Diplodus annularis* (0.27 mm day^{-1}).

Hatch Sizes: Sizes-at-hatching (3.1 - 3.7 mm L_B) are similar to field observations where yolk sac individuals 3.0 – 3.5 mm in length have been collected. Although these sizes are smaller than those recorded for three Tripterygiids (average size at hatch = 5.85, 5.03 and 5.72 mm) off of New Zealand (Ruck, 1980), they occur within the size range (3.0 – 6.0 mm) documented for the Family Tripterygiidae in Watson, 2009.

PLD: PLD estimates for *T. delaisi* in this study ranged between 29 - 34 days with a mean of 31.75 ± 1.54. The minimum, maximum and mean values are all higher than those obtained from two previous studies conducted in the Mediterranean. One study derived a range of 17 - 18 days with a mean of 17.7 (N = 3) (Raventós & Macpherson, 2001) whilst the other study derived a range of 16 - 21 days with a mean of 17.3 ± 1.1 (N = 25) (Macpherson & Raventós 2006). Our results support recent studies which indicate that PLD is a more plastic trait than originally thought, with intraspecific variation in this trait being detected at both spatial and temporal scales for a number of species (Bay *et al.*, 2006; Di Franco & Guidetti, 2011; Di Franco *et al.*, 2013). Factors responsible for this variation include temperature, food availability, growth conditions, and variability in environmental and oceanographic features and processes (Victor, 1986; Green & Fisher, 2004; O'Connor *et al.*, 2007).Whilst there may be other differences, one of the most obvious difference between our study site and the previous study sites in the Mediterranean is the water temperature - water temperature in the Mediterranean can be expected to be several degrees higher than that along

the Portuguese west coast. This lower water temperature may affect PLD indirectly by decreasing larval growth rates leading to longer PLDs (Beger *et al.*, 2014; Teichert *et al.*, 2014) which is consistent with the results obtained in this study. There are however no growth rates for *T. delaisi* larvae from the Mediterranean for comparisons. Temperature is just one factor that can influence PLD and whilst it is beyond the scope of this study to conclusively determine the cause of this spatial intraspecific variation in PLD, it is important to note that the differences detected in this study may have implications for the processes of larval dispersal and connectivity. This is especially so for this species which has traditionally been regarded as a model species for investigating connectivity due in part to its relatively short PLD derived from the Mediterranean studies. Our contrasting results also highlight the need to exhibit caution when utilizing single point estimates (i.e. estimates based on one time sampling at a single location) as intraspecific variation may be underestimated. In general, mean PLD estimates for other triple fins in the literature is quite variable ranging from 2 - 3 months in the temperate waters of New Zealand (Kohn & Clements, 2011), to one month in Hawaii (Longenecker & Langston, 2005) and Japan (Ishihara & Tachihara, 2011), to two to three weeks in the Mediterranean (Raventós & Macpherson, 2001; Macpherson & Raventós 2006).

Size-at-settlement ranged from 10.10 to 12.25 mm L_B (Average =11.15±0.53). These values supported our field observations at Arrábida. In terms of comparisons with other Tripterygiids, Ishihara and Tashihara, 2011 estimated the settlement size of 6 Tripterygiids along the coast of Japan to range between 8.1±0.6 mm to 10.1±0.6 mm, which is comparable to our results.

Variability: Whilst there were no significant differences in larval growth rates between years, the postflexion larvae sampled in 2013 exhibited wide variability in otolith radius. This variability was due to the widening of the otolith increments closest to the edge resulting in a significant increase in otolith length in some of these postflexion larvae. This increase in otolith increment width usually occurs just before settlement. Although most of these postflexion larvae were caught at the bottom, it was not possible to determine if depth (i.e. surface vs bottom) was an influential factor because most of the postflexion larvae in that year came from a single bottom sample.

All of the early life-history parameters estimated in this study can exhibit variability in the marine environment due to a number of factors including temperature, food availability and maternal effects (Meekan *et al.*, 2003; Green and Fisher, 2004; Bergenius *et al.*, 2005; Spies *et al.*, 2014). Temperature differences between seasons in the temperate environments can increase

variability in these larval traits which can "carry-over" to the subsequent stages leading to variation in survival (Searcy & Sponaugle, 2001). Traits associated with higher post-settlement fitness and survival include, larger-size-at hatching (Vigliola & Meekan, 2002), faster larval growth rates (Searcy & Sponaugle, 2001), shorter PLD , larger size at settlement and higher condition at settlement.

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6. GENERAL DISCUSSION

An understanding of population connectivity and the scale of larval dispersal is essential for designing management and conservation measures for meta-population dynamics, fisheries, and biodiversity reserves. In demersal reef fish that are relatively sedentary as adults, connectivity tends to occur via the pelagic larval phase. This pelagic larval phase, which is variable in duration, is an extremely critical stage in the life-cycle, and inherent characteristics such as early life history traits and larval behaviour usually interact with oceanographic processes and habitat characteristics to determine patterns of connectivity. Despite its importance, determining or predicting connectivity patterns has proven to be difficult due to the interaction of so many factors. Of key importance, is a better understanding of the pelagic larval phase.

In this thesis, connectivity of the black-faced blenny, *Tripterygion delaisi* was investigated at a local scale indirectly through the use of population genetic structure. Various aspects such as larval spatial distribution, allometric growth and larval development, and early life history characteristics of the critical pelagic larval stage was then examined. The main findings are discussed in terms of how the connectivity patterns observed can be explained by these inherent "characteristics" of the pelagic larval stage.

Thesis overview

Chapter 2 describes the first connectivity study conducted for *T. delaisi* along the coast of the Atlantic mainland, and incorporating all the different developmental stages (adults, juveniles and larvae). Local scale population genetic structure of this species showed that the three sample locations, Arrábida, Sines and Cascais were highly genetically connected over a small spatial scale of approximately 100 km. These results were in contrast to what was hypothesized given the low dispersal capabilities of this species, and the physical characteristics of the area which may potentially promote local retention. This lack of population genetic structure was attributed to oceanographic processes acting over the small spatial scale of our study to promote considerable gene flow and genetic homogeneity. Unlike previous studies on this species in the Mediterranean, which found genetic differentiation on a small spatial scale due to habitat discontinuity created by deep water or expanses of sand, the stretches of sand between our sample sites did not act as a barrier to dispersal. Within this scenario of high gene flow however, several pieces of evidence indicated that limited or restricted dispersal was occurring leading to some degree or extent of local scale genetic homogeneity. These evidences included a pattern of significant isolation by distance, the identification of several genetic clusters, and spatial clustering of sites. The occurrence of

temporal genetic heterogeneity or "chaotic genetic patchiness was detected when the juvenile and larval stages were included in our analyses, likely due to variance in success of adult reproduction.

In **Chapter 3**, the spatial and temporal distribution of *T. delaisi* larvae in the Arrábida Marine Park was investigated from samples caught via two gear types, light traps and plankton nets attached to underwater scooters, over three years. For the first time, light traps were deployed inshore and offshore of the AMP. Our main findings were: (1) all the different larval ontogenetic stages of *T. delaisi* (preflexion, flexion and postflexion) were present in the nearshore environment; (2) the abundance of *T. delaisi* larvae was greater inshore compared to offshore; (3) there were differences in the ontogenetic composition of *T. delaisi* caught in the scooter and light traps, with the ratio of preflexion larvae to more developed larvae being greater in the scooter samples compared to the light trap samples, and (4) vertical differences in the ontogenetic structure of samples collected at distinct depth strata could be detected. Our results support previous studies which document a mainly inshore larval distribution for species belonging to the Family Tripterygiidae. Comparison of gear types was also in keeping with results from previous studies and highlighted the importance of using multiple gears when sampling to determine larval distribution patterns.

In **Chapter 4**, distinctive morphometric and meristic characteristics which are central to identifying this species from related taxa is described for the first time. A description of larval development and allometric growth patterns of *T. delaisi* revealed that most of the body proportions of *T. delaisi* exhibited allometric growth during larval development. Inflexion points of growth divided development into two stages; the 1st developmental phase includes the preflexion and flexion stage larvae, while the 2nd phase characterises the postflexion larvae prior to the transition from larvae to juvenile. Considering allometric growth patterns and ontogenetic descriptions together, it was hypothesized that the 1st phase primarily relates to the development of the digestive system (coiling of the gut occurs during this stage), and the swimming abilities of the larvae (which depends on the development of the myomeres in terms of length and width, and caudal fin development), and the 2nd phase involves continued locomotory development via progressive fin formation and development, and the transition from larvae to juvenile. It was also hypothesized that some of the essential sensory systems may have already been well developed on hatching possibly facilitating some active behaviour soon after hatching.

In **Chapter 5** we used otolith microstructure analyses of the sagittal otoliths of larvae and early recruits to determine several early life-history parameters for *T. delaisi*. Apart from PLD studies previously conducted in the Mediterranean which utilized lapillar otoliths, there was no

information available on life history parameters during the early life stages of this species. We examined larvae belonging to all three developmental stages (preflexion, flexion and postflexion) and there was a good match between the age of the older larvae examined and the age at settlement, derived from back-calculations from the settlement mark. PLD estimates ranged between 29 and 34 with a mean of 31.75 ± 1.54 days. These values are higher than that obtained from previous studies conducted in the Mediterranean on T. delaisi. Instantaneous growth rates, size-at-hatching and size-at-settlement are similar to those documented for other demersal reef fish.

Larval Dispersal and Connectivity Patterns

The examination of local-scale population genetic structure of *T. delaisi* showed that whilst gene flow was high between all three sites (no genetic differentiation detected), several pieces of evidence indicated that limited or restricted dispersal was occurring leading to some degree or extent of local scale genetic homogeneity. Previous connectivity studies conducted on *T. delaisi* in the Mediterranean yielded evidence of limited dispersal and connectivity in the form of high self-recruitment levels, genetic differentiation between populations, and isolation by distance (Carreras-Carbonell *et al.*, 2006, 2007; Schunter *et al.*, 2014).

In population genetic studies, the lack of genetic differentiation between populations does not exclude the occurrence of limited or restricted ecological connectivity as only a few migrants per generation is needed to genetically homogenize populations. For example, direct evidence of larval retention in the form of self-recruitment was detected amidst a scenario of high gene flow among sites for populations of the bicolor damselfish (*Stegastes partitus*) in Bahamian waters (Christie *et al.*, 2010).

Given the difficulties in detecting significant genetic differentiation in high gene flow scenarios indicative of marine systems, evidence of restricted ecological dispersal is increasingly being inferred from a significant pattern of isolation by distance (Carreras-Carbonell *et al.*, 2006; Purcell *et al.*, 2009; Christie *et al.*, 2010; Pinsky *et al.*, 2010). Based on the stepping stone model of dispersal, isolation by distance (Wright, 1943; Kimura & Weiss, 1964) is often applied to populations distributed along a coastline or chain of islands. Its theoretical basis is that, if individuals disperse locally (i.e. dispersal is restricted), individuals that are close to each other will be more genetically similar than individuals that are further apart, i.e., a pattern of decreasing genetic relatedness with geographic distance will emerge.

Characteristics of larval *T. delaisi* obtained from our study which may influence larval dispersal and connectivity include:

(1) **A mainly inshore larval distribution** (Chapter 3 - higher larval abundance detected inshore vs offshore).

(2) **An inshore occurrence of all the different larval developmental stages (preflexion, flexion and postflexion)** (Chapter 3).

(3) **Vertical ontogenetic structure** (Chapter 3).

(3) **Potential early development of some sensory abilities** (Chapter 4) – based solely on a combination of findings in this study and the literature. Studies on the development of larval sensory abilities in *T. delaisi* are needed to determine this conclusively.

(4) **Potential early development of swimming abilities** (Chapter 4) – based solely on the early development of body parts (myomeres and caudal fin) associated with locomotory skills and the literature. Studies on larval swimming abilities of *T. delaisi* are needed to determine this conclusively.

(5) **A PLD of 31.75 ± 1.54 days** – higher than that recorded from previous studies conducted in the Mediterranean (Chapter 5).

(6) **Larval and juvenile instantaneous growth rates** – in keeping with that recorded for other demersal reef fish (Chapter 5).

Larval spatial distribution

A mainly **inshore larval distribution together with the occurrence of all the different larval developmental stages inshore**, and the detection of **vertical ontogenetic** structure, indicate that *T. delaisi* larvae can be locally retained within the nearshore environment where it may be completing its larval life. Nevertheless, since larval origin is unknown, there is no direct evidence that the larvae present in the area were spawned from adults in that area. However, that is highly likely given some of the physical characteristics of the area. For example, the AMP is separated from the two nearest rocky reef habitats to the north and south, Cascais and Sines respectively by a large expanse of sand. As such it can be considered as a continental island with rocky habitat. It is important to note that although local retention is occurring and may be the dominant process, this not exclude a certain level or degree of larval dispersal away from the nearshore environment, as detected by the absence of population genetic structure.

One would expect an inshore larval distribution to be associated with local retention and limited connectivity (significant genetic structure) via a reduction in dispersal distance. Riginos and

Victor, 2001 found that *Axoclinus nigricaudus*, a species with a short PLD and an inshore larval distribution, exhibited greater population genetic structure than *Ophioblennius steindachneri*, a species with a longer PLD and offshore distribution. The differences in genetic structure was attributed to early life-history traits including larval spatial distribution. In contrast, in a study comparing the genetic connectivity patterns of 7 littoral fish species with varying dispersal potentials, the expectation that species with an extended PLD and an offshore larval distribution would exhibit greater dispersal (less genetic differentiation) than species with a shorter PLD and an inshore distribution, was not realized (Galarza *et al.*, 2009). Apart from yielding contrasting results, in both studies, isolating the effect of a single trait or characteristic on population genetic structure was impossible, and serves to highlight one of the major difficulties in attempting to predict connectivity patterns.

The detection of vertical ontogenetic structure indicates that *T. delaisi* larvae are able to exert some level of control over its vertical positioning in the water column. This vertical positioning behaviour by fish larvae can be used to mediate horizontal transport via ontogenetic vertical migrations. In general, this active vertical positioning in the water column should favor self-recruitment and local retention by allowing for greater control over horizontal transport (Sponaugle *et al.*, 2002). There is evidence that vertical ontogenetic migrations can facilitate local retention (Paris and Cowen, 2004; Paris *et al.*, 2007). For example, Paris and Cowen, 2004 showed that in the bicolor damselfish, *Stegastes partitus*, vertical ontogenetic migrations coupled with vertical stratification of the currents was responsible for retaining locally spawned larvae in the coastal waters of Barbados.

Early development of sensory and swimming abilities.

The development of sensory and swimming abilities facilitates the onset of larval behaviour which allows the larvae to exhibit some degree of control over its horizontal and vertical movement thus potentially preventing passive dispersal (advection by water currents only). It is now widely accepted that larval behaviour, including swimming, can influence the scale of larval dispersal and connectivity (Leis, 2010) contradicting the traditional view of larvae as passive (Leis 2006). Numerous studies have shown that many larvae: (1) exhibit vertical migration which may allow them to alter their horizontal position in the water and hence ultimately influence their dispersal (Paris & Cowen, 2004; Leis, 2007), (2) possess swimming capabilities (speed, endurance and orientation) which are more than sufficient to actively influence their dispersal (Leis & Carson-

Ewart, 1997; Stobutzki & Bellwood, 1997; Fisher *et al.*, 2000; Paris & Cowen, 2004; Fisher, 2005; Leis, 2007; Leis *et al.*, 2007) and (3) are capable of detecting olfactory (Atema *et al.*, 2002; Gerlach *et al.*, 2007; Paris *et al.*, 2007) and aural (Tolimieri *et al.*, 2000; Simpson *et al.*, 2004; Montgomery *et al.*, 2006) cues which may assist them in detecting, navigating, and actively selecting habitats.

Sensory and swimming abilities of the larvae may vary in terms of when it develops and in the extent of development. We did not directly examine the development of sensory and swimming abilities in *T. delaisi* larvae, however, we formulated some general hypotheses based on a combination of our findings and the existing literature. We hypothesize that on hatching, some of the essential sensory systems of *T. delaisi* are already well developed. Our hypothesis is based on the following:

(1) *T. delaisi* larvae hatch in a somewhat developed state with pigmented eyes, open mouth and visible otoliths (chapter 4). This is similar to other species with larvae hatching from demersal eggs, that are known to be larger and better developed, with more advanced sensory and swimming capabilities compared to those hatching from pelagic eggs (Thresher, 1984);

(2) the absence of rapid allometric growth of the head area in the preflexion larvae (Chapter 4). This fast growth is usually associated with the rapid development of sensory systems soon after hatching. Its absence maybe due to the fact that these systems have already been developed to a certain extent.

(3) the ability of preflexion *T. delaisi* larvae to detect and react to light stimulus from the light traps, indicate that certain sensory functions are already well developed at these stages (Chapter 3).

(4) an inshore larval distribution of all the different developmental stages (Chapter 3) – the exact mechanisms (i.e. physical or biophysical) favoring larval retention in the nearshore environment is not known. However, if it is biophysical (i.e. diel vertical migrations), many biophysical mechanisms require sufficient development of certain sensory abilities (Kingsford *et al.*, 2002).

We also hypothesize that the swimming abilities of *T. delaisi* larvae might develop fast early (preflexion) in development. This hypothesis is based on:

(1) the early development of body parts (myomeres and caudal fin) associated with locomotory skills (Chapter 4)

With regards to connectivity patterns, the general consensus is that larvae with better developed swimming and sensory abilities are more likely to promote self-recruitment and local retention than larvae with less developed sensory abilities and swimming capabilities. Fisher, 2005 examined the impacts of swimming speeds on self-recruitment and dispersal in the larvae of several coral reef fish species belonging to 11 families. Results suggested that (1) for as much as 50% of their larval life most reef fish families exhibited considerable influence over their dispersal pattern relative to ocean currents (2) swimming behaviour could potentially influence dispersal patterns on a magnitude similar to the dispersing effect of ocean currents and (3) the swimming capabilities of several reef fish families can potentially facilitate active self-recruitment.

While studies on larval swimming and sensory abilities are fairly prevalent in the literature, studies directly linking these abilities to actual patterns of larval dispersal and connectivity are few. An examination of connectivity via population genetic structure in the tropical inshore marine fish (*Eleutheronema tetradactylum*), showed that even modest larval swimming abilities may promote self-recruitment as opposed to dispersal. This fish, which exhibits low-average swimming performance in terms of speed and endurance (Leis *et al.*, 2007) compared to the larvae of other tropical species, exhibits a strong isolation by distance pattern across its geographic range in Northern Australia and significant genetic structure among populations separated by as little as 15 km, indicating that populations may be self-seeding on an ecological time-frame (Horne *et al.*, 2011). In another study, using a multi-disciplinary approach, Gerlach *et al.*, 2007 found that settling larvae were capable of using their olfactory senses to choose currents that return them to their natal reefs. This larval behaviour was associated with strong, persistent natal homing, detected by strong genetic sub-structure and larval assignment in the Apogonid, *Ostorhinchus doederleini*.

Early life history traits (ELHTs)

We provided estimates for a number of early life history parameters (size-at-hatch, size-at-settlement, pelagic larval duration, and larval and juvenile growth rates) of *T. delaisi*. Generally, many of these early life history traits (ELHTs) are not only taxon specific, but also exhibit considerable intra-specific variability due to a number of factors such as maternal effects (Berkeley *et al.*, 2004; Donelson *et al.*, 2009), food availability (Spies *et al.*, 2014) and temperature (Meekan *et al.*, 2003; Green & Fisher, 2004; Takahashi *et al.*, 2012; Spies *et al.*, 2014). Of the parameter estimates provided, pelagic larval duration is often regarded as a proxy for larval dispersal and traits such as size-at-hatch, size-at-settlement, and, larval and juvenile growth rates can affect connectivity via their influence on larval survival, settlement, and post settlement processes. Many

of these traits are also inter-connected, for example larval growth has been shown to influence PLD and size-at-settlement.

Our PLD estimates (both mean the value and minimum – maximum values) are higher than those obtained from two previous studies conducted in the Mediterranean. Raventós and Macpherson, 2001 estimated the mean PLD of *T. delaisi* to be 17.7 days with a range of 17-18 days. Estimates were based on back-calculations from the settlement-marks on the lapillar otoliths of three post-settled individuals (<43mm in standard length). In a subsequent study (Macpherson & Raventós 2006) which examined a greater number of individuals (N=25), although the mean PLD was similar, there was an increase in the minimum-maximum range to 16-21 days. Similar mean PLDs and ranges were also obtained for the other two *Tripterygion spp*. examined (**Table 6.1**).

Table 6.1: Pelagic larval duration in days, for *Tripterygion delaisi* caught in the Mediterranean and Atlantic. N= number of individuals examined, S.D. = standard deviation, Min = minimum and Max = maximum.

	N	**Mean**	**S.D.**	**Min**	**Max**	**Location**
T. delaisi[1]	3	17.7		17	18	Mediterranean
T. melanurus[1]	6	17.0		15	18	Mediterranean
T. tripteronotus[1]	4	17.3		17	18	Mediterranean
T. delaisi[2]	25	17.3	1.1	16	21	Mediterranean
T. melanurus[2]	53	17.6	2.3	15	25	Mediterranean
T. tripteronotus[2]	39	18.4	2.8	16	27	Mediterranean
T. delaisi[3]	36	31.75	1.54	29	34	Atlantic

[1]Raventós and Macpherson, 2001 [2] Macpherson and Raventós, 2006 [3] Present Study

Our results support recent studies which indicate that PLD is a more plastic trait than originally thought, with intraspecific variation in this trait being detected at both spatial and temporal scales for a number of species (Bay *et al.*, 2006; Di Franco & Guidetti, 2011; Di Franco *et al.*, 2013). Factors responsible for this variation include temperature, food availability, growth conditions, and variability in environmental and oceanographic features and processes (Victor, 1986; Green & Fisher, 2004; O'Connor *et al.*, 2007). Whilst there may be other differences, one of the most obvious differences between our study site and the previous study sites in the

Mediterranean is the water temperature - water temperature in the Mediterranean can be expected to be higher than that along the Portuguese west coast. This lower water temperature may affect PLD indirectly by decreasing larval growth rates leading to longer PLDs (Beger *et al.*, 2014; Teichert *et al.*, 2014) which is consistent with the results obtained in this study.

Under the traditional view of passive dispersal and demographically open populations, the expected relationship is that as PLD increases, dispersal distance should increase leading to greater connectivity. Studies to date have, nevertheless, yielded variable and in some cases contrasting results (Shulman & Bermingham, 1995; Riginos & Victor, 2001; Shanks *et al.*, 2003; Bay *et al.*, 2006; Galarza *et al.*, 2009; Shanks, 2009).

Doherty *et al.*, 1995, examined genetic differentiation in 7 species of fish with varying PLDs and life history traits between two regions of the Great Barrier Reef, 1000 km apart. Study species were carefully chosen so as to obtain a range of dispersal capabilities. Five out of the seven species examined exhibited significant genetic differentiation (F_{ST}) between regions. One out of the two species that did not exhibit genetic differentiation had the longest PLD among the 7 species examined. Overall, it was estimated that PLDs greater than 1 month should facilitate enough gene flow to homogenize genetic differences over a distance of 1000 km and that PLD was a good indicator of larval dispersal and connectivity. Contrasting results were obtained when the relationship between PLD and genetic structure was examined for 8 species of fish belonging to the family Pomacentridae. A relationship between PLD and genetic structure was detected; however, when the single species exhibiting direct development was excluded, this relationship was lost even though PLD varied between 11 – 28 days among the 7 remaining species (Bay *et al.*, 2006).

Presently, the overall consensus is, PLD can be regarded as a proxy for connectivity in the absence of direct estimates, however this relationship is not as straight forward as originally thought and must be treated with caution as other factors, both abiotic and biotic, may come into play. Our PLD estimates of 29-34 days is similar to that recorded for triplefins in Hawaiian and Japanese waters (one month - Longenecker & Langston, 2005; Ishihara & Tachihara, 2011), but much shorter than that recorded for several triplefin species in the temperate waters of New Zealand (Kohn & Clements, 2011). A review of the literature shows that demersal reef species with PLD values similar to *T. delaisi* can exhibit both significant self-recruitment as well as larval dispersal (Jones *et al*, 1999; Almany et al., 2007).

Several of the other early life history traits examined can also influence connectivity patterns by influencing settlement and post-settlement processes. Faster growth rates during the larval stages is often associated with shorter PLDs (Sponaugle and Pinkard, 2004; Shima and

Findlay, 2002; Mizusawa *et al.*, 2004), and higher planktonic survival rates. In general, larger-size-at hatching (Vigliola & Meekan, 2002), faster larval growth rates (Searcy & Sponaugle, 2001), and shorter PLD are traits associated with higher post-settlement fitness and survival at settlement

In conclusion, *T. delaisi* in Atlantic waters exhibits several early life history traits which can promote restricted dispersal and connectivity. Many of these same early life history traits however can also promote larval dispersal. An understanding of larval dispersal and connectivity patterns of *T. delaisi* in our study area as well as a general understanding of connectivity can be enhanced by the following:

6.1 Future Directions

(1) A better understanding of the small-scale local processes occurring in the nearshore environment throughout our study area, and the mechanisms which may be acting to promote larval retention.

Local processes occurring in the nearshore environment along the stretch of coastline sampled in our study is relatively unknown. For demersal fishes such as *T. delaisi* which inhabit mainly nearshore rocky habitats, local processes can be influential. Nearshore environments tend to be characterized by shallow depths, the influence of continental shelf features, freshwater inputs, and a multitude of diverse local hydrodynamic processes (tides, fronts, eddies, internal waves, bores, and coastal boundary effects) which make water flow in these environments very complex and different/distinct from the open ocean (Pineda *et al.*, 2007). Physical mechanisms/processes that can function to facilitate or promote larval retention can be numerous and quite diverse ranging from the interaction between topographical features and coastal circulation to create zones of retention (Largier, 2003; Roughan, 2005), to internal tidal bores promoting the shoreward transport of planktonic organisms (Pineda and Hare, 2007).

(2) A better understanding of the Coastal Boundary Layer

A prominent feature of the nearshore environment which has been receiving increasing attention due to its potential role in facilitating larval retention in upwelling regions, is the coastal boundary layer (CBL) – an area of reduced current velocity that extends beyond the surf zone (i.e. within 1 km from shore) (Nickols *et al.*, 2012). Not well studied due to the difficulties of accessibility, high larval abundances have been detected within this layer, with differences at the

inner and outward margins. Many aspects of this layer, including its spatial and temporal variation in width which may have consequences for dispersal, is relatively unexplored. Additionally, given that this is where the pelagic larvae of most demersal reef species complete development, the incorporation of the CBL into high resolution models would result in more realistic models of dispersal and connectivity.

(3) A better understanding of the sensory and swimming abilities of *T. delaisi*.

There is no information available on the sensory or swimming abilities of this species. Given the potential role of these two traits in facilitating larval behavior, which may promote either larval retention or dispersal, studies in these two areas will enhance our understanding of larval dispersal and connectivity in this species. Areas of priority should include the timing of ontogenetic development of both sensory and swimming abilities and swimming capabilities in terms of critical speed and endurance.

(4) The application of models to understand the interaction between numerous factors

Understanding connectivity patterns is complicated given the interaction of numerous physical and biological factors. Over time, models both physical and biophysical have been applied to connectivity studies. There is still however a need to incorporate models more routinely into connectivity studies. Additionally a greater suite of larval behaviours and life history traits need to be incorporated into these models. Small scale local process which are of important to nearshore reef species such as *T. delaisi* also needs to be included with greater prevaldence.

(5) The utilization of a combination of approaches

Given the complexity of marine connectivity, the best approach would be to use two or more methods in combination (Leis *et al*., 2011) so that the results of one method may be validated by another. This approach is especially useful when attempting to decipher patterns of demographic connectivity using population genetics, since the exchange of a few individuals per generation can homogenize genetic structure but be of no consequence to demographic connectivity (Waples & Gaggiotti, 2006). The few studies that have utilized a combination of approaches have found it to be advantageous, providing a more comprehensive description of connectivity (*e.g.* Jones et al, 2005, Almany *et al*., 2007 and Planes *et al*., 2009).

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